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Nuclease activity decreases the RNAi response in the sweetpotato weevil *Cylas puncticollis*

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ABSTRACT

RNA interference (RNAi) refers to the process of suppression of gene expression in eukaryotes, which has a great potential for the control of pest and diseases. Unfortunately, the efficacy of this technology is limited or at best variable in some insects. In the African sweet potato weevil (SPW) *Cylas puncticollis*, a devastating pest that affects the sweet potato production in Sub-Saharan Africa (SSA), the RNAi response was highly efficient when dsRNA was delivered by injection, but it showed a reduced response by oral feeding. In the present study, the role of nucleases in the reduced RNAi efficiency in *C. puncticollis* is investigated. Several putative dsRNases were first identified in the transcriptome of the SPW through homology search and were subsequently further characterized. Two of these dsRNases were specifically expressed in the gut tissue of the insect and we could demonstrate through RNAi experiments that these affected dsRNA stability in the gut. Furthermore, RNAi-of-RNAi studies, using *snf7* as a reporter gene, demonstrated that silencing one of these nucleases, *Cp-dsRNase-3*, clearly increases RNAi efficacy. After silencing this nuclease, significantly higher mortality was observed in dssnf7-treated insects 14 days post-feeding as compared to control treatments, and the gene downregulation was confirmed at the transcript level via qPCR analysis. Taken together, our results demonstrate that the RNAi efficiency is certainly impaired by nuclease activity in the gut environment of the SPW *Cylas puncticollis*.

1. Introduction

Since the discovery of RNA interference (RNAi) by Fire and Mello in 1998, RNAi rapidly caught great attention for the functional characterization of genes, and also for its application as a biological tool in crop protection (Bellés, 2010; Huvenne and Smagghe, 2010). For the implementation of RNAi as a pest control method, exogenous dsRNA needs to be ingested by the insect and remain stable long enough while it is taken up from the gut lumen to the midgut cells in order to trigger the RNAi process. However, not all insects are efficient in responding to RNAi. Coleopteran insects are particularly more efficient (Baum et al., 2007; Zhu et al., 2011; Ivashuta et al., 2015), while a considerably more variable response is observed in dipteran, lepidopteran and hemipteran insects (Whyard et al., 2009; Terenius et al., 2011; Christiaens and Smagghe, 2014; Taning et al., 2016; Joga et al., 2016). Different biological factors related to the variability of RNAi in insects were proposed, such as the malfunction of the RNAi machinery components, reduced dsRNA uptake by the cells, the variability of gut pH and the presence of double-stranded ribonucleases (dsRNases) in the digestive system of insects (Christiaens et al., 2018; Khajuria et al., 2018). The latter is increasingly seen as one of the major factors that limit the RNAi efficiency in recalcitrant insects by affecting the dsRNA integrity in body fluids (Terenius et al., 2011; Shukla et al., 2016; Wang et al., 2016).

The activity of dsRNases has been observed in several insect species. A DNA/RNA non-specific nuclease was identified and characterized in digestive juice from *Bombyx mori*, indicating that it is secreted in the gut lumen for nucleic acid digestion (Arimatsu et al., 2007a; 2007b; Liu et al., 2012). Additionally, this nuclease was found to be expressed in other tissues of the silkworm as well, such as epidermis, fat body, brain, silk glands, among others, presumably with additional functions than only extracellular digestion (Liu et al., 2012). DsRNases were also implicated to be active in saliva of the tarnished plant bug *Lygus lineolaris* (Allen and Walker, 2012), in hemolymph of *Manduca sexta* (Garbutt et al., 2013) and in both saliva and hemolymph in *Acyrthosiphon pisum* (Christiaens et al., 2014). Suppression of a specific *dsRNase* gene in the locusts *Schistocerca gregaria* and *Locusta migratoria*, led to a reduction of the dsRNA degrading activity (Wynant et al., 2014; Song et al., 2017). In the lepidopteran *Ostrinia furnacalis*, knockdown of the RNAi efficiency-related nuclease (REase) increased the RNAi efficiency in this

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insect, while its upregulation suppressed the RNAi effects in transgenic *Drosophila melanogaster* (Guan et al., 2018).

Intracellular dsRNases may also degrade the dsRNA and interfere with the RNAi effects in insects, such as Eri-1 homologous, SDN1-like and Nibbler proteins. However, the interaction of these nucleases with RNAi remains unclear. For instance, there is evidence that Eri-1 affects the sensitivity to dsRNA in several tissues of the nematode *Caenorhabditis elegans* (Kennedy et al., 2004), while in *T. castaneum* and *D. melanogaster*, which exhibit a robust RNAi response, Eri-1-like nuclease is categorized into another subclass and does not affect the RNAi sensitivity (Kupsco et al., 2006; Tomoyasu et al., 2008).

The African sweetpotato weevil (SPW) *Cylas puncticollis* poses a major threat to sweetpotato, a crop with great importance to improve the food security of Sub-Saharan African countries. The damage caused by SPW can result in total crop loss especially during periods of pronounced droughts. Larvae are considered the most devastating life stage due to the extensive tunneling by the larvae inside the storage roots, promoting the development of toxic compounds such as phenols and terpenes (Chalfant et al., 1990; Ebregt et al., 2007). This pest exhibits a strong RNAi response when dsRNA is injected into the hemocoel, but it does not efficiently respond when dsRNA is orally delivered (Prentice et al., 2017). In addition, the authors observed that the dsRNA was degraded in the presence of isolated gut juice from *C. puncticollis* larvae, which occurred more rapidly than the degradation patterns in *Cylas brunneus* and *Leptinotarsa decemlineata* gut juice (Prentice et al., 2017). The latter two insects were highly sensitive to RNAi when dsRNA was administered by feeding (Christiaens et al., 2016; Cappelle, 2017).

In an effort to conclude whether nucleases in *C. puncticollis* cause a lower sensitivity to RNAi, the current study aimed to identify the aforementioned nucleases in *C. puncticollis* and to investigate their tissue-specific expression profiles. These nucleases were then targeted for suppression of their expression using RNAi via injection and the effects were assessed through *ex vivo* gut juice incubation with dsRNA. Next, an “RNAi-of-RNAi” assay was performed in order to characterize the effect of the nucleases on RNAi efficacy in SPW. In these experiments, larvae were first injected with dsRNA targeting nucleases and subsequently, the treated insects were exposed to orally delivered dsRNA targeting *Snf7* as a reporter gene. These results provide a better understanding of the low sensitivity to oral RNAi in the SPW *C. puncticollis*.

2. Material and methods

2.1. Cylas puncticollis colony

A *Cylas puncticollis* colony obtained from the National Crops Resources Research Institute NaCCRI (Namulonge, Uganda) was established in plastic cages at standard laboratory conditions of 27 °C, 65% relative humidity, and a light:dark regime of 16:8 h. Insects were kept for feeding and oviposition on sweetpotato storage roots. Fresh storage roots were added every 3 days in order to obtain second instar larvae for injection and feeding assays. Larvae were removed from the roots at 7–9 days after oviposition.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the whole body of second instar *C. puncticollis* larvae. The tissues were homogenized in RLT buffer + β-mercaptoethanol using the RNeasy Mini Kit (Qiagen) following the manufacturers' instructions. The extracted RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and the RNA integrity was verified by 1.5% agarose gel electrophoresis. Then, RNA was treated with DNase I (Invitrogen) to remove remaining DNA. 1 μg of total RNA was used to synthesize complementary DNA (cDNA) using the Superscript II First strand cDNA synthesis kit (Invitrogen) following the manufacturer's protocol. Total RNA and cDNA samples were stored at −80 °C and −20 °C, respectively.

2.3. Validation of candidate reference genes and RT-qPCR primers design

Seven candidate reference genes were selected from the transcriptome data of *C. puncticollis* according to their stable expression evaluated in other insect experiments (Hiel et al., 2009; Yu et al., 2012; Niu et al., 2014). These genes were *glyceraldehyde 3 phosphate dehydrogenase 2* (*GAPDH*), *α-tubulin at 84B* (*Tub*), *ubiquitin 63E* (*Ubi*), *elongation factor1-α1* (*eEF1*), *β-actin* (*Actin*), *ribosomal protein L32* (*Rpl32*) and *peptidylprolyl isomerase A* (*PPIA*). First, primer sets were analyzed by primer annealing efficiency and melting curve using serial dilutions of cDNA and carried out in the CFX 96 real-time system and the CFX manager software (Bio-Rad). Primers with high efficiency were used to study their stability. Three different tissue samples (head, gut and body remnants), each with two biological and three technical repetitions were analyzed. Also, each sample contained tissues of five individuals. The expression stability of these genes was calculated using GeNorm (Hellemans et al., 2007). A threshold M value ≤ 0.5 was considered for selecting the candidate reference genes.

2.4. In silico nuclease identification and phylogenetic analysis

All nucleases related to extracellular dsRNA degradation were identified by tBLASTn using reference protein sequences from *S. gregaria* (Spit et al., 2017), *L. migratoria* (Wynant et al., 2014), *L. decemlineata* (Swevers et al., 2013) and *B. mori* (Liu et al., 2012) to search for homologous nuclease proteins in the transcriptome data of *C. puncticollis*. ORF finder from NCBI was used to search for open reading frames (ORF) in RNA sequences, which returns with protein translation.

For the annotation of nucleases related to dsRNA degradation, we searched for the presence of conserved domains using the SMART domain analysis tool (http://smart.embl-heidelberg.de/) and for signal peptide sequences using the SignalP 4.1 server (http://cbs.dtu.dk/services/SignalP/). To confirm their identity, we generated protein alignments with CLUSTALW using the BLOSUM 62 matrix in the software MEGA v.7. The best hits of nuclease homologs found in the Genebank (bitscore > 200) were selected for the alignments. Then, a phylogenetic tree of the protein sequence from the conserved domain was constructed using the neighbor-joining method in the software MEGAv.7 (Kumar et al., 2016). Bootstrapping was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

2.5. Synthesis of dsRNA

For the preparation of the dsRNA templates, DNA fragments were amplified by PCR using cDNA of *C. puncticollis* larvae as template. Each primer contained in the terminal 5′ the T7 promoter sequence TAATACGACTCACTATAGGG (Table S1). GFP was used as control. The PCR products were subsequently purified using the CyclePure E.Z.N.A. kit (Omega Bio-Tek) and immediately used for *in vitro* transcription using MEGAscript kit (Ambion) according to the manufacturer's instructions. Nuclease-free water (Invitrogen) was used for dsRNA elution. The dsRNA synthesis was verified by gel electrophoresis and quantified in a NanoDrop ND-1000 (Thermo Scientific).

2.6. Administration of dsRNA by injection and oral uptake

To study the involvement of nucleases in dsRNA degradation, second instar *C. puncticollis* larvae were first anesthetized with diethyl ether during 5 min and injected with dsRNA targeting different nucleases at a concentration of 0.2 μg/mg of body weight for gene silencing. Larvae of around 3 mg body weight were used, so in total ~0.6 μg dsRNA was injected per larvae. Injections were performed using a microinjector (FemtoJet, Eppendorf), an MN-151 micromanipulator (Narishige) and needles pulled from 50 μM glass capillaries using a twostep PC-10 needle puller (Narishige). After injection, larvae were placed into sweetpotato root slices of 5 × 5 cm in petri dishes and incubated at 27 °C and 65% relative humidity.

In order to perform an RNAi-of-RNAi assay, dsRNA targeting the selected nucleases were first injected independently into second instar larvae. As controls, larvae were injected with dsRNA targeting GFP (dsGFP) or water. Four days post-injection, larvae were removed from the root slices and placed into artificial diets containing dsRNA targeting *Cp-Snf7* (dsSnf7) at a concentration of 10 μg/mL of diet. The artificial diets were prepared according to the protocol by Ekobu et al. (2010) with agar concentration modified to 60 g/L of diet. The larvae injected with dsRNA targeting nucleases were transferred in either diet with dsGFP as control or diet with dsSnf7 at 10 μg/mL diet. After 7 days of oral treatment, the individuals were gently transferred into sweetpotato root slices for further evaluation around the moment of pupation or collected at different time points for RNA extraction. 50 larvae were injected per each treatment and 3 replicates were performed per treatment. GraphPad Prism v.7 software (Graphpad Software Inc., San Diego, California) was used to generate the figures and perform the statistical analyses.

2.7. *Ex vivo assay with midgut juice*

To evaluate the dsRNA stability in the digestive tract, *ex vivo* assays with gut contents from second instar *C. puncticollis* larvae were conducted to evaluate whether dsRNA is degraded in these fluids. Larvae were first starved for 6 h. Then, entire guts from 3 larvae of *C. puncticollis* were dissected and collected in cold 1.5 mL tubes with 50 μl of 1x PBS buffer (pH 7). Extra tissues were removed by two times centrifuging the tubes at maximum speed for 10 min and transferring the supernatants, which contain the gut juice, to clean 1.5 mL-tubes. 0.2 μg of dsGFP (1 μg/μl) was added in 10 μl of reaction and incubated for different time periods at 25 °C. After each time point, the reaction was stored in −20 °C to stop the enzymatic reaction. After incubation, dsRNA was extracted using the clean-up protocol of the RNeasy Mini Kit (Qiagen) and run on a 1.5% agarose gel.

2.8. *Quantitative reverse transcriptase PCR analysis*

Quantitative reverse transcriptase PCR (RT-qPCR) was performed to analyze the nuclease expression in three different tissues, head, gut, and remnant body, of second instar *C. puncticollis* larvae, ant to evaluate the gene silencing after dsRNA injection or feeding. Total RNA was extracted from 3 biological samples and used in the analysis of nuclease expression. Each sample contained 3 tissues of samples collected at different time points. Samples were homogenized in RLT buffer from RNeasy Mini Kit (Qiagen) + β-mercaptoethanol for RNA extraction and cDNA synthesis performed as mentioned in section 2.2. The RT-qPCR assay was performed in the CFX 96™ real-time system and the CFX manager software (Bio-Rad). The primers of the validated reference genes used in the analysis are described in Table S2. Appropriate controls, no-template control and no reverse transcriptase control, were also included in the assay. The relative transcript levels of the target were normalized to the amount of reference genes and calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The significance between control and treatments was verified by unpaired *t*-test in GraphPad Prism v.7 software.

3. Results

3.1. *Validation of candidate reference genes*

The five primer sets which were selected for the analysis of reference genes showed efficiency values higher than 90%. These primers amplify the housekeeping genes *Actin, Ubi, RpL32, PPIA* and *eEF1a*. GeNorm was used to evaluate the expression stability in three different tissues: head, gut and remnant body of second instar larvae. For each gene, geNorm calculates the pairwise variation with all other control genes, and the average is defined as the gene stability measure (M). In this study, we used 0.5 as a cut-off for M values, as lower M values indicate that reference genes are more stably expressed in tested samples (Vandesompele et al., 2002). Four genes met this requirement for *C. puncticollis*: *Rpl32*, *eEF1α*, *Ubi* and *PPIA* (Fig. 1A). To further improve this normalization in subsequent analysis, we selected two reference genes *Rpl32* and *eEF1α* for *C. puncticollis* according to the results of the pairwise variation analysis (geNorm V; $V_n/_{n+1}$) (Vandesompele et al., 2002). Generally, the number of added genes is limited until geNorm V value drops below 0.15. Here, the value for the combination of the two selected genes in *C. puncticollis* was 0.135 (Fig. 1B).

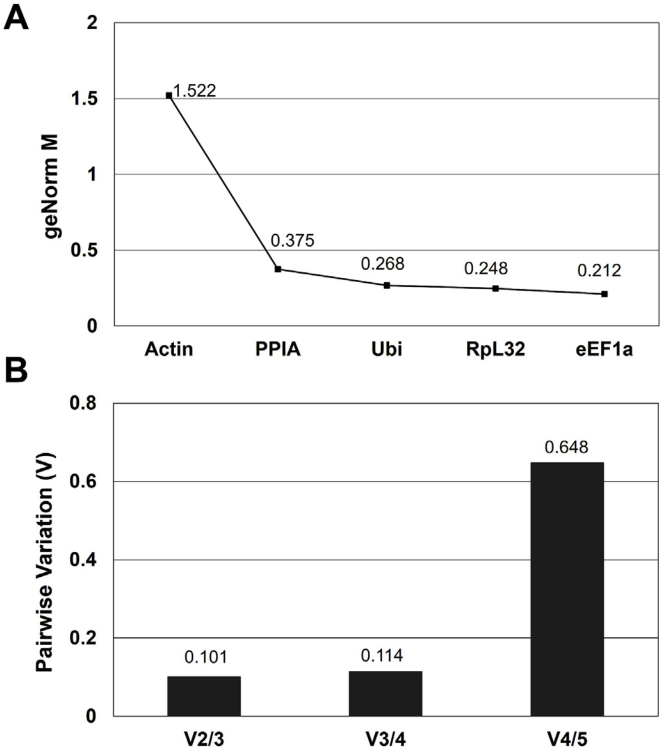


Fig. 1. Ranking of candidate reference genes in second instar *Cylas puncticollis* larvae analyzed by geNorm. Figure A represents the stability values by geNorm. Figure B represents the pairwise variation analysis to determine the number of reference genes required for the normalization in *C. puncticollis* tissues.

3.2. *Identification of nuclease homologs in the transcriptome of C. puncticollis*

After searching in the transcriptome data of *C. puncticollis* (Cp) with reference protein sequences that share more than 70% sequence similarity, three transcript sequences possibly encoding *dsRNases* were retrieved: *Cp-dsRNase-1*, *Cp-dsRNase-3* and *Cp-dsRNAse-4*. The names were given according to the protein sequence similarity with the dsRNases found in the closely related species the cotton boll weevil, *Anthonomus grandis* (Garcia et al., 2017). For *SDN-like*, also, three transcript sequences were found: *Cp-SDN-1-like*, *Cp-SDN-2-like*, *Cp-SDN-3-like*. For *Eri-1* and *Nibbler*, one possible homologue was retrieved: *Cp-Eri-1* and *Cp-Nibbler* respectively.

3.3. *Tissue expression of identified nucleases in C. puncticollis*

To study the expression of these identified nucleases in *C. puncticollis*, three different tissues were targeted, head, guts and remnant

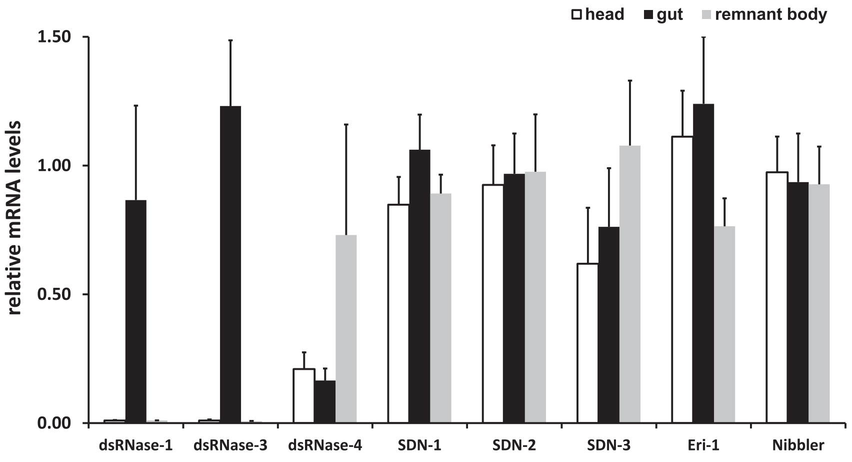


Fig. 2. Tissue transcript levels of identified nucleases of second instar *Cylas puncticollis* larvae. The analysis was performed by RT-qPCR, using as reference genes *Rpl32* and *EF1α* for the normalization of the relative expression. Each bar represents the mean of five independent samples with five pool larvae dissected tissues ± SEM.

body of second instar larvae. The expression of *Cp-dsRNase-1* and *Cp-dsRNase-3* was observed exclusively in gut tissues of *C. puncticollis*, while *Cp-dsRNase-4* was mainly expressed in the remnant body, which mostly contained fat tissue (Fig. 2). The other five nucleases, *Cp-Eri-1*, *Cp-SDN-1-like*, *Cp-SDN-2-like*, *Cp-SDN-3-like* and *Cp-Nibbler* were expressed in all selected tissues of both species.

3.4. dsRNase annotation and phylogenetic analysis

Given that *Cp-dsRNase-1* and *-3* were expressed only in gut tissues, we proceeded to annotate the *dsRNase* genes. The protein sequences of Cp-dsRNase-1, -3, -4 showed a conserved Endonuclease NS domain (DNA/RNA non-specific Nuclease (NN−) domain) (Fig. 3A), while a signal peptide was only identified in Cp-sRNase-3 suggesting that it has an extracellular activity (Fig. 3B). The three *dsRNases* genes were deposited in the NCBI database and can be accessed using the following accession numbers: MK510881: *Cp-dsRNase-1*, MK510880: *Cp-dsRNase-3* and MK510882: *Cp-dsRNase-4*.

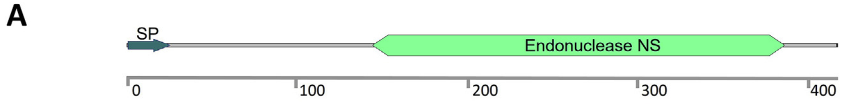
The multiple alignment containing the protein sequence of the three Cp-dsRNases, and the identified dsRNases in *C. brunneus* (Cb), *S. gregaria* (Sg), *L. migratoria* (Lm), *L. decemlineat*a (Ld), *B.mori* (Bm) and *Dendroctonus ponderosae* (Dp) confirmed the presence of a conserved endonuclease-NS domain with the active sites, namely a Mg^{2+} binding site and a substrate binding site in the *C. puncticollis* dsRNases (Fig. 4). The highest sequence identity at the amino acid level for the three Cp-dsRNases was with dsRNases from *C. brunneus* and *S. gregaria*, sharing an identity of: 39% between Cp-dsRNase-1 and Sg-dsRNase-2; 78.4% between Cp-dsRNase-3 and Cb-dsRNase-3b; and 64.7% between Cp-dsRNase-4 and Cb-dsRNase-4.

Based on the phylogenetic trees constructed with homologous proteins from insects of different orders, two main groups are formed. In the first, all coleopteran, dipteran, lepidopteran, orthopteran and crustaceans species are clustered. In the second, bacterial and mammalian species were clustered together. Cp-dsRNases formed a sub-cluster together with other coleopteran, lepidopteran, dipteran and orthopteran insects, while crustaceans were found in a second sub-cluster (Fig. 5).

3.5. Involvement of nucleases in the dsRNA degradation in C. puncticollis

As the aim of this research was to determine whether nucleases are implicated in the dsRNA degradation in *C. puncticollis*, *in vitro* produced dsRNA nucleases were injected into second instar larvae. As a preliminary assay, *Cp-dsRNase-1*, *Cp-dsRNase-3*, *Cp-dsRNase-4*, *Cp-Eri-1* and *Cp-SDN-1* were first silenced, and after 3 days of injection, the gene downregulation was evaluated by RT-qPCR analysis. Fig. 6 shows that the expression of *Cp-dsRNase-3*, *Cp-dsRNase-4*, and *Cp-Eri-1* were reduced to more than 90%, which was significant compared to the control injected with dsGFP. For *Cp-dsRNase-1* and *Cp-SDN-1-like*, the silencing was 71% and 81%, respectively, also significant compared to the control.

Once it was proven that these nucleases could be efficiently silenced using RNAi, the dsRNA stability in presence of gut juice from nuclease-silenced larvae was evaluated. At 7 days post injection with dsRNA for *Cp-dsRNase-1* (dsdsRN1), *Cp-dsRNase-3* (dsdsRN3), *Cp-dsRNase-4* (dsdsRN4), *Cp-Eri-1* (dsEri-1) and *Cp-SDN-1* (dsSDN-1), the gut juice from injected larvae was extracted and incubated with 0.2 µg of dsRNA in 10 µl of gut juice. After 15 min of incubation, dsRNA degradation was observed in the control injected with dsGFP and in the samples injected with dsSDN-1 (Fig. 7A). In the samples injected with dsEri-1, the degradation started after 30 min. In contrast, dsRNA was stable during 1 h in the samples injected with dsdsRN3. This result was consistent when the assay was repeated in different individuals (Fig. 7B). After 1 h of incubation, the dsRNA degradation in the samples injected with dsdsRN3 was reduced compared to the control injected with dsGFP. Also, samples injected with dsdsRN1 and dsdsRN4 were included and also a mix of these 3 dsRNases (dsdsRN1,3,4) (Fig. 7B). The incubation with gut juice from *Cp-dsRNase-1*-silenced larvae was variable. In these samples, the dsRNA integrity was observed in only one individual. In the *Cp-dsRNase-4*-injected larvae, the degradation of dsRNA occurred



B

	predicted domain	start	end	E-value	signal peptide [position]
Cp_dsRNase-1	endonuclease-NS	142	386	9.67E-36	no
Cp_dsRNase-3	endonuclease-NS	145	387	2.80E-32	yes [1-25]
Cp_dsRNase-4	endonuclease-NS	79	323	1.07E-28	no

Fig. 3. Identification of conserved domains and signal peptide for dsRNases identified in *Cylas puncticollis* (Cp). (A) Graphical representation of the dsRNases containing a consensus Endonuclease NS domain (B) Table showing the start and end amino acids of the predicted domain endonuclease-NS and the position of the signal peptide.

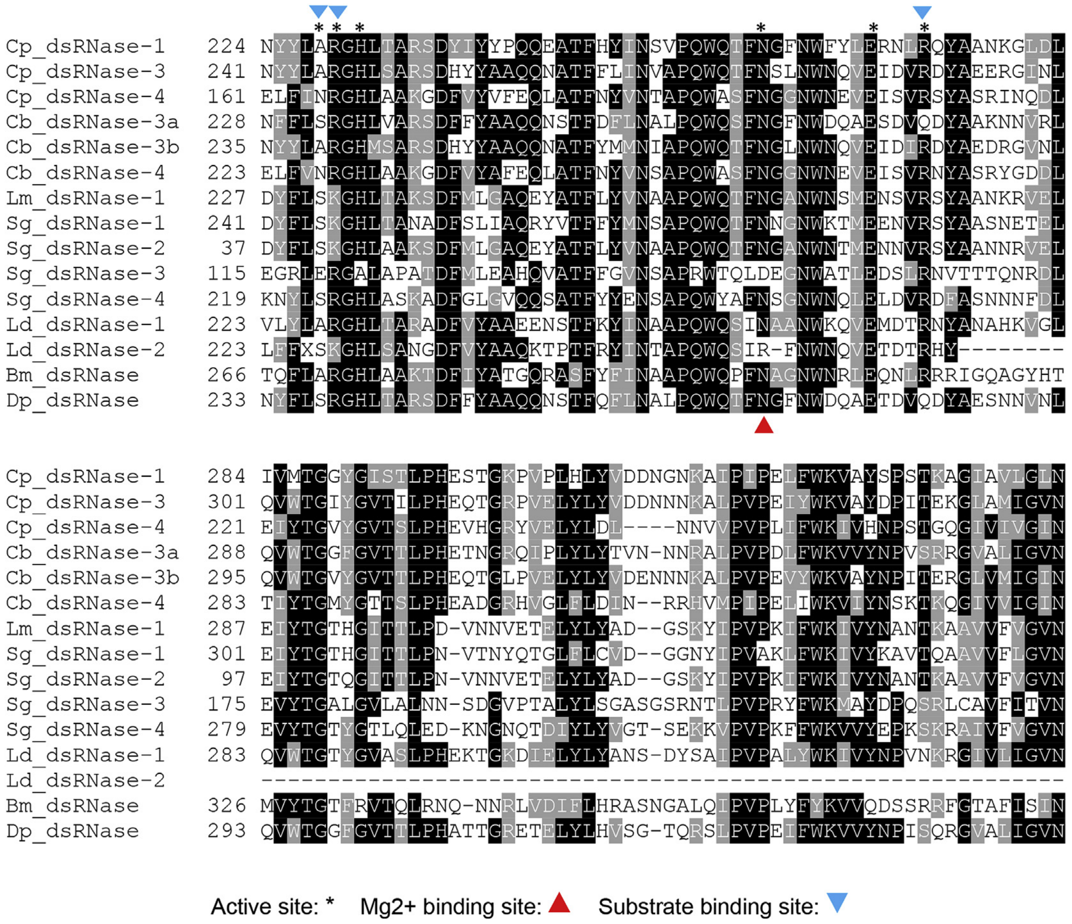


Fig. 4. Partial multiple alignment of amino acid sequences of the endonuclease-NS domain of dsRNases retrieved from *Cylas puncticollis* (Cp). Sequences of *Cylas brunneus* (Cb-dsRNase-3a, Cb-dsRNase-3b, Cb-dsRNase-4), *Locusta migratoria* (Lm-dsRNase-1, KX652408), *Schistocerca gregaria* (AHN55088 Sg-dsRNase-1; AHN55089.1 Sg-dsRNase-2; AHN55090.1 Sg-dsRNase-3, AHN55091 Sg-dsRNase-4), *Leptinotarsa decemlineata* (APF31792.1 Ld-dsRNase-1; APF31793.1 Ld-dsRNase-2), *Bombyx mori* (BAF33251 Bm-dsRNase) and *Dendroctonus ponderosae* (AEE63490.1 Dp-dsRNase) were included in the alignment. Identical residues are highlighted in black and similar amino acids are highlighted in gray. Asterisks indicate the active sites, the red triangles show the Mg^{2+} binding site, and the blue triangles indicate the substrate binding sites.

identically as in the control. When all three *Cp-dsRNases* were silenced, a higher stability of the dsRNA compared to the control was observed in two of our three biological samples. In one sample, the dsRNA was degraded after 1 h (Fig. 7B).

3.6. RNAi effects after feeding nuclease-silenced C. puncticollis insects

An “RNAi-of-RNAi” approach was performed to study whether the silencing of *dsRNases* (*Cp-dsRNase-1*, *Cp-dsRNase-3* and *Cp-dsRNase-4*) can enhance the RNAi effects on mortality of *C. puncticollis* larvae. Larvae were first injected with a specific dsRNA targeting these nucleases at concentration of 0.2 μg/mg of body weight, and then were transferred after 4 days to artificial diet mixed with dsSnf7 at a 10 μg/mL concentration. After 14 days of feeding, a significant mortality of 69 ± 10% ($P < 0.05$) was observed in the treatment of injected dsdsRN3 combined with diet containing dsSnf7 (dsdsRN3 + dsSnf7) compared to the four controls dsGFP + water (27 ± 7%), dsGFP + dsGFP (27 ± 5%), water + dsSnf7 (36 ± 9%) and dsdsRN3 + dsGFP (38 ± 2%) (Fig. 8). For the other three treatments dsdsRN1 + dsSnf7, dsdsRN4 + dsSnf7, and dsdsRN1,3,4 + dsSnf7, the mortality was 45 ± 6%, 50 ± 6% and 51 ± 6%, respectively, which was not significantly different compared to the four controls (Fig. 8).

An RT-qPCR analysis was performed to confirm the silencing of *Cp-dsRNases* and *Cp-Snf7* in the treatments dsdsRN3 + dsSnf7 (Fig. 9A and B) and the combined dsRNases dsdsRN1,3,4 + dsSnf7 (Fig. 10A–B). In the first treatment, *Cp-dsRNase-3* was significantly downregulated by 82% at day 1, 92% at day 2, 91% at day 3 and by 69% at day 4 compared to the control water + dsSnf7 (Fig. 9A), while the silencing of *Cp-Snf7* (27%) was only significant at day 1 (Fig. 9B). In the second treatment (dsdsRN1,3,4 + dsSnf7), the silencing of *Cp-dsRNase-1* and *Cp-dsRNase*-3 were significantly downregulated. The *Cp-dsRNase-1* silencing was 85% at day 2 and 77% at day 4 compared to the control water + dsSn7 (Fig. 10A), while the *Cp-dsRNase-3* silencing was 82% at day 2, 95% at day 4, and 61% at day 7 compared to the control water + dsSnf7 (Fig. 10B). Furthermore, the silencing of *Cp-dsRNase-4* (Fig. 10C) and *Cp-Snf7* (Fig. 10D) was not significant at any time point compared to the control water + dsSnf7.

4. Discussion

The present study determined whether nucleases are implicated in a refractory oral RNAi response in the SPW *C. puncticollis*, considered one of the most economically important insect pests of sweetpotato in the Sub-Sahara Africa (Okonya et al., 2016). Currently, there is no successful control method for this pest, and the application of RNAi can significantly contribute to reduce infestations caused by the SPW. Unfortunately, a challenge that RNAi technology is currently facing for pest control application is the variable RNAi efficiency observed in different insects and this is also the case for the SPW *C. puncticollis* when dsRNA is orally delivered. In earlier research, *ex vivo* assays incubating

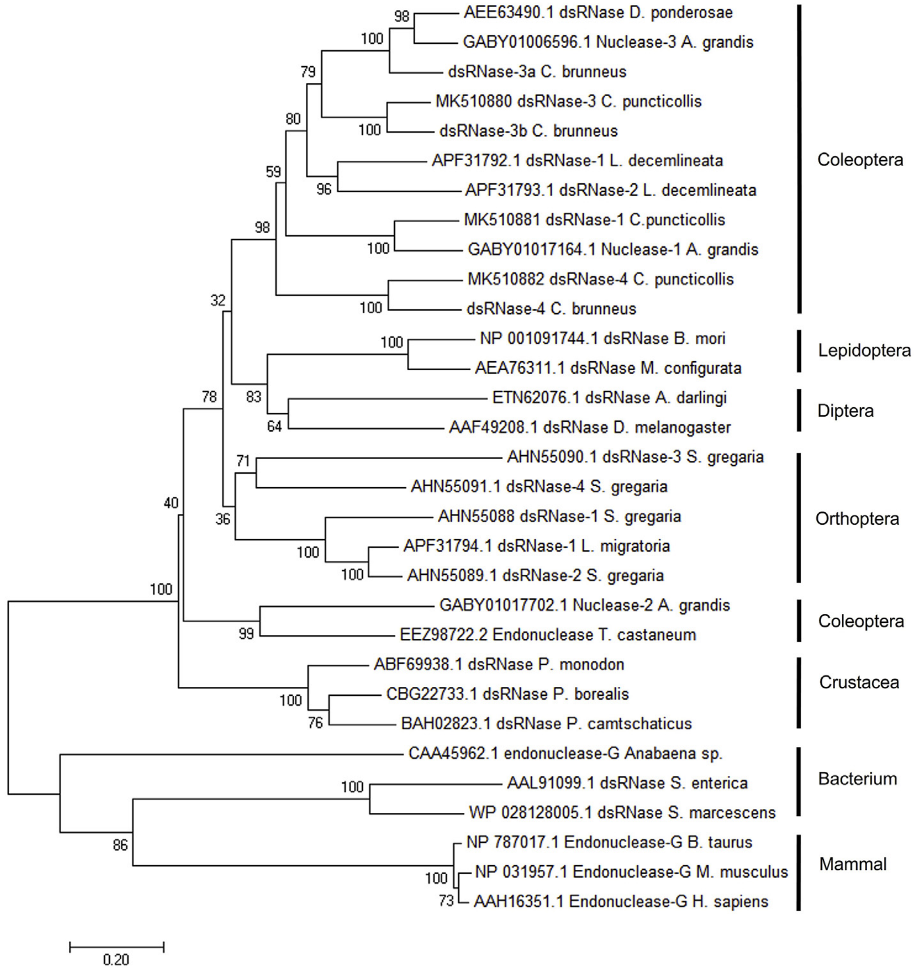


Fig. 5. Phylogenetic tree of *Cylas puncticollis* dsRNases and homologous sequences of species from the insect orders Coleoptera, Diptera, Lepidoptera, and Orthoptera, the subphylum of Crustacea and the bacterial and mammalian kingdoms. The tree was constructed using the neighbor-joining method in the software MEGA v.7. Bootstrap values are shown on the lineage of the tree and resulted from 1000 bootstrap replicates. The dsRNase homologs used in the phylogenetic trees were: *Cylas brunneus*, *Locusta migratoria*, *Schistocerca gregaria*, *Leptinotarsa decemlineata*, *Anthonomus grandis*, *Tribolium castaneum*, *Bombyx mori*, *Mamestra configurata Anopheles darlingi*, *Drosophila melanogaster*, *Dendroctonus ponderosae*, *Pandalus borealis*, *Penaeus monodon*, *Paralithodes camtschaticus*, *Salmonella enterica*, *Serratia marcescens*, *Anabaena sp.*, *Mus musculus*, *Bos taurus* and *Homo sapiens*.

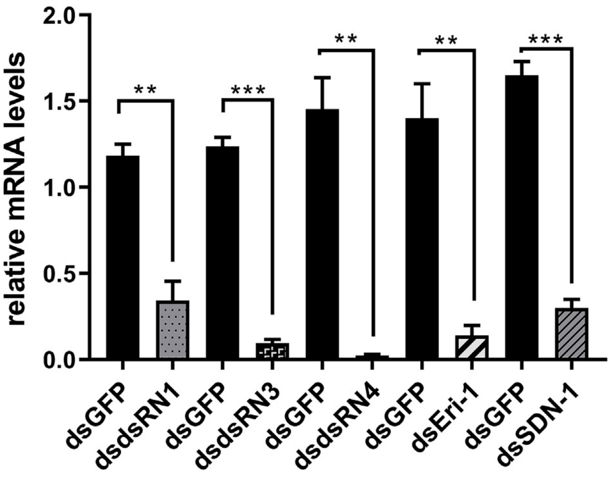


Fig. 6. Gene silencing of different nucleases at 3 days after injection in second instar *Cylas puncticollis* larvae. DsRNA targeting *Cp-dsRNase-1* (dsdsRN1), *Cp-dsRNase-3* (dsdsRN3) *Cp-dsRNase-4* (dsdsRN4), dsEri-1, and dsSDN-1 were injected at 0.2 µg/mg of body weight. The control group was injected with dsGFP at same concentration. Bars show the mean ± SEM of three independent samples. Each sample contains pooled dissected tissues from three larvae (n = 9). The *P*-values were calculated by unpaired *t*-test in GraphPad Prism v.7. Asterisks represent that controls and treatments differed significantly (*$p < 0.05$; ** < 0.005; ***$p < 0.0001$).

dsRNA in gut juice from *C. puncticollis* larvae and two other coleopteran species, *C. brunneus* and *L. decemlineata* showed that the dsRNA was degraded much faster in gut juice from *C. puncticollis* than from the other two species (Prentice et al., 2017). Here, in an effort to identify whether nucleases are the cause of the reduced RNAi efficiency in *C. puncticollis*, a search for potential dsRNA-degrading nucleases was performed in the transcriptome of this species and the impact of the identified nucleases on the lower RNAi efficiency was further characterized.

Three homologs of dsRNase were discovered in the *C. puncticollis* transcriptome. Even though three nucleases were also found in *A. grandis*, the homology with those in both *Cylas* species was not really clear and based on our phylogenetic analysis, a higher divergence was observed than we expected. We have named two *C. puncticollis* nucleases (Cp-dsRNase-1 and -3) based on the homology with those in *A. grandis*, but the third *C. puncticollis* nuclease was designated as dsRNase-4 due to its sequence being quite divergent from Ag-dsRNase-2. *Cp-dsRNase-1* and *-3* were found to be exclusively expressed in gut tissues, while *Cp-dsRNase-4* was mainly expressed in the remnant tissues which is mostly composed of fat tissue. Interestingly, the dsRNase-3 protein sequence shows a signal peptide, while this was not the case for dsRNase-1 and -4, suggesting that the former is secreted as expected, but the two latter remain inside the cells. Similar results were observed in *A. grandis*, which the most related nuclease (*Ag-Nuclease-3*) to *Cp-dsRNase-3* was also highly expressed in the guts of larvae and adults (Garcia et al., 2017). However, the expression of *Cp-dsRNase-1* and *-4* in the gut lumen is not conclusive since other protein secretion mechanisms exist (Bendtsen et al., 2004). Moreover, the SignalP 4.1 is a prediction tool, which might not recognize the presence of less

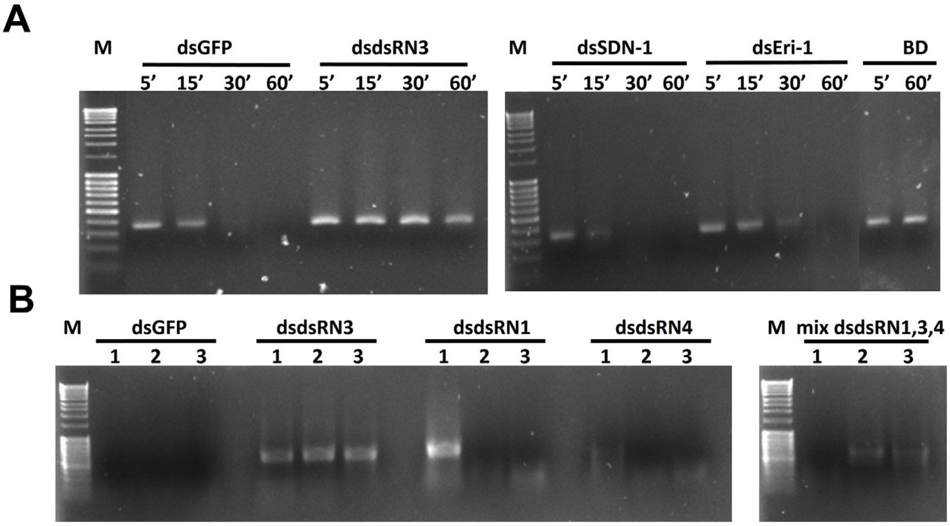


Fig. 7. ***Ex vivo*** **dsRNA degradation assay in second instar** ***Cylas puncticollis*** **larvae at 7 days after injection with dsRNA targeting different nucleases. (A)** Larvae were injected with dsRNA targeting *Cp-dsRNase-3* (dsdsRN3), *Cp-SDN-1-like* (dsSDN-1) and *Cp-Eri-1* (dsEri-1). At 7 days after injection, gut juice of two pooled larvae was extracted and 10 μl was incubated with 0.2 μg of dsRNA per well at different time points (5, 15, 30 and 60 min) and run in 1.5% agarose gel. (M) DNA ladder (Thermo Scientific). (BD) 0.2 μg of dsRNA in PBS 1X at two time points of incubation. **(B)** The dsdsRN3 assay was repeated showing the incubation at 1 h with 0.2 μg of dsRNA. This assay also includes larvae injected with dsRNA targeting *Cp-dsRNase-1* (dsdsRN1), *Cp-dsRNase-4* (dsdsRN4), and with a combination of dsRNA of the 3 *Cp-dsRNases* (dsdsRN1,3,4). Lane 1, 2 and 3 represent 10 μL of gut juice of three different samples, each with two pooled larvae. Guts were extracted 7 days after injection.

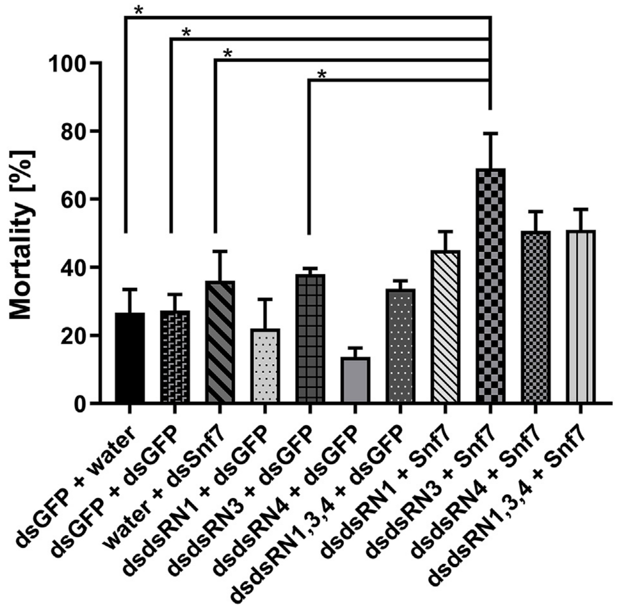


Fig. 8. RNAi effects after oral feeding of ***dsRNases*****-silenced** ***Cylas puncticollis*** **larvae with dsRNA targeting the gene** ***Cp-Snf7*** **after 14 days post-feeding at concentration of 10 μg/mL diet.** Each bar represents the mean mortality percentage ± SEM of three biological repetitions per treatment. Each repetition contains 25 individuals. Different combination of injection and oral feeding were performed for this assay: Larvae injected with dsGFP were transferred at 4 days after injection into a diet with either water (dsGFP + water) or with dsGFP (dsGFP + dsGFP). Larvae injected with water were transferred into a diet containing dsSnf7 (water + dsSnf7). Larvae injected with dsdsRN1, dsdsRN3, dsdsRN4 or a combination of these three dsdsRNs were transferred into diets with either dsGFP or dsSnf7 (dsdsRN1 + dsGFP; dsdsRN1 + dsSnf7), (dsdsRN3 + dsGFP; dsdsRN3 + dsSnf7), (dsdsRN4 + dsGFP; dsdsRN4 + dsSnf7) and (dsdsRN1,3,4 + dsGFP; dsdsRN1,3,4 + dsSnf7). The statistical significance between control and treatment results was verified by one-way ANOVA. Asterisks represent that controls and treatments differed significantly (*$p < 0.05$).

conventional signal peptides. Therefore, further studies are needed to make a definite conclusion.

The *ex vivo* assays (incubation of gut juice with dsRNA) indicated that silencing *Cp-dsRNase-3* increases the dsRNA persistence in the gut juice of second instar larvae, while the degradation in the *Cp-dsRNase-1*-silenced larvae was observed to be variable, as only one individual of the three tested showed a reduction of dsRNA degradation. This variable profile was observed in repetitions of the *ex vivo* assays as well (Fig. S1), despite a very efficient silencing of *Cp-dsRNase-1* silencing (higher than 90%). Similarly, in the *ex vivo* assay of larvae treated with combined dsRNAs for the three *Cp-dsRNases*, a reduced degradation was observed in two individuals of the three tested. Likely, the effect we see in the combined treatment is caused by silencing of *Cp-dsRNase-3*.

Other nucleases reported to be involved in RNA degradation were also targeted in this study. Eri-1 is implicated in histone mRNA degradation in mammals and in negative RNAi regulation in *C. elegans* (Kennedy et al., 2004). SDN proteins were found to be involved in mature miRNA turnover, and when inactivated in *Arabidopsis*, this resulted in increased amounts of miRNA, affecting plant development (Ramachandran and Chen, 2008). Finally, Nibbler was found in *D. melanogaster* to interact with AGO1 and process miRNAs at the 3'end within the RISC complex (Han et al., 2011; Liu et al., 2011). For these nucleases, several homologs were identified in both SPW transcriptomes. As expected, none of them contained a signal peptide indicating that they are probably not secreted. Also, these nucleases were observed to be highly expressed in all three studied tissues (head, gut, remnant body) of both insects. A reduction of dsRNA degradation could however not be observed when silencing Eri-1, SDN-1 and Nibbler in the different repetitions, indicating that they are either not present in the gut juice or not involved in this degradation and thus are not affecting the RNAi efficiency. Therefore, we continued the study focusing on the dsRNases.

The dsRNA-degrading activity of Cp-dsRNase-3 was further confirmed in our RNAi-of-RNAi study. *Snf7* was used as marker gene due to its high RNAi efficacy in the closely related SPW *C. brunneus* (Christiaens et al., 2016). Here, a significantly higher mortality after feeding compared to the controls could be detected in the *Cp-dsRNase-3*-silenced larvae, but not for *Cp-dsRNase-1, -4* or the combined treatment. Despite the increase, the mortality in the dsCp-dsRNase-3 + dsSnf7 treatment was still only 42%. In a similar study in *S. gregaria*, Spit et al. (2017) reported significant silencing of specific dsRNases, but post-feeding with dsRNA targeting the essential *α-tubulin* gene did not cause insect mortality. In *A. grandis*, silencing of all three identified nucleases simultaneously did significantly increase RNAi efficacy at the transcript level, but the phenotypic effects were also modest. One of the reasons for the relatively modest increase in RNAi efficacy could be the fact that the silencing of the nuclease was only partial and limited in time. And since we have no information on the half-life of this protein, it is also possible that already synthesized proteins remained active for

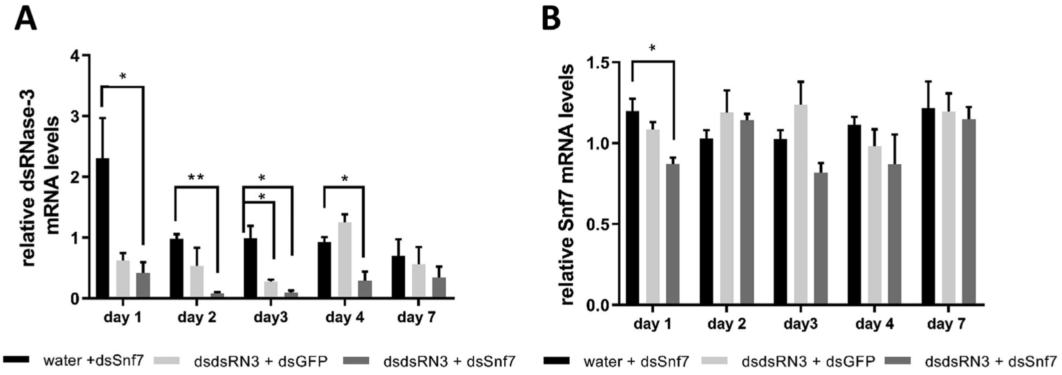


Fig. 9. Gene silencing analysis by RT-qPCR of *Cylas puncticollis* larvae injected with dsdsRN3 and then transferred into diet with dsSnf7 at concentration 10 µg/mL diet (dsdsRN3 + dsSnf7). **(A)** Represents the downregulation of *Cp-dsRNase-3* expression and **(B)** the downregulation of *Cp-Snf7* expression. Bars show the mean ± SEM of three independent samples. Each sample contains pooled dissected tissues from three larvae ($n = 9$). The significance between control and treatments was verified by unpaired *t*-test in GraphPad Prism v.7. Asterisks represent that controls and treatments differed significantly (*$p < 0.05$; ** < 0.005; ***$p < 0.0001$).

some time during the assay. Future experiments using complete knockouts, for example using CRISPR/Cas9 technology, could shed more light on this. Another possible explanation could be that other so far unidentified nucleases also play a role in dsRNA degradation or that other mechanisms, such as cellular uptake or viral infections, can affect the RNAi efficiency in this species. Inefficient or low dsRNA uptake by the epithelial cell surface of the midgut is a critical factor for oral RNAi. Previously, oral delivery with high dsRNA concentrations (30 µg dsRNA/mL diet) caused around 75% mortality in this species, indicating a functional uptake system (Prentice et al., 2017).

The SPW *C. puncticollis* is one of the few coleopteran species with a rapid dsRNA degrading activity, in contrast to several other insects of this order that are generally considered to have weak dsRNA degrading activities and to be very sensitive to oral RNAi. Coleopterans generally seem to require much longer exposure to nucleases in body or gut fluids for dsRNA degradation. Spit et al. (2017) observed that the dsRNA-degrading activity of midgut juice from *S. gregaria* is much stronger than from *L. decemlineata*. Similarly, Cao et al. (2018) compared the RNAi effects between *T. castaneum* and *A. pisum*, and saw stronger dsRNA degradation in *A. pisum* hemolymph and gut juice than in *T. castaneum*. In addition, an analysis comparing the dsRNA-degrading activity in 37 insects from 5 orders, also observed that coleopteran insects require large amounts of body fluids to impair the dsRNA integrity, suggesting that expression of dsRNases in these body fluids is low (Singh et al., 2017). In contrast, in the cotton boll weevil *A. grandis*, the RNAi efficiency was affected by the presence of nucleases in the gut lumen, similar as in our study (Garcia et al., 2017). Another coleopteran species insensitive to oral RNAi is the small hive beetle *Aethina tumida*, where RNAi was highly efficient when dsRNA was injected, but reduced effects were observed when dsRNA was orally delivered. Nuclease

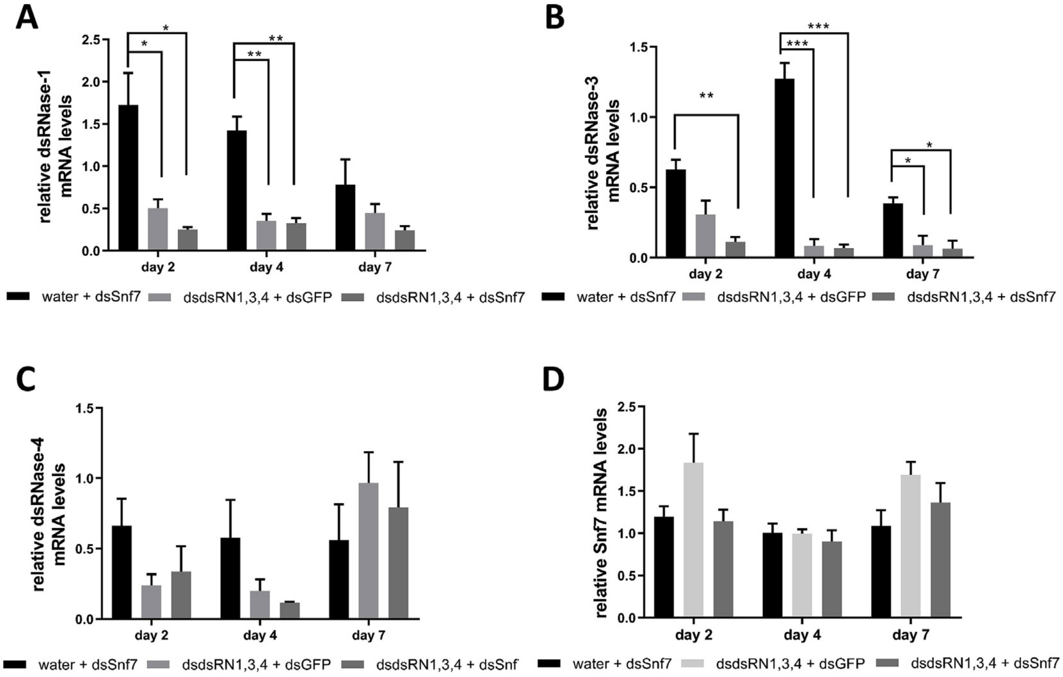


Fig. 10. Gene silencing analysis by RT-qPCR of *Cylas puncticollis* larvae injected with the combination of the three dsdsRNases (dsRN1,3,4) and then transferred into a diet with dsSnf7 (dsdsRN1,3,4 + dsSnf7). (A) Represents the downregulation of *Cp-dsRNase-1*, (B) *Cp-dsRNase-3*, (C) *Cp-dsRNase-4* and (D) *Cp-Snf7* expression. Bars show the mean ± SEM of three independent samples. Each sample contains pooled dissected tissues from three larvae ($n = 9$). The significance between control and treatments was verified by unpaired *t*-test in GraphPad Prism v.7. Asterisks represent that controls and treatments differed significantly (*$p < 0.05$; ** < 0.005; ***$p < 0.0001$).

activity was indirectly implied by the observation of dsRNA degradation in digestive secretions of *A. tumida* (Powell et al., 2017).

For RNAi application as a pest control strategy of the SPW *C. puncticollis*, dsRNA will need to be protected from the nuclease activity for oral delivery to be successful. One strategy could be the use of topical formulations, which have already demonstrated to efficiently protect the dsRNA. Nanoparticles for example, have been used as delivery agents leading to promising results in *Aedes aegypti*. One of the tested nanoparticles, carbon quantum dot (CQD), was able to significantly increase gene silencing and mortality in this insect (Das et al., 2015). Fluorescent nanoparticles (FNP) coated with dsRNA targeting the chitinase-like *Cht10* gene were rapidly engulfed into cells, and showed high efficacy in impairing *Ostrinia furnacalis* larvae development (He et al., 2013). However, because *Cylas* larvae are feeding inside the roots of the sweetpotato, a topical application of dsRNA will not cause sufficient exposure of the larvae for sufficient RNAi effects. Only adults on the surface will be exposed in such a scenario. Another alternative to overcome the dsRNA degradation could be the expression of specific dsRNAs in African sweetpotato varieties for continuous exposure. This strategy has already been proven successful in targeting several other insect species (Baum et al., 2007; Mao et al., 2011; Zha et al., 2011; Pitino et al., 2011; Kumar et al., 2014; Zhang et al., 2015).

In conclusion, our research identified a dsRNase enzyme, *Cp-dsRNase-3*, that negatively affects the RNAi efficiency in *C. puncticollis* through degradation of dsRNA in the gut lumen. The fact that only a partial rescue of RNAi efficiency was observed may indicate the presence other of non-identified nucleases with dsRNA-degrading activity. Despite *C. puncticollis* showing an efficient RNAi response by injection, the rapid dsRNA degradation observed in the digestive tract of *C. puncticollis* complicates the use of RNAi as a pest control strategy against this species. In this regard, future research will be necessary in order to protect the dsRNA and effectively deliver these molecules to SPW larvae.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.04.001.

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