Insights into the antiviral functions of the RNAi machinery in penaeid shrimp

Yannick Labreuche[a], *, Gregory W. Warr[b, c]

[a] IFREMER, Département Lagons, Ecosystèmes et Aquaculture Durable en Nouvelle-Calédonie, BP 2059, 98846 Nouméa Cedex, New Caledonia, France
[b] Division of Molecular and Cellular Biosciences, National Science Foundation, Arlington, VA 22230, USA
[c] Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA

*: Corresponding author : Yannick Labreuche, Tel.: +33 687 352588 ; fax: +33 687 351177 ;
email addresses : ylabreuc@ifremer.fr ; yannick.labreuche@ifremer.fr

Abstract:

Over the last decade, RNA interference pathways have emerged in eukaryotes as critical regulators of many diverse biological functions including, among others, transcriptional gene regulation, post-transcriptional gene silencing, heterochromatin remodelling, suppression of transposon activity, and antiviral defences. Although this gene silencing process has been reported to be relatively well conserved in species of different phyla, there are important discrepancies between plants, invertebrates and mammals. In penaeid shrimp, the existence of an intact and functional RNAi machinery is supported by a rapidly growing body of evidence. However, the extent to which this process participates to the host immune responses remains poorly defined in this non-model organism. This review summarizes our current knowledge of RNAi mechanisms in shrimp and focuses on their implication in antiviral activities and shrimp immune defences.

Keywords: Shrimp ; Innate immune response ; In vertebrate immunity ; RNA interference ; Virus

1. Introduction

Shrimp are infected by more than 20 virus-caused diseases, these viruses being classified within more than 10 families that are quite diverse in their genetic material, infection cycles, geographic distribution, host range and pathogenesis (reviewed in [1, 2]). The emergence and spread of these pathogenic agents have led to considerable economic losses, threatening the economic viability and long-term sustainability of the shrimp aquaculture industry worldwide [3]. For some countries, the economic and social impacts of these pandemics have been sometimes so important that shrimp production has never fully recovered [4]. As a consequence, the need for better control and understanding of viral diseases has prompted an increasing interest in the study of the shrimp immune system, and significant advances into the molecular bases for antiviral immunity in these animals have been gained in the last years [5, 6].

Initially identified as a natural antiviral mechanism in plants, RNA interference (RNAi) has also been recognized as a key player in generating antiviral immunity in arthropods [7, 8]. In contrast to the considerable information available for insects for example, much less is known about how crustaceans respond to viral infections. In shrimp, gene silencing through RNAi was reported for the first time by Robalino and collaborators, who observed that *in vivo* administration of dsRNAs afforded in *Litopenaeus vannamei* protection against viral infection and thus demonstrated that marine invertebrates were able to display inducible antiviral immunity in response to a virus-associated molecular structure [9, 10]. Following these observations, RNAi-based applications (to down-regulate both endogenous shrimp genes and viral genes) have generated substantial enthusiasm and enabled a wide field of research in shrimp, as reflected by the increasing number of publications on this topic. Becoming a standard laboratory practice, RNAi technology has been increasingly used to unravel gene functions *in vivo*, providing new opportunities not only to understand in greater depth the nature of shrimp biological and physiological processes, but also to decipher host-pathogen interactions. Based on these approaches, the delivery of dsRNAs or siRNAs specific for viral genes into shrimp has proved feasible and highly effective to suppress viral replication of the most intensively studied viruses, namely the white spot syndrome virus (WSSV), the yellow head virus (YHV) and the Taura syndrome virus (TSV). The practical aspects of developing RNAi-based therapies for viral diseases (i.e. choice of RNAi molecules, delivery routes/methods, targeted genes, etc) have been extensively covered elsewhere [11-14] and are outside the scope of this review. Instead, we present in this article an overview of the current knowledge of the RNAi machinery in penaeid shrimp and discuss the implication of RNAi components in the shrimp response to viral infections

2. RNAi silencing pathways : a brief overview

First described in plants and nematodes [15], and subsequently in many eukaryotes, RNAi is a highly conserved nucleic acid-based mechanism, playing roles both in the regulation of endogenous gene expression as well as in antiviral defence mechanisms in plants, animals and fungi. The basic RNAi process is frequently divided into three major steps [16]. First, the engagement of this machinery is initiated by long double-stranded RNAs (dsRNAs) produced locally or taken up by the cells. These precursors are processed by a member of the Dicer family of RNase-III-like enzymes into small effector RNA duplexes (sRNAs, 20–30 nt), among which microRNAs (miRNAs) and small interfering RNAs (siRNAs) represent the two major classes. siRNAs originate from endogenous transcripts from repetitive sequences, exogenous dsRNAs (e.g., viral RNAs) or from transcripts that can form long hairpins, while miRNAs are derived from endogenous, single-stranded noncoding RNA transcripts or introns that fold into imperfect stem-loop structures. In a second step, these duplexes are unwound

and one single-stranded RNA, called the guide strand, is preferentially loaded into a multimeric protein complex, the RNA-induced silencing complex (RISC). Third, this complex, of which an Argonaute (Ago) family protein forms the catalytic core, searches the transcriptome and, depending on the sequence homology between the potential target messenger RNAs (mRNAs) and the guide strand, induces post-transcriptional gene silencing via a variety of mechanisms. While siRNAs generally fully match their mRNA targets, miRNAs can target mRNAs that are not fully complementary, inducing mRNA degradation and/or translational repression.

3. A functional RNAi pathway in shrimp

Although RNAi pathways comprise different proteins and mechanisms in species of different phyla, core RNAi components have been described in the major branches of eukaryotes, indicating that these pathways may share both evolutionarily conserved and convergent mechanisms. dsRNA-induced gene silencing has been reported in several shrimp species, suggesting the existence of an intact RNAi machinery. Because the understanding of the molecular mechanisms underlying RNAi in shrimp may help in applying RNAi-derived techniques to these organisms, research efforts have focussed in the last decade on the identification and functional characterization of RNAi pathway components (amongst which are Dicer and Argonaute) in penaeids (Table 1). Dicer proteins are a widely conserved family, present in many organisms including plants, fungi, and the Metazoa. Depending on the organism, there may be one or more Dicer-like enzymes. The plants *Arabidopsis thaliana* and *Oryza sativa* contain four and five Dicer proteins, respectively, while metazoans contain only one (*Caenorhabditis elegans* and vertebrates), two (insects and cnidarians) or five (poriferans and placozoans) Dicer genes [17-19]. To date, only one Dicer gene has been identified in *Penaeus monodon* and *Marsupenaeus japonicus*, (*PmDcr1* and *MjDcr1*, respectively) while two Dicer-like genes (*LvDcr1* and *LvDcr2*), have been cloned in *L. vannamei* [20-22]. In plants and insects, each Dicer protein is involved in the biogenesis of different types of small-silencing RNAs with distinct characteristics [23, 24]. In insects, Dicer-1 is mainly used to produce miRNAs, while Dicer-2 participates in the processing of long dsRNAs (including those from viral origin) into siRNAs [17] (Figure 1). Structural and phylogenetic analyses of the shrimp Dicer family have established that *LvDcr1*, *PmDcr1* and *MjDcr1* encode putative proteins containing between 2473 and 2482 amino acids (aa), sharing more than 95% identity and clustering with the Dicer-1 group from other invertebrates and vertebrates, while the putative protein encoded by *LvDcr2* is 1502 aa in size and shares only 30% identity with shrimp Dcr1s, grouping with the Dicer-2 proteins characterized in insects and cnidarians. Although mRNA transcripts of shrimp Dcrs have been detected in several shrimp tissues [20, 22], it still remains to be determined whether these proteins possess specialized functional activities in the processing of small effector RNA duplexes.

To recruit Argonaute proteins and form the core of the RNA-induced silencing complex, Dicer family enzymes have been shown to act in cooperation with several dsRNA binding proteins, including the HIV-1 transactivating response (TAR) RNA-binding protein (TRBP) and a protein activator of PKR (PACT) in humans [25-27] or R2D2 and Loquacious (LOQS) in *Drosophila melanogaster* [28, 29] (Figure 1). Other accessory proteins including the eukaryotic initiation factor 6 (eIF6) have also been described as components of this multiprotein complex [30]. Several new members of the TRBP family have been characterized in *Fenneropenaeus chinensis* (*Fc*-TRBP1–3) and *M. japonicus* (*Mj*-TRBP1–3) and shown to share more than 99% sequence similarity [31, 32] (Table 1). A TRBP homologue was also recently isolated in *L. vannamei* [21]. Phylogenetic analysis and sequence alignments from multiple species demonstrated that these shrimp TRBPs are highly conserved in relationship to other members of the same family, especially in terms of

dsRNA-binding domains (dsRBDs). Homologues of eIF6 (namely *Fc*-eIF6 and *Mj*-eIF6) were also cloned in *F. chinensis* and *M. japonicus* and reported to be highly similar to the eIF6 sequences in other species [31, 32]. Similar to reports in other organisms [30], direct interactions were evidenced between eIF6 and TRBP in shrimp, suggesting their association in the same protein complex. Moreover, knockdown of shrimp TRBP and eIF6 was shown to result in a substantial loss of dsRNA-mediated silencing of other target mRNAs, which is consistent with the hypothesis that these two proteins function as part of the shrimp RNAi machinery [31, 32].

Argonaute protein family members, the key components of the RISC, were the first components of a putative RNAi pathway to be identified in penaeid shrimp [33]. As is the case with the Dicer family, there may be one or more Argonaute family members within a given organism. For instance, the yeast *Schizosaccharomyces pombe* contains only one Argonaute, while five paralogues have been found in *Drosophila*, eight in humans, ten in *Arabidopsis* and twenty-seven in *C. elegans* [34] (Table 1). These Argonaute proteins can be divided into two subclasses, namely the AGO and PIWI subfamilies. Members of the Ago subfamily are ubiquitously expressed and associate with miRNAs and siRNAs, whereas the expression pattern of PIWI subfamily members is mainly restricted to the germline, explaining why this subfamily is considered to play a tissue-specific role during development rather than a more ubiquitous role in RNAi [34]. In the black tiger shrimp *P. monodon*, two isoforms (Pm-Ago and Pem-AGO) were cloned [33, 35], while *L. vannamei* was reported to contain two different Argonaute proteins (Lv-Ago 1 and Lv-Ago 2) [36]. The corresponding genes were found to be ubiquitously expressed and their deduced amino acid sequences shown to possess the two distinctive features of the AGO subfamily, i.e. a PAZ domain involved in dsRNA binding and a PIWI domain reported to display RNase activity, suggesting thus their implication in RNAi mechanisms [33, 35, 36]. A confirmation of this hypothesis was provided by the knock-down of Pem-AGO in *P. monodon*, that was reported to partially reduce ($\approx$ 50% of the original activity) the efficiency of dsRNA-mediated silencing of an endogenous shrimp gene [35]. Considering these results, it has been hypothesized that other Argonaute family members present in shrimp may be involved in this mechanism. Supporting this assumption, the study of *Lv-ago 1* and *Lv-ago 2* transcript accumulation following dsRNA injection into shrimp revealed a strong induction of *Lv-ago 2* mRNA expression only [36], while co-immunoprecipitation and pull-down assays demonstrated that Lv-Ago 2 interacts with LvDcr2 and LvTRBP1 [21]. Taken together, these data suggest that the two Argonaute proteins characterized in *L. vannamei* may play specialized functions, Lv-Ago 2 being more specifically implicated in a "siRNAi pathway". Similar observations have been previously reported in *Drosophila*, where Ago1 and Ago2-RISC are functionally distinct and allow the silencing of different types of target RNAs by distinct mechanisms. miRNAs partition thus between Ago1- and Ago2-RISC, while siRNAs associate almost exclusively with Ago2-RISC [37] (Figure 1). Whether similar mechanisms of Argonaute specialization occur in shrimp remains to be formally demonstrated.

Many other RISC-associated proteins involved in posttranscriptional regulation have been characterized in vertebrate and invertebrate animal models, among which is the Tudor Staphylococcal nuclease (TSN) [38]. A TSN homologue (PmTSN) was recently characterized in *P. monodon* [39]. Because knockdown of PmTSN only partially reduced the efficiency of dsRNA-mediated gene silencing, it has been proposed that this protein may function as a minor component affecting RISC efficiency but dispensable for RNAi in shrimp.

As indicated by the different studies presented here, considerable advances have been made in the characterization of a RNAi machinery in penaeids. However there are still many open questions to be solved. For instance, the discovery of different sets of Dicer and Argonaute proteins in *L. vannamei* suggest the potential existence of several RNAi pathways in shrimp. Whether these pathways are redundant or whether they have specialized independent functions has yet to be determined. *In vivo* investigations of gene functions at

the organism level are thus clearly needed. It is well known that transgenic and knockout animal technology are not available or are unreliable in shrimp, but it is possible, as exemplified by different research articles cited in this review [32, 35], to silence the silencing machinery itself by injection of long dsRNA for a given target (a process also known as "RNAi of RNAi") . However, silencing of some genes presumptively associated with the RNAi pathway has been sometimes shown to result in a lethal phenotype, as observed by Labreuche and collaborators in *L. vannamei* [36], suggesting that these components may be involved in functions essential for life but which remain largely uncharacterized. To begin addressing this issue, we next focus in this review on the role of the RNAi machinery in shrimp antiviral immunity.

4. RNAi as an antiviral mechanism in penaeids ?

Mammals are frequently considered to have lost antiviral silencing activities and their innate antiviral defences thus rely mainly on the interferon response [40]. In contrast, RNAi has evolved as a potent antiviral pathway in plants and invertebrates, presumably as a means of responding to viral infections through the sensing of dsRNA, a central molecular pattern found in viral genomes, in viral replication complexes or resulting from bidirectional transcription in DNA viruses. As mentioned above, two Rnase III-related Dicers initiate the miRNA and siRNA pathways in *D. melanogaster* (DCR1 and DCR2, respectively). However, recognition of viral dsRNA relies essentially and specifically on DCR2, leading to the production of virus-derived siRNAs (also designated vsiRNAs). These vsiRNAs are transferred with the help of R2D2 (a dsRNA binding protein) to AGO2, which targets the viral RNA molecules for degradation [41, 42]. Unlike *D. melanogaster*, a functional redundancy among the Dicer-like proteins (DCLs) has been reported in *A. thaliana*, DCL4 and DCL2 producing vsiRNAs against distinct positive-strand RNA viruses in a hierarchical and redundant manner [43, 44]. To decipher the functional role(s) of Dicer and Argonaute proteins in shrimp antiviral immunity, several studies have first attempted to investigate the mRNA transcript abundances of the corresponding genes following experimental viral challenges [20-22]. The underlying idea was that if these components are effector mechanisms of the shrimp antiviral defences, their expression would be modified during the course of infection. The expression of *PmDcr1* was thus investigated and shown to be transiently up-regulated at an early stage of gill-associated virus (GAV) infection (< 48 h), but returned to normal levels later and no direct correlation could be found between levels of *PmDcr1* expression and the viral loads in shrimp [22]. The expression pattern of *LvDcr1* in hemocytes and gills of shrimp challenged with TSV was somewhat similar, being up-regulated in both tissues during the first 24 h after TSV injection and subsequently decreasing [20]. In contrast to these results, no significant difference occurred at 24 h post-challenge in the expression level of *LvDcr1* mRNA between infected animals and the PBS-injected controls following WSSV challenge [45]; however, the temporal mRNA expression of *LvDcr2* increased continuously during the first 48 h after WSSV challenge [21]. Finally, the expression of *Pm Ago* mRNA was reported to be 2–3-fold increased during the early period of viral infection but thereafter declined rapidly. Interestingly, experimental infection by a bacterial pathogen did not modify *Pm Ago* transcript abundance, leading the authors to propose that its expression was specifically associated with virus infection [33]. Although the expression patterns of the shrimp Dicer and Argonaute genes in viral response studies provide the first clues for the antiviral role of their cognate protein, it is quite difficult to draw any definitive conclusion as to whether or not these proteins play a direct role in shrimp defences. Moreover, the different outcomes observed in *LvDcr1* gene expression after viral infection [20, 45] could be due to a lack of standardized challenge protocols and bioassays, to the existence of genetic variations in the viral stocks used, and/or to the use of domesticated stocks of shrimp genetically selected for tolerance to some viruses (see [46] for a review). More direct evidence has come from PmDcr1 knockdown by injection of

sequence-specific dsRNA prior to infection with low viral titres [22]. In this experimental system, higher viral loads and more rapid mortalities occurred in PmDcr1-silenced shrimp, illustrating a significant enhancement of the animal's susceptibility to viral infection. Although the *LvDcr2* expression pattern differed from that of *LvDcr1* in *L. vannamei*, both were clearly induced following viral challenge, suggesting that shrimp Dcr1 and Dcr2 may both participate in the antiviral immune response. Whether there is a certain form of functional redundancy among these Dicer proteins in shrimp (as observed in plants), or whether LvDcr1 and LvDcr2 act by distinct mechanisms, still remains to be elucidated by knockdown experiments for example. In addition, more compelling evidence is needed to better decipher the implication of shrimp Argonautes in the RNAi-mediated inhibition of viral replication. Other putative shrimp RNAi components have been investigated for their role in antiviral RNAi. Complex changes in the expression patterns of *TRBP* and *eIF6* were thus detected in *F. chinensis* in response to WSSV challenge [31]. However, the most straightforward demonstration of the implication of TRBP in antiviral immunity came from co-injection experiments of recombinantly expressed *Fc*-TRBP1 with WSSV into shrimp, that were shown to induce an inhibition of viral replication. These results were recently confirmed in *M. japonicus* by dsRNA-mediated silencing of *Mj-TRBP* and *Mj-eIF6* which was reported to increase WSSV replication, implying a crucial role of these proteins in the antiviral defence program [32].

In invertebrates, much of the understanding of antiviral RNAi derives from studies on *D. melanogaster* and *C. elegans*, two genetically tractable animal models where disruption of gene function is a classic approach in genetic analyses. In the absence of similar technology in understudied animals such as shrimp, our knowledge about the implication of the RNAi machinery in antiviral immunity remains much more scarce in comparison. However, different studies have provided several lines of evidence clearly supporting a naturally antiviral role for RNA silencing in shrimp. Research in this field is still in its infancy and needs to be further pursued to help build a global picture of the pathway(s) controlling viral infections and transmission.

5. Antiviral cellular miRNAs in shrimp?

miRNAs are generated from miRNA-encoding genes and are 21- to 23-nucleotide ssRNAs considered to be key regulators of the expression of genes with roles in many biological processes including development, cellular differentiation, proliferation, metabolism and apoptosis [47]. They thus represent an additional level of gene expression regulation between mRNA and protein translation (see [48, 49] for a review). They are transcribed as long, hairpin-containing precursors (referred to as primary miRNAs or pri-miRNAs) by RNA polymerase II and cleaved in the nucleus by the Microprocessor, a protein complex which includes the RNase III enzyme Drosha, and its binding partner known as Pasha in *D. melanogaster* or DGCR8 in humans (Figure 1). Recently, it has been demonstrated that other proteins, including Ars2 (Arsenite-resistance protein 2), physically interacts with the Microprocessor [50]. Next, the pre-miRNAs are transported to the cytoplasm, where they are further processed by a member of the Dicer family (DCL-1 in *Arabidopsis* and DCR1 in *Drosophila*) and its cofactor TRBP (or LOQS in *Drosophila*) into mature miRNAs, and finally loaded into a RISC. Because recognition by the miRISC of a target mRNA relies on the complementary base-pairing of a 6-8 nt seed region, a single miRNA can mediate translational inhibition of hundreds of proteins at once. Since the discovery of this pathway, more than 18000 miRNAs have been annotated, according to the last miRBase database release in November 2011 (http://www.mirbase.org/), in plants and animals, among which are a few aquatic crustacean species [51, 52].

In *L. vannamei*, the identification of two Argonaute and two Dicer paralogues, combined with the fact that only *Lv-ago2* expression was responsive to dsRNAs while the substrate for LvAgo1 remained enigmatic, has led to the suggestion that a miRNA pathway may also be

operating in penaeid shrimp [36]. This hypothesis was indirectly reinforced by the report that long-hairpin mRNAs were also able to promote targeted gene silencing in *P. monodon* [53]. Finally, the first evidence has come last year from a small RNA cloning approach in the kuruma shrimp *M. japonicus*, that has allowed for the first time the identification in shrimp of 35 miRNAs of which 15 were shown to display high homologies with known miRNAs from other Metazoa [54]. Following this discovery, homologues of two proteins involved in miRNA biogenesis (namely LvArs2 and LvPasha) have been characterized in *L. vannamei*, their deduced amino acid sequence sharing in both cases more than 40 % identity with their insect orthologues [55]. In *D. melanogaster*, Ars2 plays a role in several modes of RNA silencing, regulating the siRNA pathway by associating with DCR2 and modulating its activity *in vitro*, but also controlling the miRNA pathway by interacting with Pasha and stabilizing pri-miRNAs [50]. Similarly to the observations reported in this insect model, it was demonstrated by means of co-immunoprecipitation assays that the shrimp LvArs2 interacts with LvPasha and LvDcr2 [55]. Although still largely unclear, the emerging picture suggests that, as in other arthropods [56], multiple small RNA regulatory pathways function in shrimp.

In a broad range of eukaryotic species, much of the understanding of the cellular processes modulated by the miRNA pathway has focused on development. However, a role for miRNAs in the antiviral defense network has gradually been proposed [57, 58]. In *M. japonicus*, the expression level of the 35 identified miRNAs was examined upon WSSV infection. Thirteen were reported to be upregulated, and 15 downregulated after viral challenge, leading the authors to propose their putative implication in the shrimp immune response [54]. Several publications have reported in other organisms similar modifications in the host miRNA expression profile following viral infection [59-61]. The problem with such approaches is the difficulty of determining if the observed changes are elicited by the pathogen itself to enhance its infectious cycle, or instead if they occur to prevent viral replication, participating thus in the host immune responses. Indeed, it is well known that viruses have evolved multiple mechanisms to interfere with host cell regulation and render the infected cells amenable to viral replication, producing thus foreign molecules that may trigger systemic warnings and responses [62]. As stated by the authors of these studies, additional experiments have thus to be conducted to explore the individual role(s) of these shrimp miRNAs. Following the approach developed to investigate the implication of candidate genes in the RNAi antiviral response, the expression patterns of *LvArs2* and *LvPasha* were also examined in shrimp stimulated by the injection of poly(C-G), a commercial dsRNA analogue [55]. Although the mRNA transcript abundance of both genes was modified following the immune stimulation, the results are insufficient to answer the question of the implication of these components in the shrimp immune response. It is worth reiterating that functional studies based on reverse genetic approaches will be required to fully understand the mechanistic role of these proteins in shrimp physiology and immune defence.

6. dsRNA-induced antiviral immunity in penaeids

As mentioned earlier, long dsRNAs are recognized by the host cells as a pathogen-associated molecular pattern (PAMP). In vertebrates, dsRNA sensing rapidly initiates host immune defenses via complex intracellular signaling pathways which culminate in the induction of inflammatory responses mediated by various transcription factors, including IFN regulatory factors (IRFs) and nuclear factor (NF)-κB [63-65]. Similar innate responses to dsRNA were assumed for a long time to have been absent from the arthropod immune system (including that of insects, shrimp and other crustaceans), mainly because of the lack of invertebrate homologues of IFNs and IFN receptors. However, while using dsRNA-mediated gene silencing to investigate the role of specific host genes in shrimp immune response, Robalino and collaborators fortuitously observed that administration of dsRNA of arbitrary sequence also elicited in *L. vannamei* a partial protection from viral infection [9].

Similar results were subsequently reported in different shrimp species by several authors (see [13, 66, 67] for reviews), suggesting the existence of a possible evolutionary link between innate antiviral immunity in vertebrates and invertebrates. The recent characterization of an interferon-like protein in *P. japonicus* [68] and the cloning of a γ-interferon-inducible lysosomal thiol reductase homologue (an enzyme shown to be induced by interferon-γ) in *P. monodon* [69], together with the observed improved survival following "vaccination" [70, 71], further support the idea that the traditional views of innate immune responses in shrimp may need to be reconsidered.

To date, the mechanisms underlying the dsRNA-induced antiviral immunity in shrimp remain largely unknown. In vertebrates, the general non-specific antiviral response was initially considered as being initiated by long dsRNAs only, and shorther RNA duplexes (such as siRNAs) were therefore used for specific gene manipulation in order to circumvent these side effects [72]. However, it was later reported that siRNAs could also activate cells of the immune system and induce the production of cytokines [73]. In *L. vannamei*, the non-specific effect of arbitrary dsRNAs was reported to be length-dependent since only > 50 bp-dsRNAs, but not siRNAs, could generate antiviral protection during a viral challenge [10, 36]. Nevertheless, discrepancies in the ability of siRNAs to promote this side effect have also been reported in shrimp [74]. In vertebrates, the extent to which the innate immune response may be activated is known to be dependent on the siRNA sequence but also on the type of delivery system employed for cell transfection [75, 76]. It remains to be explored in shrimp whether similar considerations may applied to explain the conflicting results sometimes observed between studies.

To further understand the mechanistic details of the innate response to dsRNA in penaeids, several approaches have been explored, among which is the identification of the receptor(s) involved in dsRNA recognition. In vertebrates, antiviral responses are well-characterized and initiated through the detection of viral nucleic acids by pattern recognition receptors (PRRs) that belong to two families, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the transmembrane Toll-like receptors (TLRs), detecting viral components outside of cells as well as in cytoplasmic vacuoles after phagocytosis or endocytosis (see [77] for a review). Among the 10 TLRs identified in humans, only 4 are involved in the detection of nucleic acids derived from viruses and bacteria : TLR7 and TLR8 recognize sRNAs, TLR9 ssDNAs, while TLR3 senses dsRNAs independently of its base sequence [78]. Searches of shrimp sequences have not yet yielded homologues for RLRs, whereas a set of TLRs have been identified in *L. vannamei* [79, 80], *F. chinensis* [81], *P. monodon* [82] and *M. japonicus* [83]. It was hypothesized that these shrimp TLRs may contribute to dsRNA sensing, and the role of LvToll (later renamed LvToll1 following the characterization in *L. vannamei* of two novel Toll-like receptors, LvToll2 and LvToll3), was thus investigated by a reverse genetic approach [84]. However, the results were inconclusive. To date, most of the information about shrimp TLR functions comes mainly from correlative expression analyses. Although recent work has shown that relative expression of *LvToll1*, *LvToll2* and *LvToll3* was upregulated after viral challenge [80], it is still far from clear whether one of these receptors may act as a dsRNA sensor activating specific signaling pathway(s).

In vertebrates, once PRRs detect dsRNAs, a chain of events is activated to promote inhibition of protein synthesis, transcriptional induction of interferon and other cytokines, and ultimately, cell death. Several distinct pathways responsible for each of these events have been described and reported to display some degree of crosstalk between them [85]. For instance, the RNA-activated protein kinase (PKR), one of the most studied components of the IFN-induced antiviral pathways, is activated by dsRNAs and exerts its antiviral functions by translational regulation [86]. Interestingly, its activity has been shown to be modulated by multiple viral and cellular factors, among which are TRBP and PACT, two components of the RNA-induced silencing complex (RISC) [87]. PACT association with PKR was thus reported to activate PKR in the absence of dsRNAs [88], whereas interaction of TRBP with PKR leads

to a strong inhibition of the kinase by direct binding [89]. In shrimp, Robalino and collaborators have proposed the concept that the mechanisms responsible for dsRNA-induced innate immunity and RNAi in the antiviral response may crosstalk and interact functionally, facilitating the host antiviral defence [10, 66]. Supporting this hypothesis, it was later shown that non-specific dsRNAs trigger induction of *Lv-ago 2* mRNA expression in *L. vannamei*, suggesting the existence of potential functional overlap or link(s) between non-specific activation of antiviral immunity and the RNAi mechanism [36]. Considering the recent characterization of several shrimp TRBPs [21, 31, 32], it would be interesting to investigate whether these components may also be involved in general antiviral immunity as described in mammals. Indeed, as previously mentioned, dsRNA-mediated silencing of *Mj-TRBP* was clearly shown to promote viral replication [32]. It remains to be precisely determined if this increased number of WSSV DNA copies results solely from an impairment of the RNAi machinery through a potential destabilization of Dicer following TRBP depletion or may, to some extent, be an indirect consequence from a decreased efficiency of the non-specific antiviral immune response due to a potential dual role played by this protein.

Although a sequence-independent antiviral response has been described in insects, such as the sandfly *Lutzomyia longipalpis* [90] or the silkworm *Bombyx mori* [91], there are no similar reports to date in commonly studied model organisms with fully sequenced genomes, such as *Drosophila*. This may in part explain why the question of the mechanisms underpinning this phenomenon has raised relatively limited interest in the scientific community. The development of functional genomic and proteomic approaches in penaeid shrimp (see [5] for a review) should help obtaining new molecular information and thus fill the knowledge gap between marine invertebrates and other well-developed biological models.

7. Interplay between viral infections and the RNAi machinery

To counteract host defences, replicate in host cells and thus achieve systemic infection, viruses have evolved several original strategies that frequently rely on the subversion of the host immune signalling pathways for their own benefit. Among these strategies is the production of viral suppressors of RNAi (also known as VSRs) to inhibit various stages of the silencing process. This phenomenon was first described in plant viruses [92], but many other plant as well as animal viruses displaying the same ability have rapidly been identified (reviewed in [93, 94]). Encoded by ORFs within or overlapping with other conserved viral genes, the VSRs characterized so far are all dsRNA-binding proteins that greatly differ not only in their primary sequence and structure but also in their function, targeting different key steps of the RNAi pathway. Some VSRs have thus been reported to suppress siRNA production, while others act by either sequestering siRNAs to prevent their incorporation into the RISC or preventing short- and long-distance spread of the RNA silencing effect. Finally, VSRs have also been proposed to alter the function of host miRNAs (reviewed in [93, 95]).

Given the suspected importance of RNAi as an antiviral defence mechanism in shrimp, it may be hypothesized that shrimp viruses may similarly encode VSRs to counteract the host antiviral responses. This assumption was verified quite recently in *L. vannamei* when Robalino and collaborators reported for the first time the inhibition of dsRNA-mediated silencing in WSSV-infected shrimp [66]. Interestingly, this inhibitory effect was shown to take place preferentially in certain tissues such as the hepatopancreas, an organ considered as a primary site for the production of immune response factors [96]. Although it occurred more occasionally, a similar suppression of gene silencing was also observed by the authors in the hepatopancreas of TSV-infected shrimp. To date, the exact mechanisms supporting the VSR activities of these two viruses remain to be determined. Moreover, additional experiments are needed to investigate whether other shrimp viruses may display the same ability. For instance, the Mourilyan virus (MoV), that infects *P. monodon* and *M. japonicus* shrimp in

Australia, comprises 4 RNA segments, one of which encodes a putative small non structural protein (NSs2) [97]. Because bioinformatic analyses showed that NSs2 is similar in size to the NSs proteins of several arthropod–borne bunyaviruses known to display RNAi suppression activity, a similar function was suspected in shrimp. However, knockdown of MoV NSs2 expression did not reduce the viral loads nor modify targeted silencing of other genes [98]. Further studies are thus needed to decipher the NSs2 function in MoV pathogenesis. Criteria to predict RNA silencing suppressor activity in viral proteins have been recently provided based on the characteristics possessed by VSRs of plant and animals viruses [93, 99]. They include the presence of dsRNA-binding domains as well as the detection of glycine-tryptophane/tryptophane-glycine repeats (GW/WG) within the protein sequence. In depth analyses of available sequenced genomes from shrimp viruses may thus represent a first step toward the identification of potential VSR activities in shrimp viruses.

8. Concluding remarks

Although the lack of whole genome sequences in penaeid shrimp represents a serious impediment to the understanding of the molecular immune responses, an increasing number of sequence information is now available in public databases, allowing in the past few years the identification of several core components of the machinery for RNA-mediated silencing in shrimp. However, our knowledge of this biological system is still far from complete and many important questions remain to be addressed in the future: how many types of RNAi pathways are there exactly in shrimp ? What are their roles ? How these pathways are regulated ? Do they cooperate ? The rapid development of functional genomics and proteomics in shrimp should undoubtedly help to discover new players of silencing pathway(s) and decipher their biological implication(s).

One other major challenge in shrimp will be to understand how these RNAi components participate in the fine-tuning of innate immune signaling pathways. Despite the evidence presented here for the important role of RNAi in antiviral defences, additional experiments supporting this scenario are needed. One of the landmarks of the antiviral RNAi response is the production of virus-derived siRNAs from viral dsRNAs. It would be particularly interesting to determine whether infected animal's tissues contain significant amounts of vsiRNAs, since this would indicate that viruses are both inducers and targets of the shrimp antiviral silencing machinery.

Finally, it is clear that shrimp immune responses, like all complex cellular phenomena, will need to be understood not just in terms of the properties of the individual molecular components involved, but ultimately in terms of the interactions of those components in the integrated functional networks that determine the behavior of cells. Such approaches are in their infancy in shrimp [100-102] but should ultimately provide a basis for generating predictive hypotheses to guide future research in crustacean host-virus interactions.

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Figure

Figure 1. Schematic representation of the miRNA and siRNA pathways in *Drosophila* and comparison with a putative model for RNA interference mechanisms in penaeid shrimp.
In flies, the miRNA and siRNA pathways are substantially separated. Small RNA duplexes are produced by different Dicer enzymes (Dcr1 and Dcr2) and predominantly sorted to functionally distinct Argonautes (AGO1 and AGO2) [37], although recent studies have revealed some level of cross talk between these pathways [28]. In shrimp, it remains to be determined whether LvDcr1 and LvDcr2 possess such specialized functional activities in the processing of small effector RNA duplexes. Previous studies have established that LvAgo2 physically interacts with LvDcr2 and LvTRBP [21], suggesting that they may belong to the same molecular pathway. Because knockdown of shrimp TRBP impairs dsRNA-mediated silencing of other target mRNAs [32], and considering the fact that *Lvago2* expression only responds to dsRNAs [36], we propose in this model that these proteins may be required for gene silencing and the generation of siRNAs from exogenous dsRNAs.
In flies, endogenous primary-miRNA (pri-miRNA) transcripts are processed into precursor miRNAs (pre-miRNAs) in the nucleus by a protein complex formed by Drosha and its binding partners (Pasha and Ars2). The pre-miRNAs are subsequently exported in the cytoplasm and cleaved by Dcr1 into mature miRNAs, which are finally incorporated into an AGO1-dependent RISC to mediate translational repression of target transcripts. Homologues of proteins involved in miRNA biogenesis (LvArs2 and LvPasha) have been identified in shrimp [55], and several miRNAs characterized [54]. Moreover, LvArs2 have been shown to interact with LvPasha and LvDcr2 [55], suggesting that this protein may also function in the production of miRNAs, similar to the role played by its homologue in flies. However, it remains to be formally demonstrated whether a miRNA pathway may also be operating in this organism.
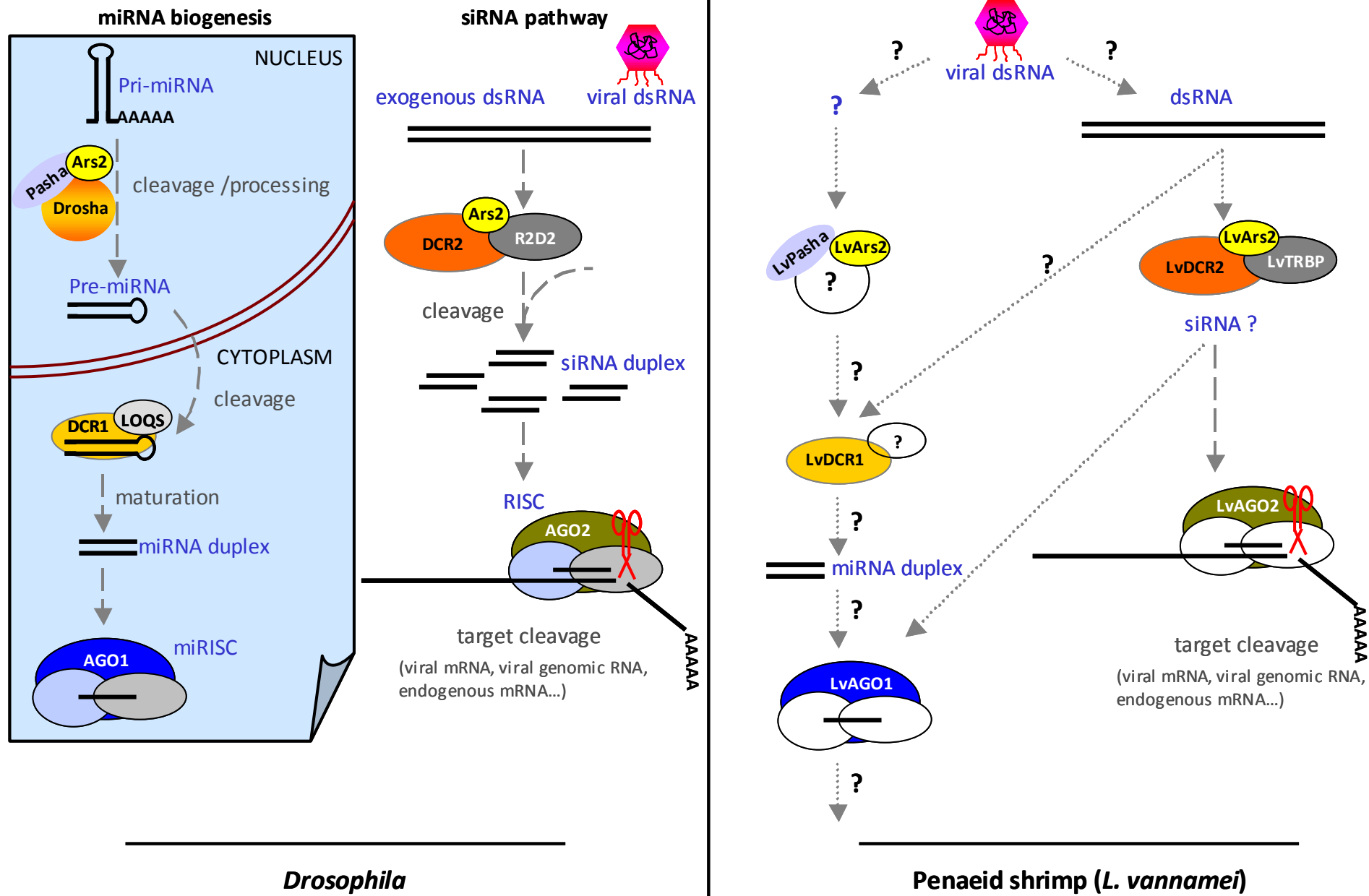
Figure 1

Table

Table 1. Key proteins implicated in RNAi pathways of various organisms and known homologues in shrimp (modified from [16]). Nd: no avalaible data. Abbreviations: AGO, Argonaute; ALG, Argonaute-like; AUB, Aubergine; DCL, Dicer-like; DCR, Dicer; DGCR8, DiGeorge Critical Region 8, DRSH, drosha; ERGO, Endogenous RNAi deficient Argonaute; HYL-1, hyponastic leaves-1; LOQS, loquacious; PACT, protein activator of PKR; PASH, pasha; PRG, piwi-related gene; RDE-1, RNAi-deficient-4; TRBP, transactivating response (TAR) RNA-binding protein.

protein	mode of action	Plant	Nematode	Fruitfly	Human	Penaeid shrimp			
						P. monodon	*L. vannamei*	*M. japonicus*	*F. chinensis*
Rnase III	production of small RNAs in the RNAi and miRNA pathways	DCL1 DCL2 DCL3 DCL4	DCR-1 DRSH-1	DCR-1 DCR-2 DROSHA	DICER1 DROSHA	Pm Dcr1	LvDcr1 LvDcr2	MjDcr1	nd
Argonautes AGO subfamily	components of RISC	AGO 1-7 AGO10 3 others	ALG-1 ALG-2 3 others	AGO1 AGO2	AGO 1- 4	Pm-Ago / PemAGO (isoform)	Lv-Ago1 Lv-Ago2	MjAgo	nd
PIWI subfamily	germ cell development	None	ERGO-1 PRG-1 PRG-2	AGO3 PIWI AUB	HILI HIWI HIWI2 PIWIL3	nd	nd	nd	nd
dsRNA-binding domain-containing cofactor of Rnase III	stabilizing Dicer	HYL1	PASH-1 RDE-4	PASHA R2D2 LOQS	DGCR8 TRBP PACT	nd	LvTRBP LvPasha	Mj-TRBP	Fc-TRBP
Arsenite resistance gene (Ars2)	enhancing Drosha processing	SERRATE	E01A2.2	DmArs2	Ars2	nd	LvArs2	nd	nd