RNA Interference and Its Potential Applications in Aquatic Animal Health Management

2

Vikash Kumar, Suvra Roy, Bijay Kumar Behera, and Basanta Kumar Das

Abstract

In the past two decades, with the discovery of RNA interference (RNAi) or post-transcriptional gene silencing (PTGS), the modern molecular biology field has been boosted with its immense applications. This rapid and powerful silencing method is useful in studying the gene function as well as in therapeutic applications for disease treatment. The RNAi or PTGS is a biological process of mRNA degradation induced by complementary double-stranded (ds) small interfering RNA (siRNA) sequences that mediate suppression of target protein-coding gene expression and provide resistance to both exogenous and endogenous microbial nucleic acids. This sequence-specific natural gene silencing mechanism has revolutionized experimental biology. RNAi technology has important practical applications in aquatic animal health, including functional genomics, therapeutic intervention, and other areas. Here in this chapter, we introduced the RNAi or PTGS mechanisms and the current understanding of gene silencing in aquatic animals in both fish and shellfish and will propose key areas of aquaculture fields where gene silencing could be applied.

Keywords

RNA interference · Post-transcriptional gene silencing · Disease resistance · Aquatic animal health management

V. Kumar (✉) · S. Roy · B. K. Behera
Aquatic Environmental Biotechnology and Nanotechnology (AEBN) Division, ICAR-Central Inland Fisheries Research Institute (CIFRI), Barrackpore, India

B. K. Das
ICAR-Central Inland Fisheries Research Institute (CIFRI), Barrackpore, India

S. K. Gupta, S. S. Giri (eds.), *Biotechnological Advances in Aquaculture Health Management*, https://doi.org/10.1007/978-981-16-5195-3_2

2.1 Introduction

RNA interference (RNAi) or post-transcriptional gene silencing (PTGS) is a naturally occurring biological regulatory mechanism first described in *Caenorhabditis* sp. model (Fire et al. 1998). Following this ground-breaking discovery, molecular biology has advanced tremendously for both in vitro and in vivo studies. RNAi mechanism has been described in all eukaryotic species ranging from unicellular organism to complex species from both animal and plant kingdoms (Ghildiyal and Zamore 2009). RNAi is essentially an endogenous cellular mechanism utilized by the host to prevent the transcription of foreign genetic material and inhibits the accumulation of microbial pathogens. RNAi is triggered by gene-specific double-stranded RNA (dsRNA), leading to post-transcriptional silencing of specific genes by inhibiting the messenger RNA (mRNA) expression or translational in a cell or organism (Fabian et al. 2010; Fire et al. 1998; Meister and Tuschl 2004; Schuster et al. 2019). The basic concept of RNAi-mediated silencing pathways is the association of double-stranded (ds) small RNAs of ~21–22 base pairs nucleotides, having characteristic 2 nt 3′ overhangs, with a Argonaute protein superfamily to form RNA-protein core complex termed the RNA-induced silencing complex (RISC) (Elkayam et al. 2012; Kim and Rossi 2008; Nguyen et al. 2016; Schirle et al. 2016). In eukaryotes, three classes of small RNAs exist: (1) small interfering RNAs (siRNAs), (2) microRNAs (miRNAs) and (3) PIWI-interacting RNAs (piRNAs) (Ketting 2011). Interestingly, these small RNAs guide Argonaute proteins onto target RNAs molecules via Watson-Crick base pairing that results in silencing in gene. The siRNAs, miRNAs or piRNAs pathways follow the basic principle of post-transcriptional gene silencing; however, the general mechanism for effector functions and small RNA biogenesis is relatively different. For instance, the piRNA biogenesis is dicer independent, whereas siRNAs and miRNAs depend on RNase-III dicer enzymes for processing of double-stranded RNA (dsRNA) precursors into small RNAs (Carthew and Sontheimer 2009).

Aquaculture, farming of aquatic animals and plants, continues to dominate the food-producing sector in the world (Kumar 2020; Kumar et al. 2021; Roy 2020; Roy et al. 2019, 2020). However, due to the global demand increase, the pressure for intensification and expansion of aquaculture systems has rendered aquaculture business fragile and created ideal ground for disease outbreaks, having a devastating impact on socio-economic development worldwide (Kumar et al. 2016, 2019a, b, 2020a, b, 2021; Tran et al. 2020). Moreover, in recent years, the application of RNAi technique has become a potential tool to investigate the functionality of gene of interest and suppress the infection or replication of many pathogens that cause severe economic losses in aquafarming. Therefore, there is an urgent need to thoroughly understand the cost-effective RNAi technology to manage heath of aquatic animals and avoid severe mortality and production losses in aquaculture.

2.2 Mechanism of RNAi Technology

The genetic studies on two model organisms, e.g. *Drosophila* sp. and *Caenorhabditis* sp., have helped in the understanding of mechanism underlying RNAi (Reshi et al. 2014). However, the RNAi mechanism was first recognized in plants, when post-transcriptional gene silencing using small specific RNA oligonucleotides of 20−25 bases was studied. The RNAi-mediated gene silencing involves mRNA expression and translational repression by non-specific binding (imperfect) of small RNAs to the 3' UTR region of target mRNA molecule. Further studies using biochemical and molecular genetics reveal that RNAi occurs via a two-stage process involving an initiation stage and an effector stage (Fig. 2.1) (Hammond et al. 2001).

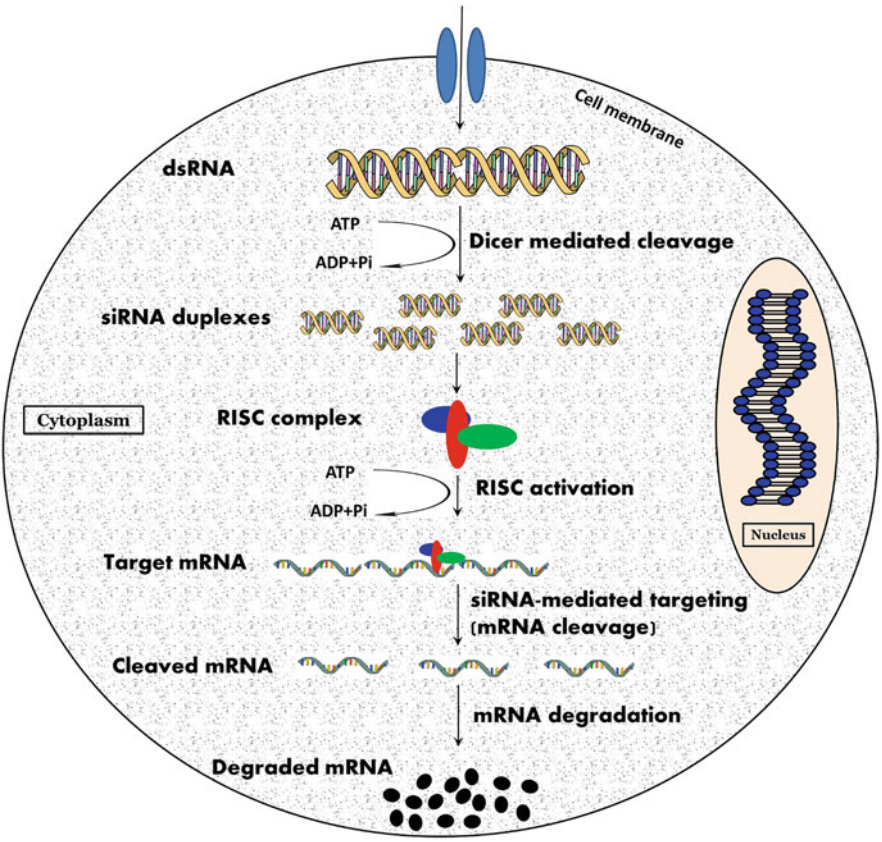

Fig. 2.1 Schematic representation of RNAi molecular mechanism in aquatic animals

2.2.1 Initiation Stage

The initiation stage was reported to take place inside cell cytoplasm. Briefly, a protein molecule known as dicer (ribonuclease III) specifically recognizes the dsRNA sequences and generates 21−23 nucleotides in length small interfering RNAs (siRNAs) molecules (Fig. 2.1) (Reshi et al. 2014).

2.2.2 Effector Stage

The generated double-stranded small interfering RNAs were unwound into single-stranded molecules by a protein complex (containing dicer, Argonaute proteins, etc.) known as RNA-induced silencing complex (RISC). Among two strands, the sense strand is released, while antisense strand is retained by RISC. The antisense strand further guides RISC onto the complementary mRNA molecules, resulting in degradation of target mRNA (Fig. 2.1) (Lingel and Izaurralde 2004). The endonucleases bind to the mRNA region homologous to the siRNA and carry out degradation, resulting in inhibition of expression or translation of target molecules (Zamore et al. 2000).

2.3 RNAi Technology in Aquatic Animal Health Managements

Aquatic animals including both fish and shellfish are susceptible to a variety of pathogens, posing a constant threat to the aquaculture production and severe ecological and economic losses. However, the development of RNAi technology, as a promising gene knockdown tool, has shown promising results in both health and disease management of aquatic animals (Kumar et al. 2018). Fishes were the first vertebrate in which RNAi knockdown of gene expression using long dsRNA was performed. The comprehensive RNAi study was first done in embryos of rainbow trout (*Oncorhynchus* sp.), carrying a green fluorescent protein (GFP) expressing the vector. The siRNAs were able to reduce the number of strongly fluorescent embryos by 60% compared to the negative control embryos, thus providing the first evidence of an effective siRNA-mediated gene silencing in fish (Boonanuntanasarn et al. 2003). The major proteins involved in the RNAi pathway, including dicer and Argonaute, have been identified in fish and shrimp species, e.g. *Danio rerio* (zebrafish), *Gobiocypris rarus* (rare minnow), *Penaeus monodon* (giant tiger prawn), *Litopenaeus vannamei* (whiteleg shrimp), *Marsupenaeus japonicus* (kuruma shrimp) and *Fenneropenaeus chinensis* (Chinese white shrimp), confirming the existence of RNAi machinery in aquatic animals (Table 2.1) (Nguyen et al. 2016; Saksmerprome et al. 2009).

There are reports which highlight that RNAi has become promising tool to study the function of genes in a cell or animals. Additionally, the RNAi-based sequence-specific dsRNA has shown to be effective against severe aquatic animal pathogens. For instance, genes from shrimp pathogens such as yellow head virus (YHV), white

Table 2.1 Major core RNAi genes identified in aquatic animals

Species	Genes	Tissue	References
D. rerio	Dicer	Fertilized eggs	Andrews et al. (2014)
	Dicer 1	Retina	Akhtar et al. (2015)
	Argonaute 2	Fertilized eggs	Kretov et al. (2020)
G. rarus	Argonaute 2	Gill, heart, intestine, kidney, liver, muscle and spleen	Su et al. (2009)
L. vannamei	Dicer 1	Fertilized eggs	Yao et al. (2010)
	Dicer 2	Gill, hepatopancreas, heart, intestine, stomach, nerve, pyloric caecum and epidermis	Chen et al. (2011)
	Argonaute 1	Gill	Labreuche et al. (2010)
	Argonaute 2	Gill	
P. monodon	Dicer 1	Lymphoid organ and haemolymph	Su et al. (2008)
	Dicer 2	Gill, haemolymph and haemocyte but not in tissue muscle	Li et al. (2013)
	Argonaute 1	Lymphoid organs, muscle, gill, hepatopancreas, ovary and nerve cord tissue	Dechklar et al. (2008)
	Argonaute 2	Lymph tissue, gill, haemocytes, eyestalk, heart, ovary, epidermis, stomach, brain, muscle, hepatopancreas, intestines and nerve	Yang et al. (2014)
	Argonaute 3	Haemocytes, lymphoid organ, ovary, heart, hepatopancreas, nerve cord, brain gill, thoracic ganglia, and eyestalks	Phetrungnapha et al. (2013)
	TRBP[a]	Ovaries and lymphatic organs	Yang et al. (2013)
F. chinensis	TRBP[a]	Haemocytes	Wang et al. (2012)
M. japonicus	TRBP[a]	Haemocytes	

[a]*TRBP* transactivation response RNA-binding protein

spot syndrome virus (WSSV), and Taura syndrome virus (TSV) have been targeted by dsRNA, resulting in enhanced survival of shrimp species (Tirasophon et al. 2007; Westenberg et al. 2005; Yodmuang et al. 2006). The detailed application of RNAi technology in aquatic animals is summarized below.

2.3.1 Functional Genomics

RNAi technique can be applied to study the physiological status of different aquatic species. For instance, RNAi technique is being used to determine the process of moulting, reproductive functions (gonad-stimulating hormone and gonad-inhibiting

hormone) and growth by identifying the genes regulating the function of this processes (De Santis et al. 2011).

Additionally, there are several RNAi-related studies on aquatic model organisms, e.g. *D. rerio* (zebrafish) and *Artemia franciscana* (brine shrimp), which help in understanding of animal physiology. Zebrafish, a cyprinid freshwater fish, share very high physiological and genetic similarities with higher vertebrate, including the digestive tract, brain, vasculature, musculature and innate immune system. For instance, zebrafish has almost 70% of functional similarities with human genes responsible for disease, whereas the brine shrimp, an aquatic invertebrate highly osmotolerant, characteristically small and branchiopod crustacean that can be grown in gnotobiotic conditions (germ-free environment, allowing host-associated microbial communities control), serves as exceptional model organism to study the host-pathogen interactions in commercially important shrimps and other crustacean's species. RNAi studies on these model organism have unravel the mechanisms of stress resistance, controlling the process of cell division, by identifying the genes involved in the differentiation, development and reproductive processes (Kumar et al. 2018; Nguyen et al. 2016).

2.3.2 Sex Control

The RNAi technique to control the sex of aquatic animals has remarkably high practical application in aquaculture sector. For instance, an important shellfish species, *Macrobrachium rosenbergii* (giant freshwater prawn), have a very high economic value; however, due to higher growth rate and bigger in size, only male population is preferred for culture. In this prawn species, a highly expressed androgenic gland specific gene, namely, insulin-like AG (Mr-IAG), plays important role in male sex differentiation (Ventura and Sagi 2012). Interestingly, injecting the male prawn juvenile, at an early developmental stage, with Mr-IAG-specific dsRNA, resulted in in vivo silencing of Mr-IAG gene and functional and full sex reversal of male population into neo-females. Subsequently, if the neo-female population were crossed with untreated males, the resulted progenies will be all male population (Ventura et al. 2009). The above experiment demonstrates that RNAi technology is useful in maintaining all male population in prawn species without changing the genome structure, and hence it could become a promising strategy to regulate the sex in other aquatic animals, without creating transgenic animal species.

2.3.3 Disease Management

The fish and shrimp species are constantly exposed to pathogenic microbes in aquatic environment due to which disease outbreak caused by bacteria, viruses and parasites is often observed in aquaculture system. Moreover, with the development of RNAi technology, the understanding on immune mechanism and role of genes potentially involved in tolerance against pathogenic microbial infection has

significantly increased. This has created a positive impact on developing health management protocols for inhibiting the proliferation of pathogenic microbes and improved host survival. However, still RNAi technique is mostly done in smaller scale, and application for commercial disease management is very limited in aquaculture. The RNAi technology used for antimicrobial strategies in aquaculture is summarized below.

2.3.3.1 Antibacterial

The RNAi technique helps in understanding the functional role of genes and underlying immune mechanism, imparting tolerance in aquatic species against pathogenic bacterial infection. In aquatic species, using RNAi, it was shown that prophenoloxidase, p38 mitogen-activated protein and crustin play important role in the multiplication of pathogenic bacteria. For instance, RNA-mediated knockdown of prophenoloxidase inactive precursor resulted in significantly increased bacterial load in shrimp species (Fagutao et al. 2009). Similarly, knockdown of p38 mitogen-activated protein kinases leads to significant lower expression of antimicrobial peptide genes (PEN4, crustin and ALF2) and higher mortality of shrimp species against pathogenic bacterial pathogens (*Vibrio alginolyticus* and *Staphylococcus aureus*) (Yan et al. 2013). In another study, the role of *M. japonicus* crustin (MjCRS) was determined by RNAi technique, and results showed that MjCRS is an important antibacterial defence peptide and silencing results in upregulation of pathogenic bacteria numbers and decreased survival of shrimp species (Hipolito et al. 2014).

2.3.3.2 Antiviral

RNAi technique has been widely used as a powerful tool to identify the genes that participate in viral replication and protect the aquatic animals from viral infection (Wang et al. 2013). Through sensing the viral nucleic acid, RNAi-mediated immunity is triggered to inhibit the replication of the RNA and DNA viruses and knocking down the virus-specific genes or downregulating host genes that are related with viral replication mechanisms. In aquatic animals, siRNA- and miRNA-mediated RNAi antiviral immunity is being reported (Ongvarrasopone et al. 2011).

In siRNA-mediated RNAi silencing, the exogenous microbial dsRNA sequences were cleaved by dicer protein into a siRNA duplex. The siRNA duplex generally possesses 3′ OH, 5′ phosphate (PO_4) and 3′ dinucleotide overhangs molecules. Afterwards, the siRNA duplex combines with Argonaute protein and forms a precursor RNAi-induced silencing complex (pre-RISC). In complex, one passenger strand from duplex is cleaved by Argonaute protein. Later, the Argonaute protein and guide strand in RISC target the complementary strand of mRNAs, resulting in inhibition of translation (Saksmerprome et al. 2013). In shrimp, siRNA-mediated post-transcriptional silencing is reported as important antiviral immune mechanism that leads to inhibition of viral proliferation and infection by degradation of viral mRNA. For example, the viruses after entry and replication inside host produce dsRNA molecules, which initiate an siRNA-mediated antiviral mechanism. The viral dsRNA is processed by dicer2 protein, which cleaved the strand and produces

siRNAs molecules. Afterwards, the siRNAs bind with Argonaute protein and form RISC, resulting in degradation and inhibition of viral replication. Interestingly, it has been found that siRNA contains a 2nd−7th seed region that is responsible for initial recognition of target molecule and a 12th−17th nt supplementary region that helps in binding to the target molecule (Gong and Zhang 2021).

Moreover, miRNAs molecule is derived from the spliced short introns or endogenous noncoding RNA transcripts that are folded into incomplete stem-loop structures. In miRNA-mediated RNAi silencing, the primary miRNAs (pri-miRNAs) are transcribed by the RNA polymerase from genome and later cleaved into approximately 70 nucleotide precursors, called pre-miRNAs, by Drosha protein. Simultaneously, the pre-miRNAs bind with exportin 5 and exported to cell cytoplasm, where pre-miRNAs are processed by Dicer protein into miRNA duplexes. The miRNA duplex binds with Argonaute protein and forms pre-RISC. Later, after the passenger strand is removed from duplex, the RICS containing Argonaute protein and guide strand targets the complementary strand resulting in post-transcriptional gene silencing of target mRNA (Gong et al. 2018). Interestingly, the miRNA 2nd−7th bases are the key domain that recognizes target mRNA through Watson-Crick base pairing, which is generally known as seed sequence. In aquatic animals, miRNAs are reported as a key regulator for variety of biofunctions, e.g. cell development, metabolism, differentiation, proliferation, immunity and apoptosis (He et al. 2015). There are also growing evidence that miRNA plays critical role in antiviral immunity in aquatic organisms by targeting multiple genes through individual miRNA or one gene by several miRNAs. It has been also demonstrated that miRNA promotes apoptosis and cellular phagocytosis by targeting the specific genes, resulting in suppression of viral proliferation and infection.

2.3.3.3 Antiparasitic

An important application of RNAi, apart from being antibacterial and antiviral tool, is its considerable potential for the development of parasitic control technologies. Being very sequence-specific in action, it may be possible to develop species-specific dsRNA for parasites. In mammals, RNAi has been used to suppress gene expression in the intra-mammalian life stages (adults and schistosomula) of *Schistosoma mansoni*, which affects more than 200 million people worldwide and is responsible for 300,000 deaths annually (Da'dara and Skelly 2015).

In aquaculture, the first evidence of gene silencing mediated by dsRNA in a fish parasite was reported in 2007 (Ohashi et al. 2007). Since then, the RNAi technology has been mostly applied to study gene function of various parasites responsible for disease outbreaks in aquaculture. Despite being considered a suitable and capable tool for functional genomics, very little research has been done on its use in the manipulation of the gene function of fish parasites. RNAi techniques have been successfully used to study the microsporidian parasite (*Heterosporis saurida*) of lizardfish (*Saurida undosquamis*) and *Myxobolus cerebralis* (causative agent of whirling disease infection) in the salmonid fishes. The study highlights that RNAi could be a promising tool to enlighten better about the gene functions in parasites for

targeted drug delivery, silencing gene expression and reducing the role of vectors to transmit disease by parasites.

2.4 Delivery Strategies

For successful RNAi-induced silencing, suitable and specific systems are required for delivering RNAi molecules to their target host cell. Depending on the species and organ, different dsRNA delivery strategies can be used, such as electroporation, microinjection, oral pathway, lipid nanoparticles, polymer-based systems and protein-based systems. However, numerous extra- and intracellular biological barriers to RNAi delivery exist in an organism necessitating the smart designing of delivery strategies (Attasart et al. 2010; Timmons and Fire 1998; Tseng et al. 2000). A variety of delivery strategies have been developed for the successful delivery of siRNA molecules, both in vivo and in vitro conditions. Some of the delivery strategies used in aquatic species are discussed below.

2.4.1 Electroporation

Electroporation is another microbiology technique, which utilizes electrical field in order to increase the permeability of the cell membrane, allowing introduction of chemicals, drugs or DNA molecules into the cell. The first experiment on electroporation delivery method to introduce foreign DNA was conducted on *P. monodon* in 1999 (Tseng et al. 2000). Afterwards, several works have been done to validate the method of electroporation-based introduction of foreign DNA using *Artemia* model system (Arenal et al. 2000). Electroporation technique was also employed to deliver siRNA molecule into embryos of model shrimp (*Artemia sinica*) to knock down the As-sumo1 gene. These studies support the electroporation technique to deliver nucleic acid into embryos of fish and shellfish at earlier life stages. Additionally, electroporation can be performed with significantly large numbers of zygotes or embryos at the same time.

2.4.2 Microinjection

Microinjection, utilizing glass micropipette to inject a liquid substance, is a direct method to introduce DNA into either cytoplasm or nucleus. The microinjection method has also been used widely to introduce nucleic acids, including dsRNA, into fishes and shellfish, at different developmental stages. Studies on aquatic animals, e.g. *Daphnia magna*, *Macrobrachium rosenbergii*, *L. vannamei*, *P. monodon* and *A. franciscana*, have reported that microinjection of siRNA resulting in significant induced post-transcriptional silencing in host animals (Chimwai et al. 2016; Han et al. 2019; Kumar et al. 2018).

2.4.3 Oral Delivery

Oral delivery of RNAi therapeutic molecule, either naked or conjugated with a polymer or in the form of bacteria that contain the specific dsRNA/siRNA or in the form feed, has been successfully applied to many aquaculture species (Sarathi et al. 2008). Antiviral activity of RNAi molecule incorporated in nanoparticulate feed has been reported in shrimp species, targeting the WSSV vp28 gene. The study demonstrated that gene knockdown by RNAi incorporated feed results in significant increased protection of shrimp species against viral challenge (Ufaz et al. 2018). In another study, oral delivery of RNAi molecule against WSSV infection in *P. monodon* leads to reduced percentages in cumulative mortality and delayed average time of death (Thammasorn et al. 2015).

2.4.4 Vector-Based Delivery

Vectors are tools commonly used by molecular biologists to deliver genetic material into the cells. This process can be performed in both in vivo (living organism) or in vitro (cell culture) condition. Vector-based delivery methods include those derived from viruses and plasmids. Viral vectors are recognized as efficient delivery systems for RNAi technology (Qayoom and Mushtaq 2020). However, induction of strong immune response and the risk of vector integration with host genome are often associated this method. These delivery methods are still in laboratory use and require further work and validation before they can be used in commercial aquaculture.

2.5 RNA Interference (RNAi) Procedure in Crustaceans: A Case Study from *A. franciscana* Model System

2.5.1 Culture of *A. franciscana* for Microinjection

At first, the *Artemia* cysts were hydrated in distilled water for 1 h. Later, the cysts were decapsulated using sodium hydroxide (NaOH) and sodium hypochlorite NaOCl for 2 min; afterwards, the reaction was stopped by adding sodium thiosulphate ($Na_2S_2O_3$). The decapsulated cysts were washed and suspended in filtered 35 g/L autoclaved seawater (FASW) and incubated for 28 h at 28 °C for hatching with constant illumination of approximately 27μE/m^2 s. The developmental stage instar II (mouth is opened to ingest particles) hatched larvae were collected and daily fed with green algae (*Tetraselmis suecica*) until they grow to adults in a 28 °C controlled temperature room with approximately 27μE/m^2 s constant illumination.

It is important to mention that approximately after 2 weeks of *Artemia* culture, the females are manually separated and kept in a separate tank from male population to avoid the mating and fertilization.

2.5.2 Preparation of *A. franciscana* hsp70 and gfp dsRNA

The dsRNA specific to the *A. franciscana hsp70* gene transcript is amplified using gene-specific primers, and each primer included with T7 promoter (5′ TAATACGACTCACTATAGGG 3′) at their 5′ ends (forward primer, 5′ GATGCAGGTGCCATTGC 3′ and reverse primer, 5′ AGCTCCTCAAAACGGGC 3′). In addition, a green fluorescence protein (gfp) fragment was also amplified to prepare dsRNA of *gfp*, which could serve as a negative control. Here also T7 promoter was included at their 5′ ends (forward 5′ *AGA*GCGCTTCTCGTTGGGG 3′ and reverse 5′ *AGA*CCTGAAGTTCATCTGC 3′). After PCR, the purified product was incubated overnight at 37 °C with T7 enzyme mix, reaction buffer and four ribonucleotides (ATP, CTP, GTP and UTP). Afterwards, the dsRNA was incubated with RNAse or DNase I for 1 h at 37 °C for 1 h for nuclease digestion to remove any ssRNA and DNA template that did not anneal.

The dsRNA of *hsp70* and *gfp* was quantified using nanodrop spectrophotometer and further subjected to agarose gel electrophoresis (1.5%) in order to check the efficiency and integrity of duplex formation.

2.5.3 Microinjection of Artemia Females with dsRNA

The *hsp70* and *gfp* dsRNA were mixed separately in 1:10 ratio (v/v) with phenol red (0.5%) in Dulbecco's phosphate-buffered saline (DPBS). The diluted *hsp70* or *gfp* dsRNA (250 nL of solution containing approximately 80 ng dsRNA) was injected to egg sacs of adult *Artemia* female with a FemtoJet® microinjector using Femtotips II microinjection capillary tips while viewing under stereomicroscope.

The females were injected with each *hsp70* and *gfp* dsRNA. Injected females were kept for 2 h, and animals which remained healthy, retained dye and could swim properly were used in the further experiments. The injected females were transferred to 6-well plate (1 female/well) containing sea water (35 ppt). In each well, a healthy adult male was transferred, and the pairs were fed daily with green algae, *Tetraselmis suecica*. The plates were maintained in a 28 °C controlled temperature room with approximately 27μE/m^2 s constant. After 5 days, larvae were collected from each mating pairs, injected with dsRNA *hsp70* and *gfp*, for further analysis.

2.5.4 Validation of RNAi Microinjection Efficiency

2.5.4.1 Survival of *A. franciscana* Larvae

The *Artemia* larvae collected from single female injected with either *hsp70* or *gfp* dsRNA were transferred to sterile falcon tubes (50 mL) containing 30 mL seawater. Subsequently, the larvae were challenged at 10^7 cells/mL with pathogenic bacterial pathogen strain. The survival of *Artemia* larvae was recorded after 48 h of pathogen addition.

2.5.4.2 Detection of *hsp70* mRNA in Brine Shrimp Larvae

The larvae from injected *Artemia* females with dsRNA either *hsp70* or *gfp* were collected in sterile falcon tubes (50 mL) containing 30 mL seawater. Subsequently, the larvae were washed with distilled water and resuspended in eppendorf tubes (1.5 mL) with RLT buffer. Later, the larvae were homogenized, and according to the manufacturer's protocols, the total RNA was isolated using RNA extraction kit. Equal amounts of larvae total RNA (determined by nanodrop spectrophotometer) were used for synthesis of cDNA, using first-strand synthesis qRT-PCR kit. Subsequently, cDNA product (5μL) was used for *hsp70* PCR amplification using set of forward and reverse primers. The PCR products were transferred in 1.5% agarose gel (w/v), stained with nucleic acid gel stain (GelRed™) and visualized by imaging system (ChemiDoc MP).

2.5.4.3 Detection of Hsp70 Protein in Brine Shrimp Larvae

As mentioned before in detection of hsp70 mRNA protocol, the larvae from dsRNA injected females were collected in sterile eppendorf tubes (1.5 mL) and homogenized in equal volume of loading buffer (5μL). Later the samples were vortexed, heated at 95 °C for 5 min, and 15μL of each sample were transferred in SDS-PAGE gel (10%). A positive control, i.e. HeLa (heat shocked) cells were loaded in one well. Later, the gels were then stained with Coomassie Biosafe to visualize the quality of samples. Subsequently, the gels are transferred to polyvinylidene fluoride membrane (PVDF) for antibody probing. At first, the membrane was incubated with blocking buffer at room temperature for 60 min and then with primary antibody (monoclonal anti-Hsp70 antibody). Later, the membrane was incubated with secondary antibody (anti-mouse IgG). Finally, western ECL substrates (chemiluminescence reagent) were added to membrane for 5 min, and the bands were visualized in imaging system (ChemiDoc MP).

2.6 Conclusion and Future Perspective

RNAi has undergone an information explosion in the last two decades, and it is now established that as much as 5% of the human genome is dedicated to encoding and producing, the >1000 microRNAs (miRNAs) regulate at least 30% of our genes. It is now understood that RNAi is a promising tool that controls the vital processes of a biological organism, such as cell growth, tissue differentiation, heterochromatin formation and cell proliferation. RNAi can successfully result in the knockdown of single or multiple genes, providing a quick and convenient method to analyse the gene function. More specifically, small interfering RNAs (siRNA) of ~20−22 bp dsRNA molecules having a characteristic 2 nt 3′-overhangs allow recognition and subsequent binding of RNAi machinery, ultimately leading to a homology-dependent degradation of the target indigenous mRNA. The possibilities of dsRNA molecules production are evolving from in vitro to in vivo synthesis with lower cost, allowing more practical applications in broader scale. Additionally, the newly developed dsRNA delivery techniques allow manipulations at the cellular and

tissue level especially, and studies have shown initial success in dsRNA delivery simultaneously on many individuals. RNAi can now be applied to control the sex of a population and has shown possibility for widespread application, enhancing the animal production and prevention of microbial diseases in aquatic animals. In conclusion, RNAi technology promises great potential for use in both research and applied sciences and could become a core component in aquatic animal health management.

Acknowledgements Authors are thankful to ICAR-Central Inland Fisheries Research Institute (ICAR-CIFRI) for ample help and support.

References

Akhtar S, Patnaik SR, Kotapati Raghupathy R, Al-Mubrad TM, Craft JA, Shu X (2015) Histological characterization of the Dicer1 mutant zebrafish retina. J Ophthalmol 2015:1–8. https://doi.org/10.1155/2015/309510

Andrews OE, Cha DJ, Wei C, Patton JG (2014) RNAi-mediated gene silencing in zebrafish triggered by convergent transcription. Sci Rep 4:1–8. https://doi.org/10.1038/srep05222

Arenal A, Pimentel R, Guimarais M, Rodriguez A, Martinez R, Abad Z (2000) Gene transfer in shrimp (Litopenaeus schmitti) by electroporation of single-cell embryos and injection of naked DNA into adult muscle. Biotecnol Apl 17:247–250

Attasart P, Kaewkhaw R, Chimwai C, Kongphom U, Namramoon O, Panyim S (2010) Inhibition of Penaeus monodon densovirus replication in shrimp by double-stranded RNA. Arch Virol 155:825–832. https://doi.org/10.1007/s00705-010-0649-5

Boonanuntanasarn S, Yoshizaki G, Takeuchi T (2003) Specific gene silencing using small interfering RNAs in fish embryos. Biochem Biophys Res Commun 310:1089–1095. https://doi.org/10.1016/j.bbrc.2003.09.127

Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136:642–655. https://doi.org/10.1016/j.cell.2009.01.035

Chen Y-H, Jia X-T, Zhao L, Li C-Z, Zhang S, Chen Y-G, Weng S-P, He J-G (2011) Identification and functional characterization of Dicer2 and five single VWC domain proteins of Litopenaeus vannamei. Dev Comp Immunol 35:661–671. https://doi.org/10.1016/j.dci.2011.01.010

Chimwai C, Tongboonsong P, Namramoon O, Panyim S, Attasart P (2016) A formulated double-stranded RNA diet for reducing Penaeus monodon densovirus infection in black tiger shrimp. J Invertebr Pathol 134:23–26. https://doi.org/10.1016/j.jip.2016.01.003

Da'dara AA, Skelly PJ (2015) Gene suppression in schistosomes using RNAi. In: Peacock C (ed) Parasite genomics protocols. Springer, New York, pp 143–164. https://doi.org/10.1007/978-1-4939-1438-8_8

De Santis C, Wade NM, Jerry DR, Preston NP, Glencross BD, Sellars MJ (2011) Growing backwards: an inverted role for the shrimp ortholog of vertebrate myostatin and GDF11. J Exp Biol 214:2671–2677. https://doi.org/10.1242/jeb.056374

Dechklar M, Udomkit A, Panyim S (2008) Characterization of Argonaute cDNA from Penaeus monodon and implication of its role in RNA interference. Biochem Biophys Res Commun 367:768–774. https://doi.org/10.1016/j.bbrc.2008.01.031

Elkayam E, Kuhn CD, Tocilj A, Haase AD, Greene EM, Hannon GJ, Joshua-Tor L (2012) The structure of human argonaute-2 in complex with miR-20a. Cell 150:100–110. https://doi.org/10.1016/j.cell.2012.05.017

Fabian MR, Sonenberg N, Filipowicz W (2010) Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79:351–379. https://doi.org/10.1146/annurev-biochem-060308-103103

Fagutao FF, Koyama T, Kaizu A, Saito-Taki T, Kondo H, Aoki T, Hirono I (2009) Increased bacterial load in shrimp hemolymph in the absence of prophenoloxidase. FEBS J 276:5298–5306. https://doi.org/10.1111/j.1742-4658.2009.07225.x

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806–811

Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an expanding universe. Nat Rev Genet 10:94–108. https://doi.org/10.1038/nrg2504.Small

Gong Y, Zhang X (2021) RNAi-based antiviral immunity of shrimp. Dev Comp Immunol 115:103907. https://doi.org/10.1016/j.dci.2020.103907

Gong Y, Ju C, Zhang X (2018) Shrimp miR-1000 functions in antiviral immunity by simultaneously triggering the degradation of two viral mRNAs. Front Immunol 9:2999. https://doi.org/10.3389/fimmu.2018.02999

Hammond SM, Caudy AA, Hannon GJ (2001) Post-transcriptional gene silencing by double-stranded RNA. Nat Rev Genet 2:110–119

Han B, Kaur VI, Baruah K, Nguyen VD, Bossier P (2019) High doses of sodium ascorbate act as a prooxidant and protect gnotobiotic brine shrimp larvae (Artemia franciscana) against Vibrio harveyi infection coinciding with heat shock protein 70 activation. Dev Comp Immunol 92:69–76. https://doi.org/10.1016/J.DCI.2018.11.007

He Y, Ju C, Zhang X (2015) Roles of small RNAs in the immune defense mechanisms of crustaceans. Mol Immunol 68:399–403. https://doi.org/10.1016/j.molimm.2015.07.008

Hipolito SG, Shitara A, Kondo H, Hirono I (2014) Role of Marsupenaeus japonicus crustin-like peptide against Vibrio penaeicida and white spot syndrome virus infection. Dev Comp Immunol 46:461–469. https://doi.org/10.1016/j.dci.2014.06.001

Ketting RF (2011) The many faces of RNAi. Dev Cell 20:148–161. https://doi.org/10.1016/j.devcel.2011.01.012

Kim DH, Rossi JJ (2008) RNAi mechanisms and applications. BioTechniques 44:613–616. https://doi.org/10.2144/000112792

Kretov DA, Walawalkar IA, Mora-Martin A, Shafik AM, Moxon S, Cifuentes D (2020) Ago2-dependent processing allows miR-451 to evade the global MicroRNA turnover elicited during erythropoiesis. Mol Cell 78:317–328.e6. https://doi.org/10.1016/j.molcel.2020.02.020

Kumar V (2020) Acute hepatopancreatic necrosis disease (AHPND) in shrimp: virulence. University of Ghent, pathogenesis and mitigation strategies

Kumar, Roy S, Meena DK, Sarkar UK (2016) Application of probiotics in shrimp aquaculture: importance, mechanisms of action, and methods of administration. Rev Fish Sci Aquac 24. https://doi.org/10.1080/23308249.2016.1193841

Kumar V, Baruah K, Nguyen DV, Smagghe G, Vossen E, Bossier P (2018) Phloroglucinol mediated Hsp70 production in crustaceans : protection against Vibrio parahaemolyticus in Artemia franciscana and Macrobrachium rosenbergii. Front Immunol 9:1091. https://doi.org/10.3389/fimmu.2018.01091

Kumar V, De Bels L, Couck L, Baruah K, Bossier P, Van den Broeck W (2019a) PirABVP toxin binds to epithelial cells of the digestive tract and produce pathognomonic AHPND lesions in germ-free Brine Shrimp. Toxins (Basel) 11:717. https://doi.org/10.3390/toxins11120717

Kumar V, Viet D, Baruah K, Bossier P (2019b) Probing the mechanism of VP AHPND extracellular proteins toxicity purified from Vibrio parahaemolyticus AHPND strain in germ-free Artemia test system. Aquaculture 504:414–419. https://doi.org/10.1016/j.aquaculture.2019.02.029

Kumar V, Roy S, Baruah K, Van Haver D, Impens F, Bossier P (2020a) Environmental conditions steer phenotypic switching in acute hepatopancreatic necrosis disease-causing Vibrio parahaemolyticus, affecting PirAVP/PirBVP toxins production. Environ Microbiol 22:4212–4230. https://doi.org/10.1111/1462-2920.14903

Kumar V, Roy S, Behera BK, Bossier P, Das BK (2021) Acute hepatopancreatic necrosis disease (AHPND): virulence, pathogenesis and mitigation strategies in shrimp aquaculture. Toxins 13 (8):524

Kumar V, Wille M, Lourenço TM, Bossier P (2020b) Biofloc-based enhanced survival of Litopenaeus vannamei upon AHPND-causing Vibrio parahaemolyticus challenge is partially mediated by reduced expression of its virulence genes. Front Microbiol 11. https://doi.org/10.3389/fmicb.2020.01270

Kumar V, Baruah K, Bossier P (2021) Bamboo powder protects gnotobiotically-grown brine shrimp against AHPND-causing Vibrio parahaemolyticus strains by cessation of PirAB VP toxin secretion. Aquaculture 736624. https://doi.org/10.1016/j.aquaculture.2021.736624

Labreuche Y, Veloso A, de la Vega E, Gross PS, Chapman RW, Browdy CL, Warr GW (2010) Non-specific activation of antiviral immunity and induction of RNA interference may engage the same pathway in the Pacific white leg shrimp Litopenaeus vannamei. Dev Comp Immunol 34:1209–1218. https://doi.org/10.1016/j.dci.2010.06.017

Li X, Yang L, Jiang S, Fu M, Huang J, Jiang S (2013) Identification and expression analysis of Dicer2 in black tiger shrimp (Penaeus monodon) responses to immune challenges. Fish Shellfish Immunol 35:1–8. https://doi.org/10.1016/j.fsi.2013.03.370

Lingel A, Izaurralde E (2004) RNAi: finding the elusive endonuclease. RNA 10:1675–1679. https://doi.org/10.1261/rna.7175704

Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431:343–349. https://doi.org/10.1038/nature02873

Nguyen DV, Christiaens O, Bossier P, Smagghe G (2016) RNA interference in shrimp and potential applications in aquaculture. Rev Aquac 1–12. https://doi.org/10.1111/raq.12187

Ohashi H, Umeda N, Hirazawa N, Ozaki Y, Miura C, Miura T (2007) Expression of vasa (vas)-related genes in germ cells and specific interference with gene functions by double-stranded RNA in the monogenean, Neobenedenia girellae. Int J Parasitol 37:515–523. https://doi.org/10.1016/j.ijpara.2006.11.003

Ongvarrasopone C, Saejia P, Chanasakulniyom M, Panyim S (2011) Inhibition of Taura syndrome virus replication in Litopenaeus vannamei through silencing the LvRab7 gene using double-stranded RNA. Arch Virol 156:1117–1123. https://doi.org/10.1007/s00705-011-0952-9

Phetrungnapha A, Ho T, Udomkit A, Panyim S, Ongvarrasopone C (2013) Molecular cloning and functional characterization of Argonaute-3 gene from Penaeus monodon. Fish Shellfish Immunol 35:874–882. https://doi.org/10.1016/j.fsi.2013.06.025

Qayoom U, Mushtaq Z (2020) RNAi technology in fish and shellfish-status and prospects: a review. Agric Rev:1–9. https://doi.org/10.18805/ag.r-2041

Reshi ML, Wu JL, Wang HV, Hong JR (2014) RNA interference technology used for the study of aquatic virus infections. Fish Shellfish Immunol 40:14–23. https://doi.org/10.1016/j.fsi.2014.06.008

Roy S (2020) Modulating innate immune memory in brine shrimp (Artemia franciscana) and in giant freshwater prawn (Macrobrachium rosenbergii). University of Ghent

Roy S, Kumar V, Bossier P, Norouzitallab P, Vanrompay D (2019) Phloroglucinol treatment induces transgenerational epigenetic inherited resistance against vibrio infections and thermal stress in a brine shrimp (Artemia franciscana) model. Front Immunol 10:2745. https://doi.org/10.3389/fimmu.2019.02745

Roy S, Bossier P, Norouzitallab P, Vanrompay D (2020) Trained immunity and perspectives for shrimp aquaculture. Rev Aquac. https://doi.org/10.1111/raq.12438

Saksmerprome V, Charoonnart P, Gangnonngiw W, Withyachumnarnkul B (2009) A novel and inexpensive application of RNAi technology to protect shrimp from viral disease. J Virol Methods 162:213–217. https://doi.org/10.1016/j.jviromet.2009.08.010

Saksmerprome V, Thammasorn T, Jitrakorn S, Wongtripop S, Borwornpinyo S, Withyachumnarnkul B (2013) Using double-stranded RNA for the control of Laem-Singh virus (LSNV) in Thai P. monodon. J Biotechnol 164:449–453. https://doi.org/10.1016/j.jbiotec.2013.01.028

Sarathi M, Simon MC, Venkatesan C, Hameed ASS (2008) Oral administration of bacterially expressed VP28dsRNA to protect Penaeus monodon from white spot syndrome virus. Mar Biotechnol 10:242–249. https://doi.org/10.1007/s10126-007-9057-6

Schirle NT, Kinberger GA, Murray HF, Lima WF, Prakash TP, MacRae IJ (2016) Structural analysis of human Argonaute-2 bound to a modified siRNA guide. J Am Chem Soc 138:8694–8697. https://doi.org/10.1021/jacs.6b04454

Schuster S, Miesen P, van Rij RP (2019) Antiviral RNAi in insects and mammals: parallels and differences. Viruses 11:1–32. https://doi.org/10.3390/v11050448

Su J, Oanh DTH, Lyons RE, Leeton L, van Hulten MCW, Tan S-H, Song L, Rajendran KV, Walker PJ (2008) A key gene of the RNA interference pathway in the black tiger shrimp, Penaeus monodon: identification and functional characterisation of Dicer-1. Fish Shellfish Immunol 24:223–233. https://doi.org/10.1016/j.fsi.2007.11.006

Su J, Zhu Z, Wang Y, Jang S (2009) Isolation and characterization of Argonaute 2: a key gene of the RNA interference pathway in the rare minnow, Gobiocypris rarus. Fish Shellfish Immunol 26:164–170. https://doi.org/10.1016/j.fsi.2008.10.002

Thammasorn T, Sangsuriya P, Meemetta W, Senapin S, Jitrakorn S, Rattanarojpong T, Saksmerprome V (2015) Large-scale production and antiviral efficacy of multi-target double-stranded RNA for the prevention of white spot syndrome virus (WSSV) in shrimp. BMC Biotechnol 15:5–8. https://doi.org/10.1186/s12896-015-0226-9

Timmons L, Fire A (1998) Specific interference by ingested dsRNA. Nature 395:854

Tirasophon W, Yodmuang S, Chinnirunvong W, Plongthongkum N, Panyim S (2007) Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antivir Res 74:150–155. https://doi.org/10.1016/j.antiviral.2006.11.002

Tran PTN, Kumar V, Bossier P (2020) Do acute hepatopancreatic necrosis disease-causing PirABVP toxins aggravate vibriosis? Emerg Microbes Infect 9:1919–1932. https://doi.org/10.1080/22221751.2020.1811778

Tseng FS, Tsai HJ, Liao IC, Song YL (2000) Introducing foreign DNA into tiger shrimp (Penaeus monodon) by electroporation. Theriogenology 54:1421–1432. https://doi.org/10.1016/S0093-691X(00)00464-7

Ufaz S, Balter A, Tzror C, Einbender S, Koshet O, Shainsky-Roitman J, Yaari Z, Schroeder A (2018) Anti-viral RNAi nanoparticles protect shrimp against white spot disease. Mol Syst Des Eng 3:38–48. https://doi.org/10.1039/C7ME00092H

Ventura T, Sagi A (2012) The insulin-like androgenic gland hormone in crustaceans: from a single gene silencing to a wide array of sexual manipulation-based biotechnologies. Biotechnol Adv 30:1543–1550. https://doi.org/10.1016/j.biotechadv.2012.04.008

Ventura T, Manor R, Aflalo ED, Weil S, Raviv S, Glazer L, Sagi A (2009) Temporal silencing of an androgenic gland-specific insulin-like gene affecting phenotypical gender differences and spermatogenesis. Endocrinology 150:1278–1286. https://doi.org/10.1210/en.2008-0906

Vikash Kumar, Suvra Roy, Bijay Kumar Behera, Peter Bossier, Basanta Kumar Das, (2021) Acute Hepatopancreatic Necrosis Disease (AHPND): Virulence, Pathogenesis and Mitigation Strategies in Shrimp Aquaculture. Toxins 13 (8):524

Wang S, Chen AJ, Shi LJ, Zhao XF, Wang JX (2012) TRBP and eIF6 homologue in Marsupenaeus japonicus play crucial roles in antiviral response. PLoS One 7:1–10. https://doi.org/10.1371/journal.pone.0030057

Wang PH, Wan DH, Chen YG, Weng SP, Yu XQ, He JG (2013) Characterization of four novel caspases from Litopenaeus vannamei (Lvcaspase2-5) and their role in WSSV infection through dsRNA-mediated gene silencing. PLoS One 8:1–14. https://doi.org/10.1371/journal.pone.0080418

Westenberg M, Heinhuis B, Zuidema D, Vlak JM (2005) siRNA injection induces sequence-independent protection in Penaeus monodon against white spot syndrome virus. Virus Res 114:133–139. https://doi.org/10.1016/j.virusres.2005.06.006

Yan H, Zhang S, Li C-Z, Chen Y-H, Chen Y-G, Weng S-P, He J-G (2013) Molecular characterization and function of a p38 MAPK gene from Litopenaeus vannamei. Fish Shellfish Immunol 34:1421–1431. https://doi.org/10.1016/j.fsi.2013.02.030

Yang L, Li X, Huang J, Zhou F, Su T, Jiang S (2013) Isolation and characterization of homologous TRBP cDNA for RNA interference in Penaeus monodon. Fish Shellfish Immunol 34:704–711. https://doi.org/10.1016/j.fsi.2012.11.022

Yang L, Li X, Jiang S, Qiu L, Zhou F, Liu W, Jiang S (2014) Characterization of Argonaute2 gene from black tiger shrimp (Penaeus monodon) and its responses to immune challenges. Fish Shellfish Immunol 36:261–269. https://doi.org/10.1016/j.fsi.2013.11.010

Yao X, Wang L, Song L, Zhang H, Dong C, Zhang Y, Qiu L, Shi Y, Zhao J, Bi Y (2010) A Dicer-1 gene from white shrimp Litopenaeus vannamei: expression pattern in the processes of immune response and larval development. Fish Shellfish Immunol 29:565–570. https://doi.org/10.1016/j.fsi.2010.05.016

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, Panyim S (2006) YHV-protease dsRNA inhibits YHV replication in Penaeus monodon and prevents mortality. Biochem Biophys Res Commun 341:351–356. https://doi.org/10.1016/j.bbrc.2005.12.186

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101:25–33. https://doi.org/10.1016/S0092-8674(00)80620-0