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Short versus long double-stranded RNA activation of a post-transcriptional gene knockdown pathway

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ABSTRACT
RNA interference (RNAi) utilizes a conserved cellular autoimmune defense mechanism involving the internalization of dsRNA into cells and the activation of a set of RNAi related genes. Using RNAi, complete sex reversal is achievable in males of the prawn Macrobrachium rosenbergii by knocking down the transcript level of an insulin-like androgenic gland hormone (Mr-IAG) through injections of dsRNA of the entire Mr-IAG ORF sequence (dsMr-IAG – 518bp). Interestingly, in-vivo knockdown success and dsMr-IAG lengths seemed to correlate, with long dsRNA being the most effective and short dsRNA fragments showing no effect. However, little is known about the RNAi machinery in M. rosenbergii. We discovered the Mr-Dicer and Mr-Argonaute gene families, associated with the major knockdown pathways, in our M. rosenbergii transcriptomic library. In response to dsMr-IAG administration, only post-transcriptional pathway-related gene transcript levels were upregulated. In addition, a passive dsRNA channel (a SID1 gene ortholog) that allows external dsRNA to enter cells was found. Its function was validated by observing Mr-SID1 specific upregulation dependent on dsRNA lengths, while attempted loss-of-function experiments were lethal. Our results, which suggest differential systemic responses to dsRNA lengths, provide evidence that the above RNAi-based manipulation occurs via the post-transcriptional pathway. The temporal nature of the latter pathway supports the safety of using such RNAi-based biotechnologies in aquaculture and environmental applications. Unlike reports of RNAi driven by the administration of small dsRNA fragments in-vitro, the case presented here demonstrates length dependency in-vivo, suggesting further complexity in the context of the entire organism.

Introduction

RNA interference (RNAi) has gained wide acceptance as an effective tool not only in molecular and gene function research, but also in a variety of technological applications. RNAi is based on an endogenous cellular defense mechanism against double-stranded RNA (dsRNA) viruses. Potent and specific interference at the post-transcriptional-mRNA level by using synthetic dsRNA complementary to endogenous sequences was first observed in Caenorhabditis elegans. Gene expression interference through dsRNA is achievable following in-vivo dsRNA administration through feeding, immersion and injection. Post administration, dsRNA is delivered into the cytoplasm from the extracellular matrix either by SID1, a specific dsRNA passive channel, or through endocytosis. In the cytosol, long dsRNA fragments are processed by Dicer2, a ribonuclease III–related enzyme, into 21–22-bp fragments widely known as siRNAs. These siRNAs are incorporated with the Argonaute protein (Ago), the main component of the multiprotein RNA-induced silencing complex (RISC). In its unwound state, the antisense of the dsRNA facilitates specific identification of the mRNA targeted for endonucleolytic cleavage.

In general and similar to other non-coding RNAs (ncRNAs), dsRNA molecules are involved in many cellular pathways for regulating eukaryote gene expression during various life stages. These ncRNAs take part in different cellular pathways and are all associated with Ago protein family members. Interference with gene expression takes place not only through the post-transcriptional, siRNA pathway, it can also occur at the transcriptional level, via the miRNA pathway, in which small ncRNAs are processed into miRNA molecules. Such miRNAs could regulate gene expression in the post-transcriptional pathway involving Dicer2 and Ago2. On the other hand, miRNA is also recognized by nuclear proteins, such as Dicer1, resulting in the generation of siRNAs that interact with Ago1, the main component of the RNA-induced initiation transcriptional silencing (RITS) complex. These proteins recruit nascent chromatin and chromatin modifying complexes resulting in chromatin remodeling and heterochromatic silencing. Ago family proteins are also involved in ncRNAs associated with transposable element regulation pathways, in which Ago3, a protein that plays an important role in transcriptional regulation, participates.
Using the RNAi method, a biotechnological application was developed in the aquaculture field for the culture of preferred all-male monosex populations of the giant freshwater prawn, *Macrobrachium rosenbergii.*22,23 This species exhibits a dimorphic growth pattern, in which males grow faster and reach larger final sizes than females at harvest, rendering the culture of all-male populations economically beneficial.24,25

In crustaceans, sex differentiation is regulated by the androgenic gland, a male specific endocrine organ. The activity of the gland is mediated in decapod crustaceans through the insulin-like androgenic gland hormone (IAG).26,27 Administration of dsRNA complementary to the entire open reading frame (ORF) sequence of *M. rosenbergii-IAG* (*Mr-IAG ORF ~ 518-bp*) causes a dramatic knockdown that leads to an approximately 99% decrease in transcript level.22,28 At the phenotypic level, *Mr-IAG* knockdown causes the cessation of spermatogenesis and the inhibition of secondary masculine sex character development.28 Furthermore, dsMr-IAG injection at a critical juvenile stage causes a full and functional sex reversal of genetic males (ZZ) into ‘neo-females’. When crossed with regular males, such neo-females produce the preferred all-male progeny.22

As in many other eukaryotic species, in several crustacean species the above-mentioned knockdown pathways are highly conserved and have important roles in gene transcriptional and post-transcriptional regulation, viral defense and the suppression of transposable elements.29,30 The current study examined the main RNAi associated genes, members of the Mr-Dicer family based on putative amino acid sequences showing cluster deviations for both Dicer1

**Results**

**Effect of dsRNA fragment lengths shorter than the full ORF on Mr-IAG knockdown efficiency**

Attempts to knock down *Mr-IAG* expression levels using shorter dsMr-IAG fragments showed a clear correlation between dsRNA fragment length and *Mr-IAG* knockdown efficiency, i.e., the longer the dsRNA fragment, the greater the knockdown of *Mr-IAG* expression levels (Fig. 1). Of the four lengths tested, only the 250-bp dsRNA fragment caused a significant knockdown effect similar to the entire ORF (~99% decrease compared with the negative control, $F_{(2,15)} = 18.871$, P-value < 0.0001). The relative *Mr-IAG* expression levels while using the 250-bp fragments were significantly different from that of the negative control and strikingly similar to the effects of the entire ORF sequence (P-value < 0.001 and P-value = 0.93, respectively). A pair of dsRNA fragments of 100-bp (Serial no. 100_2 and 100_5, Fig. S1A) caused ~75% decrease in expression, which was significantly different from expression levels in both the negative control group and the entire ORF group (P-value = 0.034 and P-value < 0.001, respectively).

Attempts to improve the knockdown efficiency of the 100-bp fragments by covering the entire ORF sequence with a mixture of 100-bp fragments did not yield better results (See Fig. S2). A similar mixture of 75-bp dsRNA fragments caused a minor decrease in *Mr-IAG* transcript level, but statistically, that result was similar to those of both the negative control and the entire ORF injected groups (P-value = 0.37 and 0.178, respectively).

**Presence and phylogeny of knockdown-pathway-related genes in *M. rosenbergii***

To understand the difference in *M. rosenbergii* in the activation of RNAi and its dependence on dsRNA length compared with its activation in other RNAi pathways, we examined the main RNAi associated genes, members of the *Dicer* and *Argonaute* families. Mining our *M. rosenbergii* transcriptomic library resulted in multiple sequence alignments with the Mr-Dicer family based on putative amino acid sequences showing cluster deviations for both Dicer1
and Dicer2 (Fig. 2A). *M. rosenbergii* Dicer1 (Mr-Dicer1) showed homologies to *Marsupenaeus japonicus* Dicer1, *Penaeus monodon* Dicer1, *Litopenaeus vannamei* Dicer1 and *Drosophila melanogaster* Dicer1 (83%, 71%, 70% and 67%, respectively). Mr-Dicer2 showed homologies to Lv-Dicer2, Pm-Dicer2 and Dm-Dicer2 (51%, 51% and 27%, respectively). Homology of 32% was found between the Mr-Dicer paralogs.

Phylogenetic analysis based on multiple amino acid sequence alignments of 3 Ago candidates demonstrated deviations into only two clusters: cluster A for Ago2 and Ago3, and cluster B for Ago1 (Fig. 2B). Mr-Ago1 showed significant homology to Ago1 orthologs from other crustacean species such as *Panulirus interruptus* Ago1, Mj-Ago1 and Lv-Ago1 (99%, 98% and 98%, respectively) and to Dm-Ago1 (86% homology). The cases of Argonaute2 and Argonaute3 were difficult to differentiate by phylogenetic analysis. Homologies of 43% and 38% were noted between Mr-Argonaute paralogs of Mr-Ago1 to Mr-Ago2 and Mr-Ago3, respectively. A higher, homology (48%) was found between Mr-Ago2 and Mr-Ago3.

To elucidate which knockdown pathway was activated as a response to dsMr-IAG manipulation, dsMr-IAG was administered and RNAi associated gene induction (Dicer and Ago families) was tested. Mr-IAG expression levels were significantly lower, ~99% of control (t(13) = 18.608, p-value < 0.0001), as a result of dsMr-IAG injection (Fig. 3A). Comparing the expression levels of genes related to the RNAi mechanism between the group injected with dsMr-IAG to a non-injected group demonstrated that Mr-Dicer2 was significantly upregulated in the injected group (t(16) = -4.873, p-value < 0.01) (Fig. 2B) while Mr-Dicer1 expression levels were similar to those in the non-injected group (p-Value = 0.128). Similarly, in the Ago gene family, only one candidate, Mr-Ago2, was upregulated in response to dsMr-IAG injection (p-value < 0.001) (Fig. 3B), while Mr-Ago1 and Mr-Ago3 expression levels were similar to those in the non-injected group (t-test, t(13) = -1.091, P-value = 0.295 and Mann-Whitney U-test, P-value = 0.779, respectively).

**Mr-SID1, knockdown pathway and dsRNA length**

Mr-SID1, which encodes for a passive channel with specificity to dsRNA molecules, was found by comparing the *L. vannamei* SID1 amino acid sequence to our *M. rosenbergii* transcriptomic library, resulting in a transcript with 52% homology. This transcript (Mr-SID1) was fully sequenced, and 11 transmembrane regions were predicted in its putative protein sequence by a bioinformatics hydrophobicity evaluation (Fig. S3A). Phylogenetic analysis demonstrated that SID1 is conserved between different arthropod species (Fig. S3B).

With the aim of confirming the function of Mr-SID1, its expression levels were measured in response to administration of dsRNA and DNA (both of GFP sequence) and PBS as a negative control (Fig. 4A). Mr-SID1 was upregulated only in the presence of dsRNA (F(2,19) = 7.19, p-value = 0.004), showing the specificity of Mr-SID1 to dsRNA. Furthermore, loss of function of Mr-SID1 showed a lethal effect after two consecutive injections of dsMr-SID1 and dsGFP (Fig. 4B). In all the control groups, including PBS, dsGFP and dual injections of two exogenous dsRNA (dsGFP + dsRB), high survival rates were demonstrated (100%, 100% and 87%, respectively). All the dsMr-SID1 and dsGFP injected individuals died during the 24 h following the second injection.

To conclude the study, expression levels of Mr-SID1 and the other components of the RNAi mechanism were tested in response to different fragment lengths of dsMr-IAG based on the same length dependency experiments shown in Fig. 1. The correlation clearly showed that dsMr-IAG fragment length was positively correlated with Mr-SID1 upregulation (Fig. 5A). A significant induction of Mr-SID1 expression levels was measured by dsMr-IAG fragment lengths of 100-bp (P-value = 0.01) and 250-bp (P-value = 0.002), and this
activity was similar to the upregulation of Mr-SID1 expression levels following injections of the entire ORF (P-value = 0.003 and 0.027, respectively). The expression of a mixture of 19-bp fragments did not cause Mr-SID1 induction, whose expression levels were similar to that of the negative control. The expression levels of Mr-SID1 following the injection of 75-bp fragments of dsMr-IAG were different from those of both the negative control and the entire ORF (P-value < 0.001, for both).

The same correlation shown above with respect to Mr-SID1 and different dsMr-IAG fragment lengths was also found for Mr-Dicer2 with the exception of the induction caused by 100-bp dsRNA fragments (Fig. 5B). The expression of Mr-Dicer2 in response to 100-bp fragments of dsRNA were significantly higher than the entire ORF injected (P-value = 0.041), and those of both the 100-bp fragment and the entire ORF were significantly higher than that of the non-injected negative control group (P-value < 0.001, both). Administrations of 75-bp and 250-bp fragments of dsRNA elicited higher Mr-Dicer2 expression levels than those of the non-injected group (P-value = 0.005 and P-value < 0.001, respectively) and, in contrast to the results obtained for the 100-bp fragments, showed similar Mr-Dicer2 expression levels to that obtained in response to injection of the entire ORF (p-value = 0.7 and 0.98, respectively). In response to administration of the mixture of 19-bp fragments, the expression levels of Mr-Dicer2, similar to those of Mr-SID1, were not affected, and its expression levels were similar to those in the non-injected group (p-value = 0.99).

As a reference, we used the expression levels of Mr-Dicer1 from the miRNA pathway. This gene remained at the same low levels in all the treatments, thus demonstrating the absence of a correlation with dsMr-IAG fragment length (Fig. 5C).

Discussion

The Mr-IAG knockdown pathway

Full and functional sex reversal of male crustaceans into females caused by the use of RNAi demonstrates the vast potential of RNAi-based biotechnologies in aquaculture and environmental applications. The use of such a biotechnology was previously suggested to be safe based on the temporal nature of the intervention and its lack of any overtly apparent long-term consequences. These studies presented evidence based on social structure, growth performance and masculine reproductive characters at the anatomic and molecular levels. Moreover, our observations that the elapsed times until disappearance of the exogenous dsRNA from prawn tissues and the return of Mr-IAG transcript levels to normal during the post silencing period were comparable, suggesting that RNAi-based technology is temporal and indeed safe. The current study, which examined the mechanism of the knockdown pathway, provided additional support for the temporal nature of the RNAi-based production of all-male cultures and rejects the possible involvement of permanent or epigenetic interference processes on the genome associated with the transcriptional silencing pathway.

Three known RNAi pathways, i.e., micro RNAs (miRNA), short interfering RNA (siRNA) and PIWI interacting RNA (piRNA), are associated with Argonaute protein family members. In the current study, the Dicer and Ago homologs were discovered and fully sequenced in M. rosenbergii. The clustering of each homolog to other members from related species further supports the correct identification of the distinct Mr-Dicer and Mr-Ago members allowing the examination of the expression of each homolog transcript level to dsMr-IAG administration. Only transcripts of Mr-Dicer2 and Mr-Ago2, essential components for RNAi response and post-transcriptional silencing, were significantly increased after dsMr-IAG injection. Other transcripts in these families, such as Mr-Ago1 and Mr-Dicer1 associated with nuclear transcriptional silencing or Ago3 associated with piRNA, were not affected by the introduction of dsMr-IAG. Since the Dicer2 and Ago2 proteins are known to be involved in the post-transcriptional pathway where they exert temporal cytoplasmic effects, the results of the present study support both the temporal nature of the effects of dsMr-IAG administration and the safety of its use in aquaculture and environmental applications.
lower the Mr-IAG transcript levels. A similar tendency was demonstrated in D. melanogaster.\textsuperscript{6} To gain insight into this phenomenon, different dsMr-IAG fragment lengths were evaluated for their effects on the expression levels of genes associated with the post-transcriptional knockdown pathway.

To investigate the mechanism of action, Mr-SID1, a passive transmembrane channel that specifically delivers dsRNA into somatic cells,\textsuperscript{5,7} was sequenced and aligned to orthologs from arthropod species. Mr-SID1 is the main gate for passive dsRNA uptake into the cell and indeed its transcript levels were only upregulated in the presence of dsRNA. The results are supported by those of Shih and Hunter,\textsuperscript{7} who examined the differences in membrane conductance in SID1-expressing cells in the presence of dsRNA and DNA and showed that conductance changed only in the presence of dsRNA. Based on the amino acid homology of Mr-SID1 to other species with the same predicted 11 transmembrane regions and based on the results of the functional test of Mr-SID1, we suggest that Mr-SID1 is the dsRNA-gated channel in M. rosenbergii.

Yet SID1 is not the only way known for dsRNA delivery into the cytosol. Many metazoan cells can deliver an exogenous dsRNA via receptor mediated endocytosis, an uptake mechanism that seems to be evolutionarily conserved.\textsuperscript{8} To determine whether Mr-SID1 is the main avenue for the delivery of dsRNA into M. rosenbergii cells, we attempted to knock down SID1, after which we planned to try SID1 knockdown in parallel with dsMr-IAG administration. The latter experiment, however, could not be performed since the effect of dsMr-SID1 administration was lethal unlike the cases of other documented RNAi-associated genes.\textsuperscript{40,41} It should be noted that dsMr-SID1 was designed based on a wide M. rosenbergii transcriptomic library\textsuperscript{42} and found to be specific to the Mr-SID1 sequence, thereby minimizing the probability of off-target effects. However, due to the lack of a full genome sequence in our study organism, lethality caused by off-target effects could not be completely ruled out. In addition, while we also cannot rule out the possibility that dsRNA was internalized through endocytosis, we suggest that SID1 is a major component in dsRNA trafficking. This is supported by results from SID1 non expressing D. melanogaster S2 cells, in which dsRNA is endocytotically delivered,\textsuperscript{8} while the expression of SID1 in those cells increases the efficiency of gene knockdown.\textsuperscript{6} In the M. rosenbergii system, the presence and differential activity of Mr-SID1 seem to suggest that it plays a crucial role in regulation of systemic RNAi and the cell’s defense mechanism against viruses.

The present study showed a positive correlation between injected dsMr-IAG fragment length and Mr-SID1 transcript induction level. In contrast to our findings, another study that examined C. elegans SID1 expression in D. melanogaster S2 cells did not find it to be selective to dsRNA length.\textsuperscript{43} The authors of the latter article suggested that dsRNA length dependency probably does not occur through SID1.\textsuperscript{43} It is thus suggested that the tendency of Mr-SID1 transcripts to respond to dsRNA lengths is regulated by factors downstream, and not upstream, of the dsRNA delivery, and that it is not self-regulated by the transmembrane channel itself. It is also suggested that dsRNA delivery depends on the dsRNA retention ability of the Dicer-RISC complex. Insofar as SID1 is a passive channel, dsRNA retention by Dicer-RISC is essential to allow the

\textbf{Post-transcriptional knockdown pathway and dsRNA length}

The current study also focused on the ability to knock down Mr-IAG using fragments with lengths shorter than the entire ORF. Gene knockdown in various organisms was demonstrated using short dsRNA fragments (siRNA) for loss of function procedures, mostly in-vitro.\textsuperscript{37-39} Thus, we attempted to knock down Mr-IAG in-vivo using siRNA. These attempts failed, eliciting the need to examine the correlation between dsRNA length and knockdown success. A clear relationship between dsMr-IAG lengths and Mr-IAG transcript levels was found, in which the longer the administered dsMr-IAG fragment, the
intra-cellular dsRNA concentration to decrease, resulting in more dsRNA molecules passing into the cell through SID1 according to intra- and extra-cellular dsRNA concentrations.\textsuperscript{43} Mr-Dicer2 transcript levels also changed in response to ds\textsuperscript{Mr-IAG} length but without a clear, linear tendency as was found for Mr-SID1. In the case of Mr-Dicer2, the highest upregulation was obtained with dsRNA fragments of 100-bp compared with the other fragment sizes. Moreover, from among the fragment lengths tested, only the 19-bp fragments failed to cause any Mr-Dicer2 upregulation. These results seem to contradict observations of knockdown success, which was better for fragment lengths of 250-bp and 500-bp. It is suggested that Mr-Dicer2 is influenced by the ability of SID1 to deliver the dsRNA molecules and that the level of induction depends on the number of molecules. Among the solutions of the different fragments at the same dsRNA concentration (in this study 5 \( \mu \text{g/g body weight} \)), that with the 100-bp fragments contains many more molecules, and this could explain the finding that the highest upregulation obtained here was for Mr-Dicer2.

Concluding remarks

For the first time, the Dicer and Argonaute genes were fully sequenced in \textit{M. rosenbergii}, which enabled us to elucidate the knockdown pathway of Mr-IAG, the target of the manipulation that causes the full and functional sex reversal of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{RNAi-associated gene expression levels following injection of Mr-IAG dsRNA of different lengths (19-bp, 75-bp, 100-bp, 250-bp, and entire ORF – 518-bp). Mr-SID (A), Mr-Dicer2 (B) and a reference gene, Mr-Dicer1, from the miRNA pathway (C). Expression levels were tested in the RNA extracted from the length dependency experiments (Fig. 1).}
\end{figure}
M. rosenbergii males into females. This study showed that the dsMr-IAG-based manipulation manifests through a temporal, post-transcriptional pathway, the well-known siRNA pathway. The temporal nature of the latter pathway further supports the safety of using such RNAi-based biotechnologies in aquaculture and environmental applications.

In this study, RNAi-based manipulation of the crustacean insulin-like androgenic hormone also demonstrated a clear dependency on dsRNA molecule length, such that dsMr-IAG fragment length was negatively correlated with Mr-IAG transcript level. Moreover, the induction of genes associated with the post-transcriptional pathway and SID1 was also differentially influenced according to dsMr-IAG fragment length. Unlike numerous clear cases of RNAi driven by the administration of small dsRNA fragments in-vitro, the case presented here demonstrates length dependency when the experiments are performed in-vivo. Our results thus support the need for further study of RNAi phenomena in the context of the entire organism.

Materials and methods

Animals

M. rosenbergii prawns of the BGU-line were maintained at Ben-Gurion University of the Negev (BGU) facilities as described by Shpak et al. The all-male progenies originating from crosses between males and ‘neo-females’ were supplied by the Tiran Shipping group through its subcontractor hatchery, Colors Ltd., Hatzeva, Israel. This hatchery holds only genetic males form the BGU line, and genetic females have never been allowed at this farm.

All dsRNA length dependency experiments were performed using small males whose morphotype was determined according to Kuris et al. It should be noted that small males are sexually active and have an active androgenic gland.46

Mr-SID1 loss of function and specific induction of Mr-SID1 by dsRNA experiments were performed using post larvae all-male progeny from the all-male hatchery mentioned above.

Synthesis of double-stranded RNA

Eight dsRNA fragments of 19-bp and two fragments of 75-bp from the Mr-IAG mRNA sequence were synthesized and supplied by BioSpring, Frankfurt, Germany (Fig. S1A). Five dsRNA fragments of 100-bp covering approximately the entire Mr-IAG ORF and one fragment of 250-bp were synthesized in-vitro in our laboratory (Fig. S1A). A pGEM®-T Easy plasmid containing the Mr-IAG open reading frame sequence served as the template for dsMr-IAG synthesis. The template was amplified by PCR, primed by two gene-specific primers with a T7 promoter site at the 5’ of one primer (T7P) (see primers and T7 promoter sequences for dsRNA synthesis in Table S1). Primer pairs were as follows: the sense strand was synthesized using primer T7P forward vs. reverse primer, while the anti-sense strand was synthesized by T7P reverse vs. forward primer. PCR amplicons were electrophoresed on a 1.3% agarose gel and visualized with ethidium bromide and UV light, excised from the gel, and purified with a Accuprep® PCR purification Kit (BIONEER Co., Daejeon, South Korea).

The TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Lithuania) was used to generate single-stranded RNA according to the manufacturer’s instructions. RNA molecules were purified by phenol-chloroform (1:1) and ammonium-acetate and precipitated with ethanol. Sense and antisense strands were hybridized by incubation at 70°C for 15 min, 65°C for 15 min and at RT for 30 min. dsRNA quality was assessed on an agarose gel and diluted to 5 μg/μl. dsRNA was kept at −80°C until used.

Mr-IAG knockdown attempts using fragments shorter than the entire ORF

Four distinct experiments were performed, one for each dsRNA length (19-bp, 75-bp, 100-bp, and 250-bp). Each experiment included a non-injected group as a negative control and an entire dsMr-IAG ORF injected group as a positive control. It is important to note that the determination of which animals would constitute the non-injected control groups was based on previous studies, which also used exogenous dsRNA or DDW injection as described in Lezer et al. and in Sharabi et al. with similar results. Each experimental group included 6–8 small M. rosenbergii males (Mean body weight = 12.5 g). Single injections of the different dsMr-IAG lengths (5 μg dsRNA/ gr body weight) were performed once, and then 48 hours post-injection, prawns were anesthetized in ice cold water, dissected and total RNA was extracted from their androgenic glands using the EZ-RNA Total RNA Isolation Kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer’s instructions. cDNA was prepared in a reverse-transcriptase reaction containing 1 μg total RNA using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Relative quantification of Mr-IAG expression levels was obtained as described in Ventura et al.47 with the FastStart Universal Probe Master (Rox; Roche Diagnostics GmbH) and Universal ProbeLibrary Probe 144 (Roche). Mr-18S (GenBank accession no. GQ131934) was used as a normalizing gene and quantified using specific primers as described in Ventura et al.47

RNAi associated gene mining

RNAi associated-genes were mined from our composite M. rosenbergii transcriptomic library, which contains more than 290 million reads assembled into 108212 contigs, fully described by Ventura, et al.47 and Sharabi, et al.42 The mining was done based on orthologs from related crustacean species using a BLAST search to reveal Dicer and Ago homologs and SID1 sequences. M. rosenbergii-Dicer1 (Mr-Dicer1) was compared with P. monodon Dicer1 (GenBank accession number ABR14013.1) and Mr-Dicer2 was compared with L. vannamei Dicer2 (GenBank accession number ACP96960.1). Mr-Argonaute1 was compared with M. japonicus Argonaute1 (GenBank accession number ADB44074.1). Mr-Argonaute2 was compared with M. japonicus Argonaute2 (GenBank accession number AB665954.1). Mr-Argonaute3 was compared with P.
monodon Argonaute3 (GenBank accession number AGC95229.1). *M. rosenbergii-SID1* was compared *L. vannamei-SID1* (GenBank accession number HM234688.1).

Putative sequences obtained from the library were computationally translated using the ExPaSy Proteomics Server (http://ca.expasy.org/tools/dna.html), and the longest open reading frame was selected. Transmembrane regions of *Mr-SID1* were evaluated using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/).

**In-vitro validation and sequencing**

Total RNA was extracted from the hepatopancreas of dsMr-IAG-injected [5μg/μl] animals, while anesthetized in ice-cold water, 48 h post-injection. Total RNA was isolated and cDNA was prepared as described above.

Putative *Mr-Dicers* (*Mr-Dicer1,2*), *Mr-Agos* (*Mr-Ago1,2,3*) and *Mr-SID1* sequences were in-vitro validated by PCR using gene specific primers (Table S2). The PCR products were cloned and sequenced as described by Ventura, et al.28 *Mr-Dicer2* and *Mr-SID1* 5' end were obtained by 5' rapid amplification of cDNA ends (RACE 5') performed with the Clontech SMART RACE kit (BD Biosciences, Palo Alto, CA) following the manufacturers protocol. PCR was performed using a gene-specific reverse primer (Table S3) and the Universal Primers Mix (UPM) provided in the kit. To confirm the obtained sequence, 5' RACE product was amplified by PCR using a reverse nested primer closer to the 5' end (Table S3) and a UPM nested primer, also provided in the kit. RACE 5' and nested 5' products were cloned and sequenced as described above. To ensure the quality of the sequence, each region of the gene was sequenced four times.

The 3' end of *Mr-Ago3* was obtained by 3' rapid amplification of cDNA ends (RACE) as described above using a gene-specific forward primer (Table S3) and a UPM provided in the kit, and the PCR products were cloned and sequenced. To confirm the obtained sequence, the 3' RACE product was amplified by PCR using a forward nested primer closer to the 3' end (Table S3). This product was cloned and sequenced as described above. To ensure the quality of the sequence, each region of the gene was sequenced four times.

**Phylogenetic analysis**

Known sequences of Dicer and Ago from related Crustacean species and Sidi1 sequences from different species were obtained from the GenBank database. A phylogenetic neighbor-joining tree was constructed using Mega 6.0 software (Molecular Evolutionary Genetics Analysis, version 6.0) according to ClustalW multiple sequence alignments of the putative amino acid sequences of Dicer, Argonaute and SID1. Bootstrap values (%) of 1000 replicates were calculated for each node of the consensus tree attained.

**Mr-IAG knockdown pathway determination**

To elucidate the *Mr-IAG* knockdown pathway, 15 small *M. rosenbergii* males (mean body weight ~15 gr) were divided into two groups, the non-injected group (n = 8) and the injected group (n = 7), whose members were injected with the entire dsMr-IAG ORF [5μg dsRNA/gr body weight] by a micro injector. Androgenic glands and hepatopancreases were dissected for total RNA extraction, 48 h post injection as described above. Total RNA was isolated and cDNA was prepared as described above. *Mr-IAG* expression levels were detected from total RNA extracted from androgenic glands to verify *Mr-IAG* knockdown success. Relative quantification of *Mr-IAG* expression levels was obtained as described above.

Relative quantification of *Mr-Dicer* (*Mr-Dicer1,2*) and *Mr-Argonaute* (*Mr-Ago1,2,3*) expression levels were tested using the total RNA isolated from the hepatopancreas, preliminarily showing high expression levels. *Mr-Dicers* and *Mr-Argonautes* expression levels were obtained using forward and reverse primers (Table S4) with FastStart Universal Probe Master (Rox; Roche Diagnostics GmbH) and Universal ProbeLibrary Probes (Roche) (Table S4). For all these relative quantifications, *Mr-18S* was also quantified by real-time RT PCR as a normalizing gene as described above.

**Double-strand RNA and Mr-SID1 induction**

To confirm the role of *Sidi1* in *M. rosenbergii*, juvenile males (n = 22) (mean weight = 0.22 gr) were divided into three groups: phosphate-buffered saline (PBSX1) injected (n = 8), DNA-GFP injected (n = 7) and dsRNA-GFP injected (n = 7). dsRNA-GFP was synthesized as described above. DNA fragments that contained the same GFP sequence were amplified by PCR. PCR products were purified using NucleoSpin® Gel and PCR Clean-up (MACHEERY-NAGEL GmbH & Co., Düren, Germany). The procedure included two injections of 5μg per g body weight each every 24 h. Prawns were anesthetized in ice cold water and their hepatopancreases were dissected 24h post second injection. Total RNA was extracted and cDNA was prepared as described above. Relative quantification of *Mr-Sid1* expression levels was performed as described above (for primers and probe, see Table S4).

**The effect of Mr-SID1 knockdown**

With the aim of examining the crucial role of *Mr-SID1* to gene knockdown in *M. rosenbergii*, the effect of *Mr-SID1* knockdown was studied. Forty juvenile males (mean body weight = 0.67 gr) were divided to four groups: DDW injected, dsGFP injected, dual injection group of dsRB and dsGFP (two exogenous dsRNA) and dual injection group of dsGFP and dsMr-SID1 (the purpose of dsGFP was to induce *Mr-Sid1* expression level). The procedure included two injections of 10μg mixed dsRNA per g body weight with 24 h interval and survival rates were monitored. Synthesis of dsGFP was done as described above. dsRB, an exogenous gene, was synthesized as described by Lezer et al.35 The synthesis of dsMr-SID1 was done as described above using specific primers (Table S1).

**RNAi-associated genes expression levels as a response to different dsMr-IAG length**

RNAi-associated gene expression levels (*Mr-SID1*, *Mr-Dicer2* and *Mr-Dicer1*) were measured using the same RNA isolated...
from the prawns that was used in the Mr-IAG knockdown attempts, with the shorter dsMr-IAG fragments described above including control groups. Mr-SID1, Mr-Dicer2 and Mr-Dicer1 were quantified by real-time RT-PCR using the above described primers and Universal ProbeLibrary Probes ve.

Statistical analysis

Final real-time RT-PCR relative quantification values (RQ) were calculated using the formula $2^{-\Delta \Delta Ct}$ (built-in feature in the ABI Prism 7300 Sequence Detection System, Applied Biosystems) for analyzing qPCR results. The RQ values in the different experiments were analyzed by Statistica v12.0 software (StatSoft, Ltd., Tulsa, OK, USA). For all multiple comparisons, the tests were selected according to assumptions of homogeneity of the variances through Levene’s test and normal distribution of the residuals as accepted. For all comparisons between two groups, the tests were selected according to the normal distribution of the samples and the homogeneity of the variances through a P-variance test.

The results of knockdown pathway elucidation experiments were analyzed using a t-test for independent variables for Mr-IAG, Mr-Dicer2 and Mr-Ago1 and the Mann-Whitney U-test for the results of Mr-Dicer1, Mr-Ago2 and Mr-Ago3. The results of evaluations of Mr-IAG response to shorter dsRNA fragment lengths were analyzed using One-way ANOVA after log(X) transformation and Post-hoc Tukey HSD for 100-bp and 250-bp experiments, One-Way ANOVA using welch correction and Tukey HSD for unequal samples for the 75-bp experiment, and Kruskal-Wallis was used for the 19-bp mixture experiment. The differences in Mr-SID1 expression levels were analyzed using One-way ANOVA after log(X+1) transformation and Post-hoc Tukey HSD. The correlation between dsRNA lengths and Mr-SID1 expression were analyzed using the Kruskal-Wallis test for the 19-bp, 100-bp and 250-bp experiments. One-Way ANOVA using welch correction and Tukey HSD for unequal samples were used to examine the correlation between 75-bp fragments and the Mr-SID1 transcript. The correlation between dsRNA length and Mr-Dicer2 were analyzed using One-way ANOVA after log(X+1) transformation and Post-hoc Tukey HSD.

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