

## Non-specific activation of antiviral immunity and induction of RNA interference may engage the same pathway in the Pacific white leg shrimp *Litopenaeus vannamei*<sup>☆</sup>

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### Abstract:

Many questions remain unanswered regarding RNAi-based mechanisms and dsRNA-induced antiviral immune responses in penaeid shrimp. In this study, we report the characterization in the white leg shrimp *Litopenaeus vannamei* of RNAi pathway associated proteins Lv-Ago 1 and Lv-Ago 2, two members of the Argonaute family of proteins, as well as Lv-sid 1, the first shrimp homologue of Sid-1, a membrane channel-forming protein implicated in the cellular import of dsRNA. To decipher their functional implication in RNAi-related phenomena, we monitored their relative expression following stimulation by specific and non-specific RNA duplexes of diverse length. The findings show that the length of small RNA duplexes plays a critical role in the activation of both RNAi-related and innate antiviral responses. They also suggest that these two mechanisms of antiviral response may activate the same pathway, requiring Lv-Sid 1 and Lv-Ago 2 induction.

**Keywords:** Shrimp; *Litopenaeus vannamei*; RNAi; dsRNA; Argonaute; Sid-1; Antiviral immunity

# 1. Introduction

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RNA interference (RNAi) is a nucleic acid-based mechanism widely conserved among all higher eukaryotes studied so far, that mediates sequence-specific targeted gene silencing. This process is initiated by double-stranded RNA (dsRNA), which is processed by a member of the Dicer family of RNase-III-like enzymes into small effector RNA duplexes (e.g. short-interfering RNAs or siRNAs, microRNAs or miRNAs, etc). The siRNAs are incorporated into a multimeric protein complex, the RNA-induced silencing complex (RISC) and related complexes, of which an Argonaute (Ago) family protein forms the catalytic core. The incorporated RNA then directs the targeted sequence-specific degradation, translational repression, and other silencing phenomena by means of complementary base-pairing. The RNAi mechanism is involved in a variety of biological phenomena including developmental processes (Grishok et al., 2001), heterochromatin remodelling (Riddle and Elgin, 2008), suppression of transposon activity (Aravin et al., 2007) and antiviral immunity (Li and Ding, 2005). Because dsRNA or siRNAs can be supplied exogenously to trigger specific gene silencing, RNAi has rapidly become the most widely used gene-silencing tool in a broad variety of eukaryotic organisms (Campbell and Choy, 2005).

In penaeid shrimp, exploiting this process is becoming increasingly important as an experimental tool to unravel gene function *in vivo*. This is exemplified by the increasing number of studies which have recently resolved gene functions involved in molting (Hui et al., 2008), osmo-regulation (Tiu et al., 2007), reproduction (Treerattrakool et al., 2008), glucose metabolism (Lugo et al., 2006) or immune responses (de la Vega et al., 2008; Shockey et al., 2009) in shrimp by using gene-specific dsRNA technology. RNAi-based applications have also offered new opportunities for experimental blockade of viral infections in shrimp by injecting animals with virus-specific RNA duplexes (as first described by (Robalino et al., 2005), providing thus a potential approach for virus control in the shrimp farming industry (Shekhar and Lu, 2009). An additional promising avenue of this technology is the observed partial protection from viral infection induced by dsRNA of diverse length, sequence, and base composition (Robalino et al., 2004). Development of methods for induction of this dsRNA-induced innate immunity could be of great interest for aquaculture.

The existence of an intact RNAi machinery in shrimp was first supported by the identification of RNAi pathway homologues such as Pem-AGO in the black tiger shrimp *P. monodon* (Dechklar et al., 2008) and Pm-Ago, another isoform of the Argonaute protein family (Unajak et al., 2006), as well as Pm Dcr1, a member of the Dicer family (Su et al., 2008). However, although a substantially impaired RNAi was observed in *Pem-ago*-depleted cells, suggesting its functional involvement in the silencing process (Dechklar et al., 2008), many questions remain unanswered regarding RNAi-based mechanism in penaeids. First, the core of this machinery and its regulation remain poorly understood. Second, it is still not known whether cross-talk and/or interactions occur between RNAi and the sequence-independent antiviral immunity observed following dsRNA injections. Finally, important efficiency discrepancies to promote gene-silencing have been reported following siRNA injections ( Li et al., 2007; Robalino et al., 2005; Westenberg et al., 2005; Wu et al., 2007; Wu et al. 2008). These inconsistencies need to be explored to improve efficacy in RNAi-based applications in shrimp aquaculture.

This study was therefore aimed at characterizing RNAi-pathway associated components in the white leg shrimp *L. vannamei*. Specifically, the present work was designed i) to decipher the functional implication of these components in both innate and RNAi-related phenomena by monitoring their relative expression following stimulation by small RNA

duplexes and ii) to determine the effect of dsRNA length on targeted genetic interference and general antiviral protection.

## 2. Materials and methods

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### 2.1. Animals and RNA extractions

*L. vannamei* shrimp from specific pathogen-free (SPF) lines were used for all experiments. Gills were collected in RNA *later* reagent (Ambion) and stored at -20°C until use. Total RNA was extracted using RNeasy columns (Qiagen) according to the manufacturer's instructions. RNA quantity, purity and integrity were verified spectrophotometrically ( $A_{260}/A_{280}$ ) and by electrophoresis on 1% agarose gels.

### 2.2. Cloning of *Lv-ago 1*, *Lv-ago 2* and *Lv-sid 1* full-length cDNAs

To isolate a cDNA of *Lv-ago 1*, specific primers (G-3524 and G-3525, Table 1) were designed on a consensus sequence of Argonaute proteins and used for PCR amplification from gill cDNA. PCR was performed as follows: initial denaturation at 95°C for 5 min; followed by 30 cycles at 95°C for 30 s; 52°C for 1 min; 72°C for 1 min. The full-length cDNA of *Lv-ago 1* was obtained by performing 5'- and 3'-RACE-PCR with the SMART™ RACE cDNA Amplification kit (Clontech/BD Biosciences) using the supplied universal primer mix in combination with either 5'RACE-primer G-3597 or 3'RACE-primer G-3595 (Table 1). Amplification profiles for RACE-PCR consisted of 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 3 min.

Based on *L. vannamei* expressed sequence tag (EST) sequences homologous to Argonaute and Sid-1 proteins, available at [www.marinegenomics.org](http://www.marinegenomics.org) (O'Leary et al., 2006), 5'RACE-primer G-3816 and 5'RACE-primer G-3780 were designed to obtain the 5'end cDNA sequences of Lv-Sid 1 and Lv-Ago 2 proteins by RACE-PCR, as described above. Finally, the full-length cDNAs of *Lv-ago 1*, *Lv-ago 2* and *Lv-sid-1* were amplified by PCR using specific primers (G-3608/G-3609, G-3822/G-3823 and G-3853/G-3854, respectively) designed at 5' and 3' extremities (Table 1) with the BD Advantage 2 polymerase mix (Clontech).

### 2.3 Phylogenetic tree constructions

A list of known sequences of the members of Argonaute and Sid-1 proteins was obtained from GenBank and EMBL databases using the BLAST (Basic Local Alignment Search Tool) program (Altschul et al., 1997). Trees were built using the MEGA 4 software (Molecular Evolutionary Genetics Analysis, version 4.0) applied to the Neighbor-Joining method (Tamura et al., 2007). Multiple alignments of amino acid sequences were created using the Piwi and multiple transmembrane conserved domains for Argonaute and Sid-1-like proteins, respectively. Bootstrap values (%) of 10000 replicates were calculated for each node of the consensus tree obtained.

## 2.4 Preparation of dsRNA

Double-stranded RNAs (dsRNA) were generated as previously described (Robalino et al., 2004). The DNA templates used for *in vitro* transcription were pCR4 vectors (Invitrogen) hosting different size fragments (50, 100, 150 and 200 bp) amplified either from the full-length CDP (CUB domain protein) cDNA (GenBank acc. no. **AY907539**) or from a 309-bp portion of the immunoglobulin u (Igu) cDNA from the duck, *Anas platyrhynchos* (**AJ312200**), using specific primers indicated in Table 1. Synthetic siRNAs with UU 3' overhangs specific for duck Ig<sub>u</sub> (Igv siRNA, GGGTTGCCCATGAGGTTCA) and for CDP (CDP siRNA 1, ACTCACCTGGCTGATGTTC; CDP siRNA 2, CACAACCAAGGAAGTATC; CDP siRNA 3, ATTCCACAGCAACAGTGCT) were purchased from Ambion. Finally, siRNA and dsRNAs were diluted to a final concentration of 250 ng/μl in sterile saline solution (10 mM Tris–HCl pH 7.5, 400 mM NaCl) and stored at –80°C.

## 2.5 Preparation of viral inoculum and experimental infection

The bioassay system, experimental animals, and white spot syndrome virus (WSSV) inoculum used here have been previously described (Prior et al., 2003; Robalino et al., 2004). Briefly, 1.0-1.5 g SPF *L. vannamei* shrimp (30 shrimp/treatment) were intramuscularly injected with 5 μg (20 μl volume) of either siRNA, dsRNA or sterile saline, and 48 h later injected again with either saline (negative controls) or a WSSV inoculum used at a  $5 \times 10^{-8}$  dilution (weight of infected tissue:volume of saline), to typically yield mortalities close to 80% of injected but otherwise untreated shrimp. Shrimp were kept in culture flasks for 10 days following infection. Cumulative mortality was recorded daily.

## 2.6 Quantitative real-time PCR

Quantitative real-time RT-PCR (qPCR) was performed on an ABI 7500 system as previously described (Labreuche et al., 2009). Amplification efficiencies for all qPCR primers were determined according to Pfaffl and collaborators (Pfaffl et al., 2002) and the specificity of the PCR amplification verified from the melting curve. Each run included the cDNA control, negative controls (total RNA treated with DNase I), and blank controls (water). The relative mRNA expression levels were determined using the two standard curve methodology (QuantiTect® SYBR Green PCR Handbook) and S3A ribosomal protein (**BF023924**) was used as the internal reference (normaliser) mRNA. Primer sequences are indicated in Table 1.

## 2.7 Statistical analyses

All numerical data were expressed as the mean ± standard error. Differences in mortality levels between treatments were analyzed by Kaplan–Meier log-rank  $\chi^2$  tests using GraphPad Prism 3.00 for Windows (GraphPad Software, USA) computer software. One-way analysis of variance (ANOVA) or the Kruskal-Wallis test was used for mRNA expression analysis. These statistical analyses were performed with Statgraphics Plus 5.0 software.

### 3. Results

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#### 3.1 Identification of RNAi components

A full-length cDNA of a *L. vannamei* putative Argonaute was first isolated by PCR and RACE, with an open reading frame (ORF) of 2820 bp, a 5' untranslated region of 84 bp, and a 3' untranslated region of 355 bp (**HM234689**). Homology searches revealed a high degree of homology between the deduced protein and other Argonaute family proteins, including *Penaeus monodon* Argonaute 1 Pem-AGO (**DQ663629**, 99% overall amino acid identity), Pm ago (**DQ343133**, 93% identity) as well as *Drosophila* Argonaute-1 (**NM 166020**, 85% identity). Therefore, this *L. vannamei*-Argonaute protein was designated Lv-Ago 1. Expressed sequence tag analyses resulted in the identification of three non-overlapping clones (**FE096200**, **FE146474** and **FE144758**) that showed strong sequence similarities to Argonaute proteins from *Caenorhabditis elegans* (BlastX *E*-value  $9 \times 10^{-24}$ ), *Gobiocypris rarus* ( $6 \times 10^{-22}$ ) and *P. monodon* ( $8 \times 10^{-26}$ ), respectively. Following reverse transcription of gill RNA, PCR reactions performed with specific primers designed at the 5' and 3' extremities of each clone indicated that these 3 EST sequences belonged to the same transcript (data not shown). Full-length cDNA cloning by RACE-PCR revealed the presence of a 2633 bp transcript (**HM234690**) containing a 2556 bp ORF and a 5'untranslated region of 77 nucleotides. Following a BlastX search, this *L. vannamei* Argonaute protein was shown to display higher similarity to *G. rarus* Argonaute 2 (**EF636801**, 40% identity) than *P. monodon* Argonaute 1 Pem-AGO (39%). Accordingly, this putative protein was designated Lv-Ago 2. Analyses of the amino acid sequence deduced from these two putative Argonaute proteins using the Conserved Domain Search Service (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) identified the presence of two distinctive domains, PIWI/Argonaute/Zwille (also named PAZ) and Piwi, that are signature motifs for the Piwi/Argonaute family of proteins (Cerutti et al. 2000). The PAZ domain is involved in dsRNA binding activity, while the Piwi domain displays RNase activity. The PAZ domain of *Lv-ago 1* and *Lv-ago 2* extended from residues 280 to 400, and 240 to 352 respectively, whereas the Piwi domain was located at residues 445-897 and 398-814, respectively (Fig. 1). To study the molecular evolution of *Lv-ago 1* and *Lv-ago 2*, Argonaute family homologous protein sequences from invertebrate organisms were used to construct a phylogenetic tree, based on Piwi domain alignments (Fig. 2). Argonaute proteins can be divided into four groups: the proteins interacting with trigger-derived sense and antisense RNAs to initiate RNAi (the RNAi pathway), the proteins implicated in the miRNA pathway, the proteins involved in transcriptional silencing, and finally the proteins involved in generation of dsRNA from aberrant RNAs. Phylogenetic tree analysis separated *Lv-Ago 1* and *Lv-Ago 2* into two clusters, *Lv-Ago 1* being clearly orthologous to *P. monodon* Argonaute 1 (Pem Ago) and more generally to the miRNA class Argonautes, while *Lv-Ago 2* clustered on a separate branch from all miRNA class members.

*L. vannamei* EST database analyses also identified 2 non-overlapping clones (**FE073135** and **FE152240.1**) displaying strong sequence similarities to Sid 1-like proteins from *Taeniopygia guttata* (BlastX *E*-value  $8 \times 10^{-11}$ ) and *Tribolium castaneum* (BlastX *E*-value  $4 \times 10^{-26}$ ) respectively. PCR reactions performed with specific primers designed at the 5' and 3' extremities of each clone indicated that these 2 EST sequences belonged to the same transcript (data not shown). Amplification by 5'-RACE PCR of this *L. vannamei* putative Sid-1 generated a 2809 bp full-length cDNA with an ORF of 2730 bp encoding a putative protein of 909 amino acids (**HM234688**). Blast search revealed that the deduced protein is

similar to *Bos taurus* Sid-1 ([NM\\_001024530](#), 26% identity,  $E$ -value  $8 \times 10^{-58}$ ) and *Pan troglodytes* Sid-1 ([XP\\_001154535](#), 26% identity,  $E$ -value  $7 \times 10^{-57}$ ). Prediction of transmembrane (TM) helices in the deduced putative structure was performed with TMHMM server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Like *C. elegans* Sid-1, Lv-Sid-1 was shown to contain 11 TM domains (Fig. 3), with a large (348 amino acids) extracellular N-terminal domain and a 150 amino acid loop residing between the first and second predicted TM helices. A scan of the sequence against the PROSITE database did not identify additional motifs or domains. Phylogenetic analysis of the carboxy-terminal TM domain (corresponding to the TM2-TM11 region) of 15 Sid-1 like proteins demonstrated a close phylogenetic relationship for all mammalian Sid-1 proteins, while Lv-Sid-1 clustered on a separate branch (Fig. 4).

### **3.2 *Lv-sid-1* and *Lv-ago 2* mRNA expression is up-regulated by dsRNA but not by siRNAs.**

Because *Lv-sid-1*, *Lv-ago 1* and *Lv-ago 2* are putative core components of a shrimp RNAi pathway, we investigated whether their expression might be transcriptionally regulated following injection of RNA duplexes of diverse length and specificity. To this end, shrimp were injected with either specific or non-specific siRNAs or dsRNA of varying lengths. As indicated in Fig. 5, *Lv-sid-1* mRNA levels were significantly induced in animals challenged with >50 bp specific (Fig. 5 A) and non-specific dsRNA (Fig. 5 B), compared to shrimp injected with saline only ( $P < 0.05$ ). Synthetic siRNAs (specific and non-specific) did not cause any significant variation in *Lv-sid-1* mRNA levels, as assessed by comparison with saline-injected animals ( $P > 0.05$ ). As indicated in Fig. 6, 100-bp specific and non-specific dsRNA led to a significant up-regulation of *Lv-ago 2* expression profiles ( $P < 0.05$ ), whereas *Lv-ago 1* mRNA levels remained stable, compared to saline-injected animals, whatever the length and specificity of tested dsRNA. Synthetic siRNAs failed to induce any significant effect on *Lv-ago 1* and *Lv-ago 2* expression levels, independently of their specificity (Fig. 6).

### **3.3 Only > 50 bp-dsRNA, but not siRNAs, induce targeted genetic interference and general antiviral protection when injected into shrimp.**

Experiments were conducted to explore the influence of dsRNA length on specific depletion of cognate mRNA. To this end, shrimp were injected with either different size dsRNA or synthetic siRNAs targeting the CDP gene. As shown in Fig. 7, injection of gene-specific dsRNA of varying lengths (ranging from 50 to 200 bp) led to a statistically significant depletion of the cognate mRNA in gills, as determined by qPCR (ANOVA,  $P < 0.05$ ), while none of the 3 CDP-specific siRNAs induced a down-regulation of CDP gene expression when compared to injection of saline only (ANOVA,  $P > 0.05$ ).

Finally, we investigated the influence of non-specific dsRNA length to mediate antiviral protection during a WSSV challenge. For this experiment, animals were injected with either duck Ig<sub>v</sub> synthetic siRNA or duck Ig<sub>v</sub> dsRNA of varying sizes (ranging from 50 to 200 bp). Confirming previously reported results (Robalino et al., 2005), non-specific siRNAs completely failed to protect shrimp from viral infection, cumulative mortality for this treatment reaching 100 % at 5 days post injection (dpi) (Fig. 8). All tested dsRNA afforded a statistically significant antiviral protection from WSSV infection 9 dpi, compared to

siRNA-injected and saline-injected shrimp (Kaplan–Meier log-rank  $\chi^2$ : 62.21,  $P < 0.0001$ ). The degree of protection did not significantly vary with the length of dsRNA injected to the shrimp (Kaplan–Meier log-rank  $\chi^2$ : 0.4753,  $P = 0.9243$ ).

## 4. Discussion

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RNAi-based methodologies and technologies are still in their infancy in penaeid shrimp and some questions need to be addressed before the whole promise of RNAi can be utilized to improve aquaculture. Among these questions, the almost certain role of the RNAi machinery in the observed sequence-specific down-regulation of endogenous or viral gene expression after dsRNA administration remained to be clarified in *L. vannamei*, as well as the receptor(s) involved in dsRNA uptake. In this study, we report for the first time in a marine crustacean the cloning and characterization of a Sid-1 homologue, Lv-Sid-1, and the identification of two putative shrimp Argonaute, Lv-Ago 1 and Lv-Ago 2, a protein family described as the principal protein component of RNA silencing pathways.

### 4.1 Shrimp Argonautes , Lv-Ago 1 and Lv-Ago 2, may be implicated in distinct pathways.

The inferred amino acid sequences of *Lv-ago 1* and *Lv-ago 2* cDNAs revealed that these putative proteins possess the two distinctive features of Argonaute family proteins, i.e. a PAZ domain involved in dsRNA binding and a PIWI domain, reported to possess RNase activity. Homology searches indicated that Lv-Ago 1 showed high sequence similarities to *Drosophila* Argonaute-1, while *Lv-ago 2* deduced aa sequence displayed higher similarities to Argonaute 2 protein members. Similarly to this observation, several paralogs have been identified in insects, nematodes, mammals and plants, suggesting specific roles associated with each one of them, when they are recruited into the RISC or effector complexes. For instance, among the several Argonaute family proteins characterized in mammals, only Argonaute 2 is catalytically competent and responsible for mRNA cleavage activity (Liu et al., 2004). Of the 27 Argonaute proteins discovered in *C. elegans*, RDE-1 binds to many classes of small RNAs (Corrêa et al., 2010), while ALG-1 and ALG-2 exclusively bind miRNAs, a class of endogenously produced  $\approx 22$ -nt RNAs, that associate Argonaute proteins to direct mRNA degradation or translational repression (Grishok et al., 2001). Phylogenetic analyses performed to determine the evolutionary relatedness of these shrimp proteins with invertebrate Argonaute-related genes demonstrated the orthologous relationship of Lv-Ago 1 with miRNA class Argonautes, while Lv-Ago 2 localized to a separate branch. An exclusive association of Argonaute gene family with either the miRNA or RNAi pathway has been reported in many organisms, and we addressed whether *Lv-ago 1* and *Lv-ago 2* display the same functions. If one or both of these shrimp Argonaute proteins was involved in the dsRNA-inducing gene silencing mechanism, their expression would be modified following dsRNA administration, as such regulation has been shown to be important for optimal efficiency of the RNAi pathway (Choudhary et al., 2007). Monitoring *Lv-ago 1* and *Lv-ago 2* transcript accumulation by qPCR following dsRNA injection into shrimp allowed us to demonstrate a strong induction of *Lv-ago 2* mRNA expression only. Taken together, these results suggest i) that these two Argonautes do not play redundant roles in sequence-specific gene silencing, and ii) that Lv-Ago 2 may be specifically implicated in the RNAi pathway. In *Penaeus monodon*,

knock-down of *Pem-ago* (which shows 99% overall amino acid identity with Lv-Ago 1) with sequence-specific dsRNA was reported to partially reduce the efficiency of RNAi for an endogenous shrimp gene (Dechklar et al., 2008). Because RNAi was still functional at a certain level ( $\approx$  50% of the original activity), it was hypothesized that other Argonaute family members may be involved in this mechanism. The data obtained in this work further support this assumption. Interestingly, a conserved D-D-H motif, shown to be essential for the “slicing” activity in the RNase H-related Piwi domain, was identified in both proteins (Rivas et al., 2005) (Fig. 1), indicating that they may both display a catalytic activity. The phylogenetic analysis and the existence of these critical residues for endonucleolytic activity in Lv-Ago 1, in relation to the observed lack of induction by exogenous dsRNA and siRNAs, suggest that Lv-Ago 1 may selectively recruit certain classes of small RNA duplexes, as reported in *Drosophila* (Baumberger and Baulcombe, 2005; Tomari et al., 2007) and in *C. elegans* (Jannot et al., 2008). Although no confirmed miRNAs have been reported to date in penaeid shrimp, they are known to be widely expressed in metazoa, playing key regulatory roles in embryogenesis, stem cell division, neurogenesis and haematopoietic cell differentiation (Bushati, 2007). Further investigations are thus needed to determine the potential existence and role of miRNAs in shrimp and whether Lv-Ago 1 may be involved in a potential miRNA-directed RNA silencing mechanism.

#### **4.2 The shrimp Sid-1 homologue, Lv-Sid-1, responds to dsRNA stimulation**

Previous studies in penaeid shrimp have reported the systemic spread of the RNAi effect throughout the entire body, although the mechanistic details of this phenomenon remained undetermined. In animals, two distinct mechanisms responsible for systemic RNAi have been described: a channel-mediated mechanism based on a multispan transmembrane protein called Sid-1 (systemic interference defective) (Winston et al., 2002) and an endocytosis-mediated mechanism based on scavenger-like pattern recognition receptors (Saleh et al., 2006). Using an expressed sequence tag analysis approach in *L. vannamei*, a *Lv-sid-1* mRNA encoding a putative protein with strong sequence similarity to Sid-1 proteins characterized in animals was found. In keeping with this protein family, Lv-Sid-1 was predicted to contain 11 transmembrane domains, suggesting that it may function as a transmembrane channel (Fig. 3). Considering the critical role of this protein in *C. elegans*, and the occurrence of systemic RNAi in most organisms exhibiting Sid-1 homologues, it has been suggested that import of silencing signals *via* this protein is an ancient conserved function (Winston et al., 2002). However, recent work in *Tribolium castaneum* reported 3 *sid-1* like genes which did not seem to be required for the systemic RNAi response (Tomoyasu et al., 2008). To provide a clue about *Lv-sid-1* function in shrimp, we therefore analyzed its relative expression following shrimp injection with RNA duplexes. Administration of dsRNA into the shrimp body cavity elicited a substantial increase of *Lv-sid-1* mRNA levels, showing that dsRNA induces the transcriptional activation of this gene, and suggesting thus its potential role as a channel for dsRNA. Knock-down experiments of Lv-Sid-1 message with sequence-specific dsRNA induced mortality in this assay system, up to 80 % shrimp dying within the first 2 days post-injection (data not shown). Interestingly, silencing of other genes obtained from *L. vannamei* libraries and presumptively associated with the RNAi pathway (like *armitage*) resulted in a similar lethal phenotype in shrimp (J. Robalino, pers. com.), suggesting that these components, including Lv-Sid-1, may be involved in other essential for live functions. Considering these data and the unavailability of clonal cell lines in marine invertebrates, further investigations of *Lv-sid-1* molecular properties are needed in *in vivo* conditions and may require expression in heterologous tissue culture systems (Feinberg and Hunter, 2003).

#### **4.3 dsRNA-induced specific gene silencing in *L. vannamei* is length-dependent**

Discrepancies in the ability of siRNAs to promote targeted genetic interference and general antiviral protection have been reported in shrimp (Li et al., 2007; Robalino et al., 2005; Westenberg et al., 2005; Wu et al., 2007; Wu et al., 2008). In this study, we clearly showed that only >50-bp specific dsRNA induced a significant depletion of cognate mRNA. Several hypotheses have been evoked to explain the inconsistencies between these results and other published data, among which the differences between methods used to select and design target sequences for RNAi. However, this size-dependent silencing effect has been reported not only in shrimp, but also in other organisms, and demonstrated to be independent of the nature of siRNA sequences (Feinberg and Hunter, 2003). This observation is confirmed by our experimental data since 3 different siRNAs were tested in this work with the same result. It has also been proposed that the lack of biological activity of siRNAs in shrimp was linked to the existence of mechanisms for uptake of long dsRNA that do not act on very short ones (Robalino et al., 2005; Westenberg et al., 2005). The absence of *Lv-sid-1* transcriptional response observed in this study following siRNA injection into shrimp tends to support this assumption. Interestingly, Shih and collaborators have recently demonstrated in *C. elegans* that the reduced silencing efficiency associated with siRNAs could not be explained by size-selective transport through Sid-1, but would rather be linked to the fact that these short RNA duplexes are poor substrates for RISC complexes (Shih et al., 2009). Here we found that injection of siRNAs completely failed to induce *Lv-ago 2* expression, further supporting this idea that siRNA may not be incorporated into the shrimp RNAi machinery, explaining thus their observed inability to induce knock-down of the target gene.

#### **4.4 the non-specific antiviral response to arbitrary dsRNA is length-dependent and may rely on RNAi pathway components**

Double-stranded RNA, a molecular structure commonly encountered during the virus replication cycle, is a well-known inducer of innate antiviral immune responses in mammals but also, and more surprisingly, in shrimp (Robalino et al., 2004; Robalino et al., 2007). In vertebrates, dsRNA is sensed by different families of pattern recognition receptors (PRRs), among which the TLRs (Takeuchi and Akira, 2010), and triggers the transcription-based antiviral interferon (IFN) response (Haller et al., 2006). For most of these PRRs, a length dependence was observed for dsRNA to bind to the receptor and elicit innate immunity (Kato et al., 2008; Okahira et al., 2005). Genes encoding homologues of interferons and IFN-regulated genes are absent in sequenced invertebrate genomes, and the mechanistic details of the shrimp immune response to dsRNA are unknown. Data presented here provide evidence for the occurrence of a length-dependent antiviral response in shrimp, and suggest the existence of immune mechanisms analogous, at least to some extent, to those described in vertebrates. Recently, a TLR (IToll) was characterized in *L. vannamei*, but shown to play no role in dsRNA-induced antiviral immunity (Labreuche et al., 2009). Searches of available EST sequences for other PRRs capable of binding dsRNA have not yet yielded homologues for these components, and further investigations are needed to uncover the mechanisms of this sequence-independent immunity. In mammals, several studies have demonstrated the existing link between the PKR pathway (a central player in the innate immune response to viral infections known, to be activated by dsRNA in a length-dependent manner), and components of the RNAi machinery (Haase et al., 2005; Laraki et al., 2008). The existence

of similar interactions between RNAi and innate immunity by dsRNA to mount antiviral protection has also been suspected in shrimp (Robalino et al., 2005). Our study, showing that non-specific long dsRNA trigger the same induction of *Lv-sid-1* and *Lv-ago 2* mRNA expression as sequence-specific dsRNA provides further support to this assumption.

## Conclusion

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The results presented in this study expand the biochemical framework for the understanding of RNAi-based mechanisms in shrimp, and reveal, through the identification of a *Lv-ago* multigene family, the probable existence of different forms of RNA silencing in this animal model. They also support the idea that the mechanisms involved in dsRNA-induced immunity and RNAi converge to mount an efficient antiviral response in crustacea. Further research is needed to find out the mechanisms influencing the activation and/or switching between these processes but also to what extent selective recruitment of these Argonaute proteins plays a role in shrimp physiology.

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# Figures

Figure 1

Hs Ago 2 PYVREFGIMVKDEMTDVTGRVLQPPSILYGGRNKAIATPVQGVWDMRNKQFHTG---IEI  
Mm Ago 2 PYVREFGIMVKDEMTDVTGRVLQPPSILYGGRNKAIATPVQGVWDMRNKQFHTG---IEI  
Bt Ago 2 PYVREFGIMVKDEMTDVTGRVLQPPSILYGGRNKAIATPVQGVWDMRNKQFHTG---IEI  
Xl Ago 2 PFVREFGIMVKDDMTDVTGRVLQPPSILYGGRSKAIATPVQGVWDMRNKQFHTG---IEI  
Gr Ago 2 PYVREFGVMVRDEMTDEVNVRVLQAPSILYGGRNKAIATPVQGVWDMRNKQFHTG---IEI  
Lv Ago 1 PYMQEFGLTISTAMMEVGRVLPKPKLQYGGRTKQALPNQGVWDMRGKQFFTG---VEI  
Lv Ago 2 PLMRQLEFTVSDRPVEMNGRVLPAENLKMMDG---TVLPEKGVWEAWNREFFKG---ATI  
Dm Ago 2 PTISRFGIRIANDFIVVSTRVLSPPQVEYHSKRFTMVKN--GSWRMDGMKFLPEPKPAHK

Hs Ago 2 KVWAIACFAPQRQCTEVHLKSFTEQLRKISR DAGMPIQGQPCFCKYAQGADSV EPMFRHL  
Mm Ago 2 KVWAIACFAPQRQCTEVHLKSFTEQLRKISR DAGMPIQGQPCFCKYAQGADSV EPMFRHL  
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Gr Ago 2 KVWAIACFAPQRQCTE LLLKAFDQLRKISR DAGMPIQGQPCFCKYAQGADSV EPMFKHL  
Lv Ago 1 RVWAVACFAPQRTVREDALRNFTQQLQKISR DAGMPIIGQPCFCKYANGPDQVEPMFRYL  
Lv Ago 2 ETWAVINYD--KFTAQKDVWAF LNSLLKMAKERGMIMNDP----VKIMDGNAP EKDFPKI  
Dm Ago 2 CAVLYCDPRSGRKMNYTQLNDFGNLII SQGKAVNISLSDSVTYRPF TDDERSLDTIFADL

Hs\_Ago 2 KNTYAGLQLV VVILPG-KTPVYAEVKRVGDTV LGMATQCVQMKNVQRTTP-QTLSNLCLK  
Mm\_Ago 2 KNTYAGLQLV VVILPG-KTPVYAEVKRVGDTV LGMATQCVQMKNVQRTTP-QTLSNLCLK  
Bt\_Ago 2 KNTYAGLQLV VVILPG-KTPVYAEVKRVGDTV LGMATQCVQMKNVQRTTP-QTLSNLCLK  
Xl\_Ago 2 KNTYTGLQLV VVILPG-KTPVYAEVKRVGDTV LGMATQCVQMKNVQRTTP-QTLSNLCLK  
Gr\_Ago 2 KYTYQGLQLV VVILPG-KTPVYAEVKRVGDTV LGMATQCVQVKNVQKTTP-QTLSNLCLK  
Lv\_Ago 1 KSTFTGLQLV CVVLPK-KTPVYAEVKRVGDTV LGMATQCVQAKNVNKTSP-QTLSNLCLK  
Lv\_Ago 2 MKDFKGIQMILVNLP SKKGDKYGRVKKMGDREFSVV TQCILSKTLKNPKP-ATVNNVLLK  
Dm\_Ago 2 KRSQHDLAIVII PQFR---ISYDTIKQKAE LQHGI L TQC I KQFTVERKCNNQTIGNILLK

Hs Ago 2 INVKLG VNNILLPQGRPPVFQQPVI FLGADVTHPPAGDGK-KPSIAAVVGSMDAHPNRY  
Mm Ago 2 INVKLG VNNILLPQGRPPVFQQPVI FLGADVTHPPAGDGK-KPSIAAVVGSMDAHPNRY  
Bt Ago 2 INVKLG VNNILLPQGRPPVFQQPVI FLGADVTHPPAGDGK-KPSIAAVVGSMDAHPNRY  
Xl Ago 2 INVKLG VNNILLPQGRPPVFQQPVI FLGADVTHPPAGDGK-KPSIAAVVGSMDAHPNRY  
Gr Ago 2 INVKLG VNNILLPQGRPLV FQQPVI FLGADVTHPPAGDGK-KPSIAAVVGSMDAHPNRY  
Lv Ago 1 INVKLGGIN SILVPGIRPKVFNEPVI FLGADVTHPPAGDNK-KPSIAAVVGSMDAHPNRY  
Lv Ago 2 INGKMG VNNNTLGR ESSTFIL TSPV MIMGADVNHPPADDRKGT PSLAAVVGSMDCFASNY  
Dm Ago 2 INSKLNGINHKIKDDPRLPMMKN-TMYIGADVTHPPSPDQRE-IPSVVGVAAASHDPYGASY

Hs Ago 2 CATVRVQQHR-----QEIIQDLAAMVRELLIQFYKSTR  
Mm Ago 2 CATVRVQQHR-----QEIIQDLAAMVRELLIQFYKSTR  
Bt Ago 2 CATVRVQQHR-----QEIIQDLAAMVRELLIQFYKSTR  
Xl Ago 2 CATVRVQQHR-----QEIIQDLSAMVRELLIQFYKSTR  
Gr Ago 2 CATVRVQQHR-----QDI IQDLATMVRELLIQFYKSTR  
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Hs Ago 2 FKPTRIIFYR DGVSEGQFQQVLHHELLAIREACIKLEKDYQPGITFIVVQKRHHTRLFCT  
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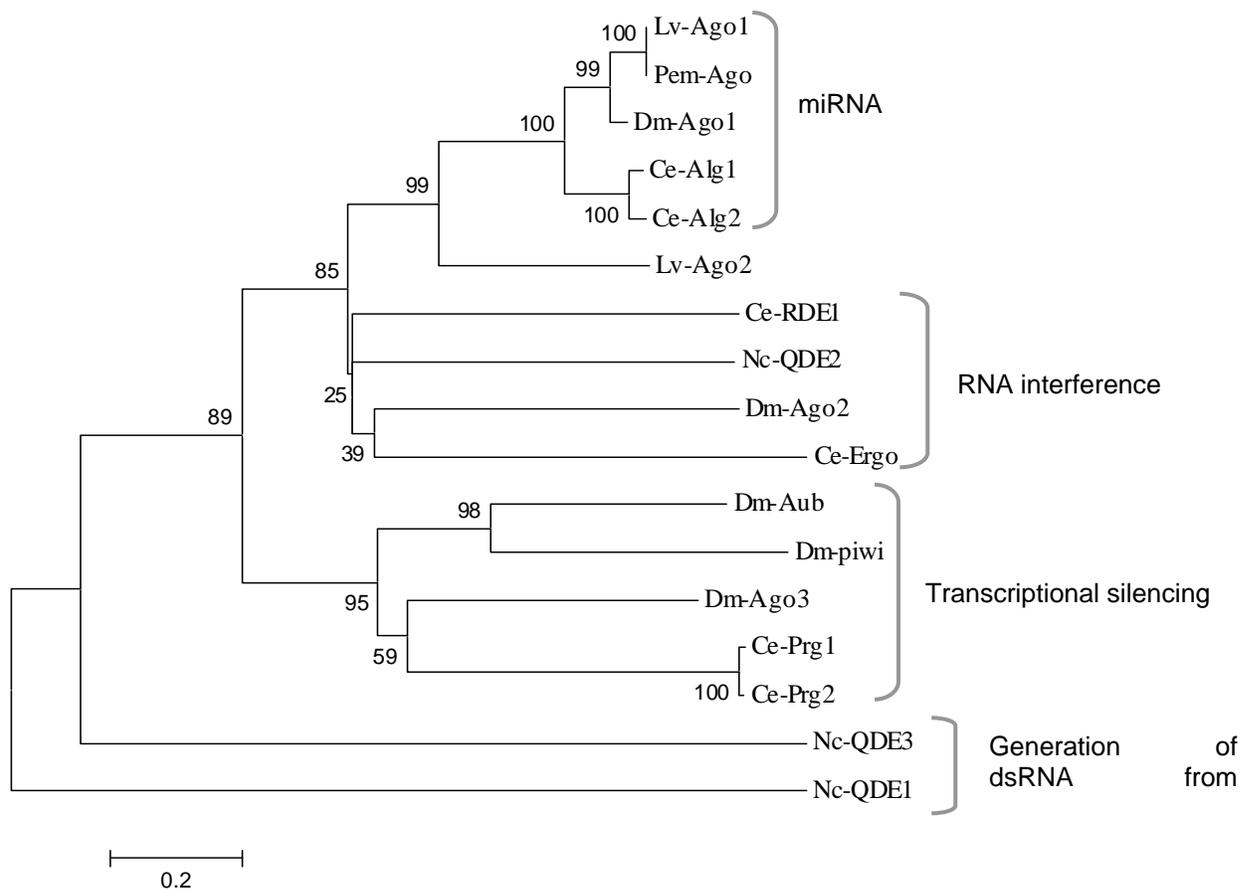
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Xl Ago 2      LQILTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHL
Gr Ago 2      LQVLTYYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHL
Lv Ago 1      LQCLTYQLCHTYVRCTRSVSIPAPAYYAHLVAFRARYHL
Lv_Ago 2     LQSMYSYAMCHTYSRCTRSVSIPAPAYYAHLAAYRAKVH-
Dm Ago 2     LQQLTYNLCHMFPRCNRSVSYAPAYLAHLVAARGRVYL

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**Figure 1.** Multiple alignment of the catalytic center of the PIWI domains from the shrimp Argonaute Lv-Ago 1 and Lv-Ago 2 and representative Ago 2 homologous proteins by ClustalW. The position of the Mg<sup>2+</sup> coordinating residues in the DDH motif, critical for slicing activity, are indicated in bold and marked with a solid circle (•). GenBank accession number are : *Drosophila melanogaster*, Dm-Ago 2 (**Q9VUQ5**); *Mus musculus*, Mm Ago 2 (**NP\_694818.3**); *Bos taurus*, Bt Ago 2 (**NP\_991363.1**); *Xenopus laevis*, Xl Ago 2 (**NP\_001086988.1**); *Homo sapiens*, Hs Ago 2 (**NP\_036286**) and *Gobiocypris rarus*, Gr Ago 2 (**ABV22635**).



**Figure 2.** Phylogenetic analysis of Argonaute proteins. Multiple alignments were created and curated in MEGA 4.0. The Neighbor-Joining tree is based on the alignment of the conserved PIWI domain. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accession number are : *Drosophila melanogaster*, Dm-Ago 1 (GenBank acc. no. [BAA88078](#)), Dm-Ago 2 ([Q9VUQ5](#)), Dm-Ago 3 ([EF688531](#)), Dm-Aub ([CAA64320.1](#)), Dm-Piwi ([AAD08705](#)); *Caenorhabditis elegans*, Ce-Alg 1 ([NP\\_510322.2](#)), Ce-Alg 2 ([NP\\_493837](#)), Ce-RDE 1 ([AAF06159.1](#)), Ce-Ergo ([NP\\_503362](#)), Ce-Prg 1 ([NP\\_492121](#)), Ce-Prg 2 ([NP\\_500944](#)); *Neurospora crassa*, Nc-QDE 1 ([CAB42634](#)), Nc-QDE 2 ([AAF43641.1](#)), Nc-QDE 3 ([AAF31695.1](#)).

Figure 3

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Ce-Sid-1      -----MIRVYLIIILMHLVIG-LTQNNSTTPSPIITSS--NSSVLVFEISSKMKMIEK 49
Lv-Sid-1      MMAPNHRGLSLTILLLLYHGVSIGSIPPEPPTTPDEIADTSTETTAITTLTETMKTPIQT 60

Ce-Sid-1      KLEANTVHVLRLLELDQSFILDL-----TKVAAEIVDSSKYSKEDGVILEVTVSNGRDSFL 104
Lv-Sid-1      TTTVTTPVTSTATTDEPVTDIPIQPETDCQAFIPKSGKCRFSKAQKVNISASNPLFKC 120

Ce-Sid-1      LKLPTVYPNLKLYTDGKLLNPLVEQDFGAHRKRHRIG-----DPHFHQNLIVTVQSR 156
Lv-Sid-1      HYNPAELSPIRFEVHLTYKHPAQNPFTVTVKQRFIFSSWTLPIILFPDHGENQKFTSIEKM 180

Ce-Sid-1      LNADIDYR-----LHVTHLDRAQYDFLKFKTGQTTKTLNSQ----KLTfVFKPIGFFLNCS 207
Lv-Sid-1      LCPPTNLQNETLEFYLTTESTQNISFILGVSNSQSYVLSMGDNVTVKATPVSPWVKLFKWD 240

Ce-Sid-1      EQNISQFHVTLYSEDDICANLITVPANESIYDRSVISDKTHNRRVLSFTKRADIFFTETE 267
Lv-Sid-1      KEDSILVTADSRDSDTVCSILALQNAKCPVYADEAEVRAGGTQFQTFTSRAGMVARR-- 298

Ce-Sid-1      ISMFKSFRI FVFIAPDDSGCSTNTRSRKSFNEKKKISFEFKKLENQS YAVPTALMMIFLTT 327
Lv-Sid-1      ENFPDGVHIIIVPLPDDDPCTLAFSERANHTSRQKSVILQVYNHATIASTWYVFLTAGA 358

Ce-Sid-1      PCLLFLPIVINIIKNSRKLAPSQSNLIS-----FSPVP 360
Lv-Sid-1      MATVISSFTALSIRMIRRNLTSETVAVEDEDEERSLLGESGHSNGTHGRIRVEEMAGVSVLS 418

Ce-Sid-1      SEQRDMDLSHDEQQNTSSELENNGEIPAAENQIVVEEITAENQ-ETSVEEGNREIQVKIPL 419
Lv-Sid-1      GGEVGGTMSEGSRRREGVSRQERPFVSASLSDGHLNRNVSNEPYGRLQIDNGSRDLQPLPYA 478

Ce-Sid-1      KQDSL SLHGQMLQYE-----VAIILP VLM 443
Lv-Sid-1      VPSTYAPHHAVLNFSASFNPRLVCWVKRLAVYELRTADAQVAEIGFQNNVLI MAVFTALP 538

Ce-Sid-1      HTAIEFHKWTSTMANRDEMCFHNHACARPLGELRAWNNIITNIGYTYLGAIFIVLSICR 503
Lv-Sid-1      TTELVRSYLKLLLYHGQEDQCFNRSRCLTAFGTLPDFARVFTNIGYLLCGAAFIIVKEH 598

Ce-Sid-1      R-----GRHE YSHVFGTYECTLLDVTIGVFMVLQSIASATYHICPSDVAFQFDTPCIQ 556
Lv-Sid-1      KKFTENILRQYGANNSVGVSRHYGLFMSVGYGLFIQGVMSLYHTCPNSVTIRFDMMFVY 658

Ce-Sid-1      VICGLLMVRQWFVRH-ESPSPAYTNILLVGVVSLNFLISAFSKTS YVRFIIAVIHVIVVG 615
Lv-Sid-1      VVAVAAVVSMWGFRRHGDVTHHVYPTMVMVGMILMAEAREWVSQAFAFWTVLSLCYVFLMV 718

Ce-Sid-1      S-----ICLAKERSLGSEKIKTRFFIMAFSMG-----NFA 645
Lv-Sid-1      TNTILLTKYGVWSFSPYKMLMVWKGWRPVAEKLRNELWGSATTAKPLQIVRIVIGLVVNS 778

Ce-Sid-1      AIVMYLTLSAFHLNQIATYCFIINCIMYLMYYGCMKVLHSERITSKAKLCGALSLLAWAV 705
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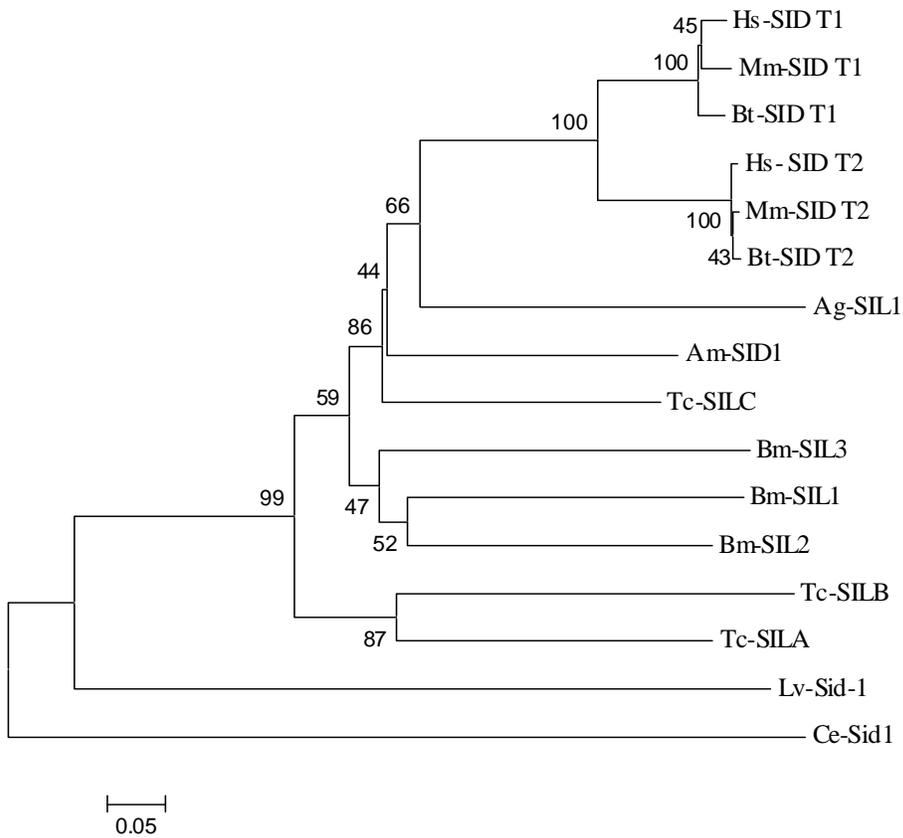
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Lv-Sid-1      ALAAFFFHSTDPEASPSMSRAKNSPCEFFGVFDTHDAWHLMSALALFTFFVGILTLDDDL 898

Ce-Sid-1      INTRKTSINIF 776
Lv-Sid-1      CHTRSDKIHVF 909

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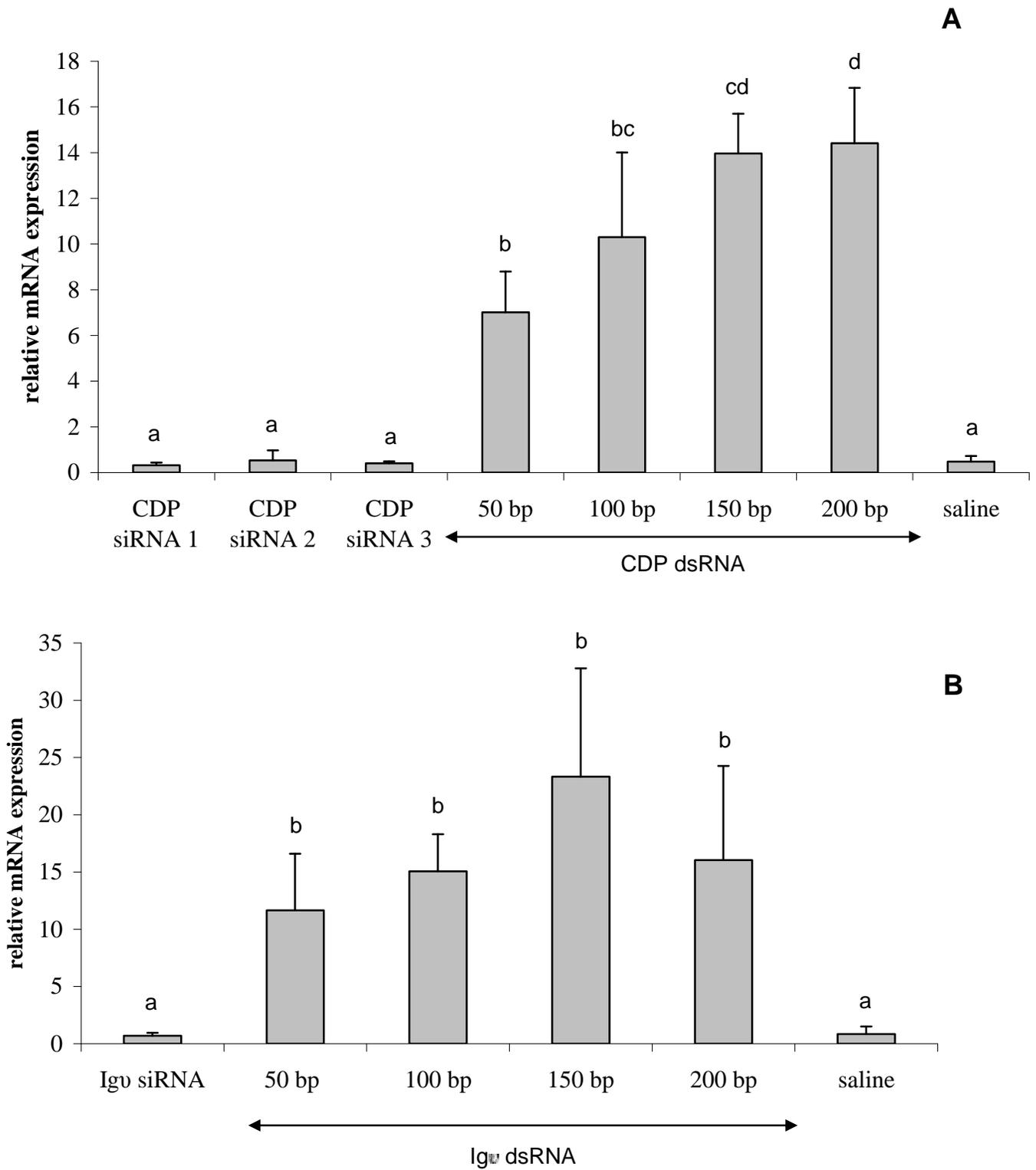
**Figure 3.** Alignment of Sid-1 transmembrane proteins. Predicted transmembrane domains from *L. vannamei* Lv-Sid-1 and *C. elegans* Ce-SID1 (**AF478687**) are respectively indicated in gray and in green. Numbers indicated on the right side represent the amino acid position in the corresponding species.

**Figure 4**



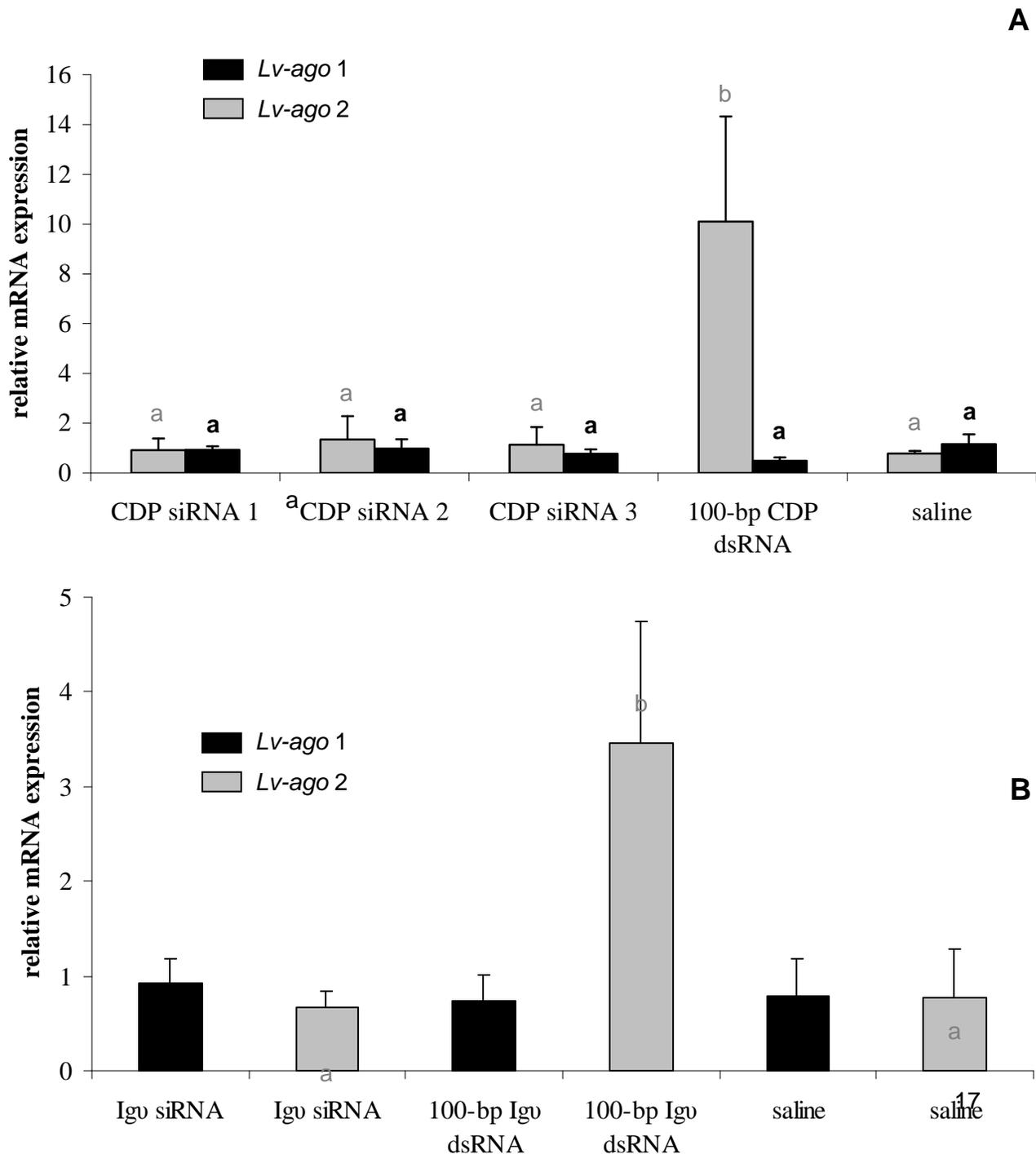
**Figure 4.** Phylogenetic tree based on amino acid sequences of multiple transmembrane (TM) domain (corresponding to TM2-TM11 portion of *C. elegans* Sid-1) from Sid-1-like genes. GenBank accession number are : *Homo sapiens*, Hs-SIDT1 (GenBank acc. no. [AAI17223.1](#)), Hs-SIDT2 ([AAI14523.1](#)); *Mus musculus*, Mm-SIDT1 ([AAH25888.1](#)), Mm-SIDT2 ([AAH06873.1](#)); *Bos Taurus*, Bt-SIDT1 ([XP\\_585013.3](#)), Bt-SIDT2 ([NP\\_001019701.2](#)); *Aphis gossypii*, Ag-SIL1 ([EF533711](#)); *Apis mellifera*, Am-SID1 ([XP\\_395167.3](#)); *Tribolium castaneum*, Tc-SILA ([NP\\_001099012.1](#)), Tc-SILB ([NP\\_001103253.1](#)), Tc-SILC ([NP\\_001099128.1](#)); *Bombyx mori*, Bm-SIL1 ([BAF95805.1](#)), Bm-SIL2 ([BAF95807.1](#)), Bm-SIL3 ([BAF95806.1](#)); *C. elegans*, Ce-SID1 ([AF478687](#))

Figure 5

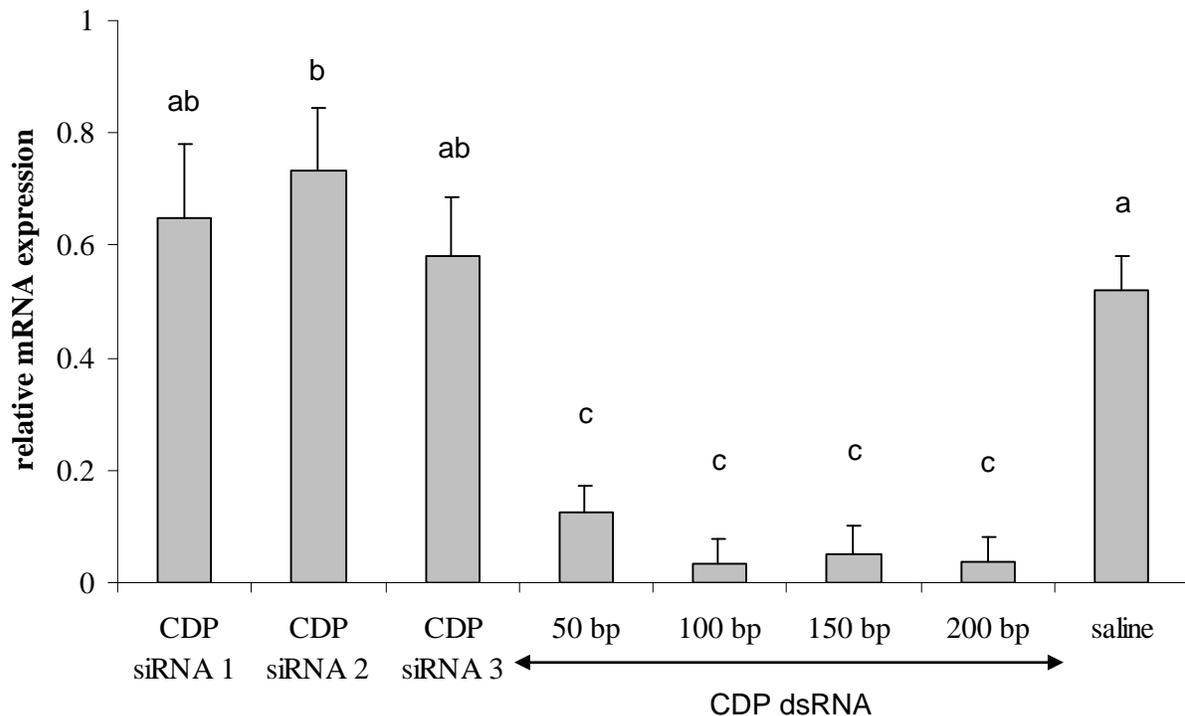


**Figure 5.** Lv-Sid-1 transcript abundance in shrimp gills 48 hrs following treatment with either specific or non-specific siRNAs or dsRNA of varying lengths (ranging from 50 to 200 bp). Shrimp (1 to 1.5 g,  $n = 10$ ) were injected with saline or 5  $\mu\text{g}$  of specific dsRNA or siRNAs targeting: CDP (CUB domain protein, [AY907539](#)), a shrimp endogenous gene (**A**), or the immunoglobulin u (Igu) gene from the duck, *Anas platyrhynchos* ([AJ312200](#)) (**B**). Five animals were randomly sampled in each group and Lv-Sid-1 expression was determined in gill tissues of each individual shrimp by quantitative real-time qPCR. Expression values are presented as relative abundance in relation to S3A ribosomal gene. Bars represent  $\pm$  standard error of the mean. Different lower-case letters indicate significant difference between treatments.

Figure 6

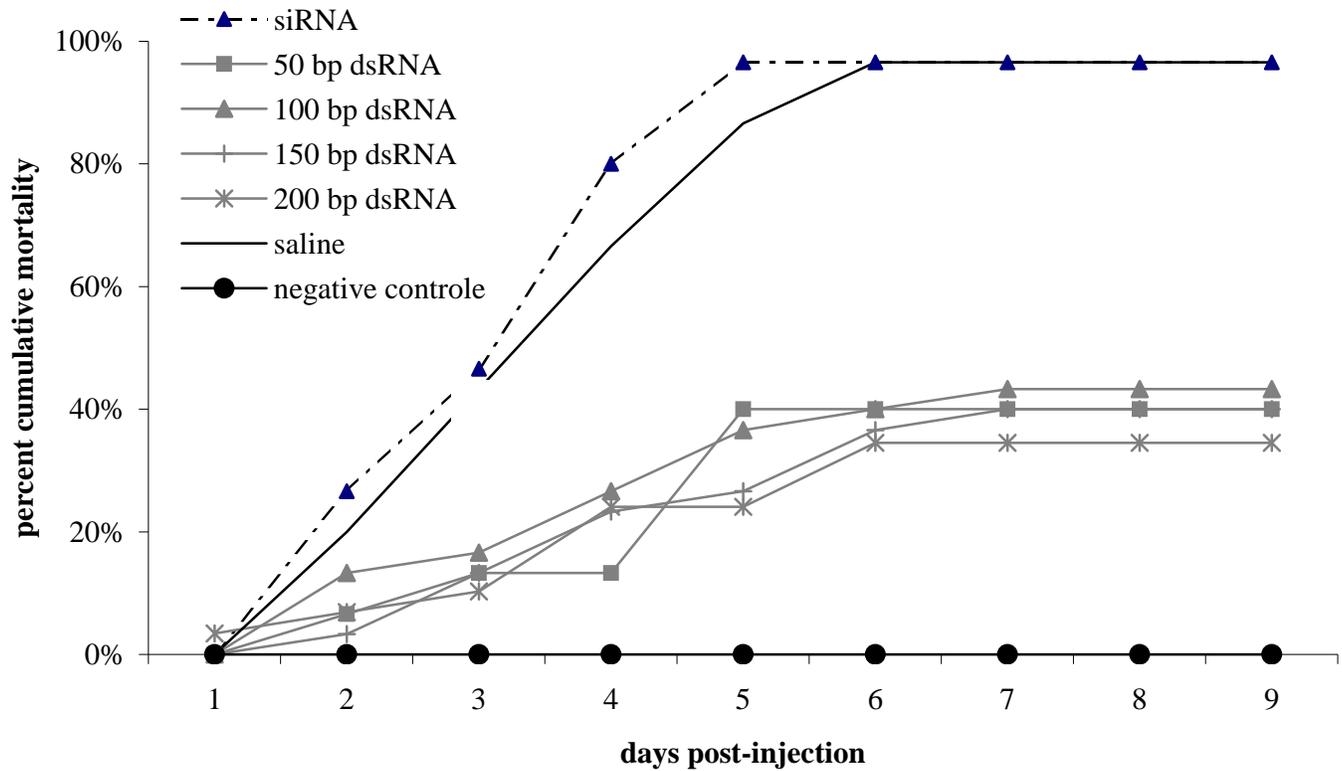


**Figure 6.** Lv-Ago 1 and Lv-Ago 2 transcript abundance in shrimp gills 48 hrs following treatment with either specific or non-specific siRNAs or dsRNA of varying lengths (ranging from 50 to 200 bp). Shrimp (1 to 1.5 g,  $n = 10$ ) were injected with saline or 5  $\mu\text{g}$  of specific dsRNA or siRNAs targeting CDP (A) or the duck immunoglobulin u (Igu) gene (B). Five animals were randomly sampled in each group. Lv-Ago 1 and Lv-Ago 2 expression values determined by quantitative real-time qPCR are presented as relative abundance in relation to S3A ribosomal gene. Bars represent  $\pm$  standard error of the mean. Different lower-case letters indicate significant difference between treatments.



**Figure 7.** CDP transcript abundance in shrimp gills following treatment with sequence-specific siRNAs or dsRNA of varying lengths (ranging from 50 to 200 bp). Shrimp (1 to 1.5 g,  $n = 10$ ) were injected on day 0 with saline or 5  $\mu\text{g}$  of siRNAs or dsRNA targeting CDP. At 48 h after this initial injection, 5 animals were randomly sampled in each group. CDP expression values determined by quantitative real-time qPCR are presented as relative abundance in relation to S3A ribosomal gene. Bars represent  $\pm$  standard error of the mean. Different lower-case letters indicate significant difference between treatments.

**Figure 8**



**Figure 8.** Shrimp mortality following treatment with non-specific siRNAs or dsRNA of varying lengths and experimental infection with WSSV. Shrimp ( $n = 30$ ) were injected intramuscularly with either saline (positive [-] and negative [•] controls) or non-specific RNA duplexes (siRNAs or dsRNA ranging from 50 to 200 bp). At 48 h after this initial injection, animals were infected with WSSV. Differences in mortality levels between treatments were analyzed by Kaplan–Meier log-rank  $\chi^2$  tests.

## Tables

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**Table 1.** List of primers used in this study. <sup>a</sup> forward and reverse primers used to perform PCR and RACE-PCR to clone *Lv-ago 1*, *Lv-ago 2* and *Lv-sid 1* full-length cDNAs. <sup>b</sup> forward and reverse primers used for measuring the expression of S3A (housekeeping gene, **F023924**) and *Lv-ago 1*, *Lv-ago 2* and *Lv-sid 1* by SYBR<sup>®</sup> Green real-time RT-PCR. <sup>c</sup> forward and reverse primers used to amplify different size fragments (50, 100, 150 and 200 bp) from the full-length CDP (CUB domain protein) cDNA (**AY907539**) or from a 309-bp portion of the immunoglobulin u (Igu) cDNA from the duck, *Anas platyrhynchos* (**AJ312200**).

**Table 1**

| <b>PCR and RACE-PCR primers<sup>a</sup></b>                            | <b>Sequence (5' → 3')</b>                   | <b>Target gene</b> | <b>Primer information</b>   |
|--|---|--------------------|---|
| G-3524   | CCA TGT TCC GTT ACC TGA AG                  | Lv-ago 1           | gene cloning  |
| G-3525   | TCC CAC AGT ACG TGG TAG TG                  |                    | gene cloning  |
| G-3597   | TGT CTG GGT CGC GAG CCG TCA CTG GCA CT      |                    | RACE  |
| G-3595   | AGT GCC AGT GAC GGC TCG CGA CCC AGA CA      |                    | RACE  |
| G-3816   | CCG TAG TTC ATA GAC TGC CAA TCG CTT CCA CCA |                    | Lv-sid 1  |
| G-3780   | GGC ATT TCC ATC CAT GAT CT                  | Lv-ago 2           | RACE  |
| G-3608   | CAC AGG AAG TCC CAT AGA ACG CCA             | Lv-ago 1           | confirming sequence   |
| G-3609   | CTT GAG GCA GAC TAG GTA AGG AGA GA          |                    | confirming sequence   |
| G-3822   | ATG CCT TGG ATA TCA GAA GTC CTT C           | Lv-ago 2           | confirming sequence   |
| G-3823   | GAG ATA TCT GCC GCA GAA CCT GCT             |                    | confirming sequence   |
| G-3853   | ATG ATG GCT CCA AAT CAC AGA GGT             | Lv-sid 1           | confirming sequence   |
| G-3854   | AAA CAC ATG AAT TTT ATC GGA GCG             |                    | confirming sequence   |
| <b>qRT-PCR primers<sup>b</sup></b>                                     |   |                    |   |
| G-3832   | GCA GAG ATG CCC CTT CAA CTT                 | CDP                | forward   |
| G-3833   | AGG TAG CCC ACG GAA GCA A                   |                    | reverse   |
| G-3644   | TGC GTC ATT TGC CAT CCA T                   | Lv-ago 1           | forward   |
| G-3645   | GCC ATC TGG AGC GGA GAA G                   |                    | reverse   |
| G-3851   | GAT GGC ATG AAG TCT GCA GTT G               | Lv-ago 2           | forward   |
| G-3852   | TGC GCA CGA CCA TCA CTA AG                  |                    | reverse   |
| G-3845   | GAA GCG ATT GGC AGT CTA TGA AC              | Lv-sid 1           | forward   |
| G-3846   | TGG AAG CCT ATC TCT GCA ACT TG              |                    | reverse   |
| G-3430   | GGC TTG CTA TGG TGT GCT CC                  | S3A                | forward   |
| G-3431   | TCA TGC TCT TGG CTC GCT G                   |                    | reverse   |
| <b>PCR primers for preparation of different size dsRNA<sup>c</sup></b> |   |                    | <b>amplicon length (bp)</b>   |
| G-2544   | GGA TCA AAC TCA CCT GGC TGA                 | CDP                | 50 bp with G-2544<br>100 bp with G-2544<br>150 bp with G-2544<br>200 bp with G-2544 |
| G-3836   | GCA GTC GGT GCA CCT CTC CA                  |                    |   |
| G-3837   | ATC AGT TCC TTG GTT GTG TTG                 |                    |   |
| G-3838   | ATG ATG TCA TAG TGG GAG G                   |                    |   |
| G-3839   | ATA GTT TCT AGC ACT GTT GC                  |                    |   |
| G-3840   | CTG GCA GGG CGG CGT GTC CT                  | Igv                | 50 bp with G-3840<br>100 bp with G-3840<br>150 bp with G-3840<br>200 bp with G-3840 |
| G-3841   | GCA ACC CTT CGT GGA CCA CCA                 |                    |   |
| G-3842   | GAT GTC CAG CTC GGG GGT CT                  |                    |   |
| G-3843   | CAG AGC CCG TCC AGT TCT TG                  |                    |   |
| G-3844   | TGT AGC AGA CGC TGA GGA GG                  |                    |   |