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ORIGINAL PAPER

Improving RNA interference in the southern green stink bug, *Nezara viridula*

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Abstract

The southern green stink bug, *Nezara viridula,* is a worldwide pest of many important crops. RNA from *N. viridula* nymphs and adults was sequenced and assembled into a transcriptome. Orthologs of genes coding for proteins involved in RNA interference (RNAi) pathways and potential target genes for RNAi-mediated control of this pest were identified by searching the transcriptome. The watery saliva, lumen and hemolymph collected from *N. viridula* showed lower dsRNase activity than that in the lumen and hemolymph collected from the fall armyworm, *Spodoptera frugiperda*, which is refractory to RNAi. Seven housekeeping genes, *60S RP L12, 40S RP S17, 18S rRNA, EF1-α, Actin, Ubiquitin* and *β-Tubulin*, were evaluated across developmental stages and dsRNA treatments and identified *60S RP L12* and *40S RP S17* as the best reference genes for use in reverse transcriptase quantitative PCR (RT-qPCR). One microgram of dsRNA targeting the orthologs of seven known RNAi target genes (*SNF7, IAP1, IAP2, IAP5, PP1, GPCR* and *ATPase1*) was tested in *N. viridula*. Three out of seven dsRNAs tested induced 67–100% mortality. Injection of dsRNA targeting *SNF7* into *N. viridula* adults caused 100% mortality, while feeding dsSNF7 induced 60% mortality in both adults and nymphs. Delivery of dsRNA by either feeding or injection efficiently knocked down target gene expression. These data demonstrate that RNAi pathway components are present in *N. viridula and* delivering dsRNA by either injection or feeding induces silencing of target gene expression and mortality, suggesting that RNAi-based methods could be developed to manage this pest.

Keywords Transcriptome · SNF7 · Double-stranded RNA · DsRNase · RT-qPCR

Key Message

- *Nezara viridula* is an economically important plant-feeding pest that damages crops.
- RNAi-based control may be an effective, species-specific and environmentally friendly molecular biopesticides.
- Transcriptome analysis suggests that the RNAi pathway might function well in this insect.
- 60S ribosomal protein L12 and 40S ribosomal protein S17 genes were identified as the most stable reference genes.
- Seven potential RNAi target genes were screened and SNF7 was identified as the most effective target gene.
- Feeding dsSNF7 induced significant mortality and target gene knockdown.

Introduction

The southern green stink bug (SGSB) [*Nezara viridula* (L.) (Hemiptera: Pentatomidae)] is a phytophagous stink bug that is believed to have originated in Ethiopia and has spread around the world (Esquivel et al. 2018). The southern green

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stink bug feeds on many important food crops causing millions of dollars of annual crop yield losses in Brazil, the USA and other countries (Esquivel et al. 2018). Chemical insecticides, which may pose ecological and environmental risks, are primary management tools used to control the southern green stink bug (Liu et al. 2018). These insecticides may have limited efficacy due to the evolution of resistance, which is a major problem as pesticides are currently the leading approach to deal with this pest (T O'Neal et al. 2018).

RNA interference (RNAi) might provide a viable strategy to control insect pests (Zhang et al. 2017). Double-stranded RNA (dsRNA) targeting insect genes delivered through multiple routes including injection, feeding or through expression in transgenic plants or microorganisms have been shown to induce RNAi response (Baum et al. 2007; Huvenne and Smagghe 2010; Zhu et al. 2011; Zhang et al. 2015). RNAi efficiency is quite variable among insects depending on the species, delivery method and the genes targeted. For example, RNAi is efficient and is systemic in most of the coleopteran insects tested so far (Baum et al. 2007; Tomoyasu et al. 2008; Zhu et al. 2011; Palli 2014). However, RNAi is inefficient in other insects (Terenius et al. 2011; Shukla et al. 2016). Differences in the degradation of dsRNA by dsRNases, intracellular transport and processing of dsRNA are thought to be the significant contributors to differential RNAi efficiency among insects (Christiaens et al. 2014; Shukla et al. 2016). dsRNAs are taken up by coleopteran cells and processed into siRNAs, resulting in the silencing of target genes. Recent studies by Yoon et al. (2018) identified a dsRNA binding protein, Staufen C, as an essential player in dsRNA processing and RNAi initiation in the Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) and a cell line, Lepd-SL1, derived from this insect. In contrast, lepidopteran cells take up dsRNAs, but these dsRNAs are not processed into siRNAs due to their accumulation in the endosomes (Shukla et al. 2016; Yoon et al. 2017).

In the brown marmorated stink bug [BMSB; *Halyomorpha halys* Stål (Hemiptera: Pentatomidae)], injection of dsRNA targeting the *catalase* (*CAT*) gene induced a decrease in its expression. However, this decrease in its expression did not result in mortality (Bansal et al. 2016). The effect of RNAi in *H. halys* was demonstrated by delivering dsRNA targeting the homeotic gene *Sex combs reduced* (*Scr*) to adult females (Lu et al. 2017). The delivery of gene-specific dsRNAs targeting *Juvenile hormone acid O-methyltransferase* (*JHAMT*) and *vitellogenin* (*Vg*) by green bean-mediated delivery method induced RNAi response in *H. halys* nymphs (Ghosh et al. 2017; 2018). Further, Mogilicherla et al. (2018) showed that feeding dsRNA targeting *Baculoviral inhibitors of apoptosis proteins repeat-containing protein 7-B-like* (*IAP*), *Charged multivesicular body protein 4b* (*Sucrose non-Fermenting Protein 7, SNF7*) and *Serine/threonine-protein phosphatase PP1-beta catalytic subunit* (*PP1*) genes to *H. halys* nymphs caused more than 70% mortality. In the Neotropical brown stink bug, *Euschistus heros*, injection of dsRNAs induced significant mortality in seven out of the 15 genes tested. Also, feeding dsRNA targeting *V-type proton ATPase catalytic subunit A* (*vATPase A*) or *muscle actin* genes formulated with a liposome transfection reagent or mixed with EDTA induced mortality (Castellanos et al. 2019). A recent study in the harlequin bug, *Murgantia histrionica* (Hahn), showed that injection of dsRNA targeting *IAP, ATPase, PP1, SRP* and *GPCR* genes into adults caused between 40 and 75% mortality and induced significant knockdown of target gene expression. Further, feeding dsRNA targeting the *IAP* gene to nymphs by plant-mediated and droplet feeding methods induced knockdown of the target gene and caused 40–55% mortality (Howell et al. 2020). Also, recent studies tested RNAi in *N. viridula*. Riga et al. (2020) found that injected dsRNA targeting *Chitin synthase* and *Acetyl-CoA carboxylase* genes induced 75% mortality. Sharma et al. (2020) tested multiple dsRNAs identified dsαCop and dsvATPase A that cause significant mortality (43–45%). Gurusamy et al. (2020) compared the transport and efficiency of dsRNA, small interfering RNA (siRNA) and short hairpin RNA (shRNA) targeting *SNF7* gene in *N. viridula* and showed that dsRNA induced higher mortality and gene knockdown when compared to siRNA and shRNA. In the present study, we sequenced RNA isolated from *N. viridula* 3rd instar nymphs and adults and assembled a transcriptome that was used to identify orthologs of genes coding for proteins in the RNAi pathway as well as target genes for RNA-mediated control of this insect. We also identified housekeeping genes for RT-qPCR quantification of mRNA levels in the *N. viridula* developmental stages, adults and their body tissues and dsRNA fed 4th instars or injected adults and their body tissues.

Materials and method

Rearing southern green stink bug

Egg masses of the southern green stink bug [SGSB; *N. viridula* (L.)] were obtained from Dr. Bryony C. Bonning's laboratory in the Department of Entomology, University of Florida, Gainesville, FL, USA. The eggs were maintained in 150 mm × 15 mm Petri dishes with a single 6 mm piece of green bean until the neonates hatched and began feeding. Approximately 100 nymphs were then carefully transferred to 20-quart Sterilite gasket boxes with an 18 cm × 18 cm square cut out of the lid and fitted with fine Amber Lumite© screen mesh for ventilation. Paper towel was used as a substrate and cardboard egg flats were vertically placed in one half of the enclosure to provide refugia and surface area for

the insects to climb and rest on. Bent pieces of plastic gutter guard mesh were placed on the other half of the enclosure as a feeding platform so that food material would not make direct contact with the substrate. Developing insects were fed a mixed diet comprised of green beans, carrot, broccoli, raw peanuts and sweet corn in equal proportions (approximately 25 g of each food item). Cages were cleaned and food material was replaced every 48 h. Eggs and insects were maintained under controlled (Percival I-36VL incubator) conditions (16 h light: 8 h dark, with 25–27 °C and 65% relative humidity).

Total RNA isolation, RNA sequencing, transcriptome assembly and annotation

Total RNA was isolated from 3rd instar *N. viridula* ($n=1$) and adults ($n=1$) using the RNeasy mini kit (Qiagen, USA) with On-column *DNase* I treatment following the manufacturer's instructions. The total RNA was quantified using NanoDrop™ ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The RNA quality was determined by agarose gel electrophoresis. The RNA samples were sequenced at the Beijing Genomics Institute (BGI), Beijing, China. De novo transcriptome assembly was performed using the Trinity assembler on a high-performance cluster (Table 1a, b). The resulting transcriptome was assessed for completeness using Benchmarking universal single-copy orthologs (BUSCO) against the Arthropoda database (Table 1c). Local BLAST for the entire transcriptome was performed using the non-redundant database from NCBI. The program BLAST2GO was used to obtain gene ontology annotations. BLAST2GO was also used to generate statistics for the local BLAST, gene ontology mapping and annotation. Sequences of *Tribolium castaneum* genes coding for proteins known to play a role in the RNAi pathway were used to identify *N. viridula* orthologs; due to the completeness of the RNAi machinery in coleopterans as well as the lack of reliable data in hemipterans or lepidopterans, we chose *T. castaneum* genes for reference. A database of *T. castaneum* RNAi genes was created, and the reads from our *N. viridula* RNA-Seq data were aligned. The resulting counts were used as the metric to measure the expression levels of RNAi genes in *N. viridula* nymphs and adults.

Collection of hemolymph, lumen contents and watery saliva from *N. viridula* and dsRNA stability assay

The hemolymph and lumen contents were collected from *N. viridula* 4th instars. The forelegs of nymphs were pricked with forceps, and hemolymph was collected into chilled 1.5 ml tubes containing phenylthiourea

Table 1 Assembly and analysis of southern green stink bug, *Nezara viridula* transcriptome (**A**) Trinity assembly stats of the transcriptome, including all isoforms for each of the transcripts of *N. viridula* (**B**) Assembly stats of the transcriptome, including only the longest isoform per gene of *N. viridula*. (**C**) BUSCO analysis of the *N. viridula* transcriptome against the arthropod BUSCO database

(**A**) Total trinity 'genes'	44,128
Total trinity transcripts	66,836
Percent GC	34.32
Stats based on ALL transcript Contigs	Contig N10: 6,017
	Contig N20: 4,516
	Contig N30: 3,668
	Contig N40: 2,951
	Contig N50: 2,392
(**B**) Stats based on ONLY LONGEST ISOFORM per 'GENE	Contig N10: 5232
	Contig N20: 3885
	Contig N30: 3023
	Contig N40: 2405
	Contig N50: 1861
Median Contig length	403 bp
Average Contig	899.27 bp
Total assembled bases	39,682,916
(**C**) Total BUSCO groups searched	1066
C:97.3% [S:60.0%,D:37.3%], F:1.6%, M:1.1%, n:1066	
Complete BUSCOs (C)	1038
Complete and single-copy BUSCOs (S)	640
Complete and duplicated BUSCOs (D)	398
Fragmented BUSCOs (F)	17
Missing BUSCOs (M)	11

(20 mg/100 µl hemolymph) (Sigma-Aldrich). The hemocytes and other cell debris were removed from the hemolymph by centrifugation at 5,000 rpm for 5 min at 4 °C, and the supernatant was transferred to a fresh tube and kept on ice for 10 min up to estimate the protein content. To collect the lumen contents, the whole alimentary canals were carefully dissected using forceps with a blunt end and placed in chilled 1.5 ml tubes containing 100 µl 1X PBS containing 20 mg phenylthiourea (Sigma-Aldrich). The tissue was gently crushed using a micropestle and centrifuged immediately for 5 min at 8,000 rpm at 4 °C. The clear supernatant was transferred to a prechilled fresh tube and kept on ice. Watery saliva was collected from 4 to 5th instars ($n = 100$) of *N. viridula*, as described previously (Mogilicherla et al. 2018). *N. viridula* nymphs were chilled on ice for five minutes, then placed ventral side up and observed under a microscope. Their rostrum was pushed into a pipet tip containing chilled 1X PBS and held in place to collect saliva as it was secreted. Saliva with PBS was transferred to a chilled 1.5-ml tube placed on ice for collection of saliva from 50 insects, and protein concentration was estimated immediately. The protein concentration of hemolymph, lumen contents and watery saliva was estimated using the Bradford assay. One microgram of GFP dsRNA in 20 µl 1X PBS was mixed with 100 and 1000 ng of proteins from hemolymph, lumen contents or watery saliva and incubated for 12 or 24 h at room temperature. The same concentration of hemolymph and lumen contents collected from the fall armyworm larvae were used to compare the activity of dsRNases (Singh et al. 2017). After incubation, the samples were run on 1% agarose gels, and the gels were stained with GelRed® (Biotium, USA) and photographed under ultraviolet (UV) light using an Alpha Imager™ Gel Imaging System (Alpha Innotech, San Leandro, CA).

Selection of housekeeping reference genes

The genes *60S ribosomal protein L12, 40S ribosomal protein S17, 18S ribosomal RNA, Elongation factor 1 alpha, Actin, Ubiquitin conjugation factor E4 A* and *β-Tubulin* were selected based on their use in the *Halyomorpha halys* and *N. viridula* insects as reference genes (Mogilicherla et al. 2018; Liu et al. 2018). The IDT PrimerQuest software (http://eu.idtdna.comwebcite) was used for designing primers. The primer sets were checked for amplification specificity and annealing temperature. The specificity of the PCR-amplified product was characterized by gel electrophoresis. Primer sets that amplified a single-specific product were chosen for RT-qPCR amplification efficiency test (Table S1).

Target gene amplification, purification and dsRNA synthesis

Target gene amplification, purification and dsRNA synthesis were performed as described previously (Mogilicherla et al. 2018). Briefly, SnapDragon (https://www.flyrnai.org/snapdragon) was used for the identification of fragments of target genes for dsRNA synthesis. The target gene fragments were PCR-amplified using gene-specific primers containing a T7 promoter sequence (TTAATACGACTC ACTATAGGG) at the 5′ end of both forward and reverse primer (Table S1). PCR conditions used were 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, finishing with an extension step at 72 °C for 10 min. The PCR templates were purified using the PCR purification kit (Qiagen Inc., Valencia, CA USA). After PCR purification, the dsRNA targeting the gene coding for green fluorescence protein (GFP, used as a control) and seven *N. viridula* target genes such as *Death-associated inhibitor of apoptosis 1* (*IAP1*), *Baculoviral IAP repeat-containing protein 7-B-like* (*IAP2*), *Apoptosis inhibitor 5* (*IAP5*), *Charged multivesicular body protein 4b* (*SNF7*), *G protein-coupled receptor 161-like isoform X2* (*GPCR*), *Serine/threonine-protein phosphatase PP1-beta catalytic subunit* (*PP1*), *Putative ATPase N2B* (*ATPase1*) were synthesized using the MEGAscript RNAi Kit (Invitrogen, Thermo Fisher Scientific, USA) following the manufacturer's instructions. Briefly, 500 ng of the purified PCR product was used as a template in 20 µl in vitro transcription reaction. The reaction mix was incubated for 16 h at 37 °C, followed by 30 min *DNase* I treatment. The dsRNA was recovered by adding 0.1 × volume of sodium acetate (3 M, pH 5.2) and 2.5 × volume of 100% ethanol and kept at − 20 °C for at least 2 h followed by centrifugation at 4 °C for 15 min. The dsRNA pellet was then rinsed with 750 µl of 75% ethanol and centrifuged again at 4 °C for 5 min. The ethanol was removed, and then, the dsRNA pellet was dried and dissolved in ultrapure distilled water. The quality of the dsRNA was checked by electrophoresis and quantified using a NanoDrop™ ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Injection of dsRNA into *N. viridula* adults

One microgram of dsRNA (i.e., 5 µg dsRNA/ µg of body weight) in two µl of nuclease-free water was injected into the thoracic region of adults using an aspirator tube assembly (Sigma) fitted with a 3.5″ glass capillary tube (Drummond). The capillary tube was constructed using a needle puller (model P-2000, Sutter Instrument, Novato, CA).

Ten adults were used for each replicate, two replicates were used for each treatment, and the experiments were repeated twice. Adults were collected on the 4th and 13th days after injection to determine knockdown efficiency, and mortality was recorded from the 7th to 13th days post-injection.

Feeding of dsRNA into *N. viridula* nymphs and adults

For feeding dsRNA, five µg of dsSNF7 or dsGFP (used as a control) was mixed with 2.5% sucrose solution (5 µl total volume) containing food coloring dye. One drop of this solution was placed in a small Petri dish, and 24–36 h starved 3rd instar nymphs ($n = 10$) or adults ($n = 10$) were released into the Petri dish containing the one drop of the dsRNA–sucrose mixture. Ten insects were used for each experiment, and three independent experiments were performed. Therefore, 30 insects were evaluated for each dsRNA. After complete consumption of dsRNA, the nymphs and adults were individually returned to a separate container with normal food material. The nymphs and adults were collected for the knockdown study on the 5th day after fed, and the mortality was recorded from 10 to 15 days post-feeding.

Isolation of total RNA and cDNA synthesis for RT-qPCR study

Total RNA was isolated from the whole insects ($n = 6$) of developmental stages (1st, 2nd, 3rd, 4th, 5th instars and adults and their gut, fat body and epidermis ($n = 6$) tissues dissected from the adults). The GFP dsRNA injected adults and fed 4th instars and their tissues ($n = 6$) were used for the reference genes validation study. The whole injects ($n = 6$) were used for the target gene injection and feeding study using TRI Reagent® RT (Molecular Research Center, Inc., Cincinnati, OH). The total RNA was treated with *DNase* I (Ambion Inc., Austin, TX) and the integrity of purified total RNA was analyzed on 1.2% agarose gels, and the concentration of RNA was determined using a NanoDrop™ ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Two micrograms of total RNA isolated from each sample was used for cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen™, Thermo Fisher Scientific, USA).

Reverse transcriptase quantitative real-time PCR (RT-qPCR)

Reverse transcriptase quantitative real-time PCR (RT-qPCR) was performed to determine the mRNA levels of housekeeping genes in all developmental stages and dsRNA treated insects and their tissues. Further, the expression of target genes in dsRNA injected and fed adults and nymphs was studied as described previously (Mogilicherla et al. 2018). Relative mRNA levels were calculated by $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001). The mRNA levels of 60S ribosomal protein L12 and 40S ribosomal protein S17 were used to normalize the target gene expression.

Statistical analysis

The stability levels of the seven-candidate housekeeping genes such as *60S ribosomal protein L12*, *40S ribosomal protein S17*, *elongation factor 1-alpha 1*, *actin*, *ubiquitin conjugation factor E4 A*, *18S rRNA* and *tubulin beta chain-like* from *N. viridula* were determined using four statistical algorithms, geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004) and RefFinder (Xie et al. 2012) tools. The corrected percent mortality of dsRNA injected adults was calculated based on Schneider-Orelli's formula (Puntener et al. 1981), and Fisher's Exact test [Two-tailed ($P < 0.001$)] was performed to check the statistical significance. For gene knockdown study, the $2(^{-\Delta\Delta}C(T))$ ($^{\Delta\Delta}$Ct) method was used for RT-qPCR data analysis (Livak and Schmittgen 2001). A two-tailed t-test was used to check the significant difference between control and treatment. Statistical significance was determined by one-way ANOVA with Turkey post hoc analysis.

Results

RNA sequencing and transcriptome assembly

The assembled *N. viridula* RNA transcriptome includes 44,128 contigs, with a median contig length of 551 bp and an N50 of 2392 bp (Table 1a, b). About 97.3% of arthropod BUSCOs were found in the SGSB transcriptome (Table 1c). To identify orthologs of genes coding for proteins involved in RNAi pathway, *N. viridula* RNA-Seq reads were mapped to a reference of red flour beetle *Tribolium castaneum* RNAi genes. The resulting count matrix in both adult and nymph transcriptomes is shown in Tables S2–S3. Orthologs of genes coding for proteins involved in the RNAi pathway for *T. castaneum* were used to identified homologs in *N. viridula* transcriptomes, suggesting that RNAi pathway might function well in this insect.

DsRNase activity in the southern green stink bug versus the fall armyworm

The stability of dsRNA in the southern green stink bug was compared to that of the fall armyworm. dsRNA stability in the hemolymph, lumen contents and watery saliva was

compared across southern green stink bug with the hemolymph and lumen contents from fall armyworm. For stability assays, the aforementioned tissues were incubated with dsGFP. Following incubation of dsGFP with 100 and 1000 ng of hemolymph or lumen contents of fall armyworm for 12 h at room temperature, we observed partial and complete degradation (Fig. 1a and b), respectively. However, the dsGFP exposed to 1000 ng of hemolymph or lumen contents of fall armyworm for 12 h showed complete degradation (Fig. 1). Additionally, 100 ng of fall armyworm hemolymph and lumen contents also caused complete degradation of dsRNA when the exposure time was increased to 24 h (Fig. 1b). In contrast, 100 or 1000 ng of watery saliva, hemolymph or lumen contents from 3 to 4th instar nymphs of southern green stink bug did not cause any degradation of dsRNA after 12 h of exposure, but caused some degradation of dsRNA after 24 h of exposure (Fig. 1b).

Identification of suitable reference genes for RT-qPCR study

After treatment with dsRNA, knockdown efficiency of a target gene is often determined by quantification of its mRNA levels using RT-qPCR. For this, a reliable reference gene is a prerequisite. Seven candidate genes (*60S RP L12, 40S RP S17, Ubiquitin, Elongation factor, Actin, 18S rRNA* and *β-Tubulin)* were selected based on previous reports (Mogilicherla et al. 2018; Liu et al. 2018). All seven genes selected (*60S RP L12, 40S RP S17, Ubiquitin, Elongation factor, Actin, 18S rRNA* and *β-Tubulin*) showed a single peak with no visible primer–dimer formation (Fig. S1). The Cq values for mRNA levels of these genes after various treatments varied from 14 to 23 cycles (Fig. 2). *Actin, 60S RP L12, β-Tubulin* and *40S RP S17* showed lower variation in their Cq values when compared to the other three genes tested (Fig. 2). The geNorm analysis identified *60S RP L12* and *40S RP S17* as the most stable genes (Table 2 and Fig. S2) across the developmental stages and dsRNA treatments. The NormFinder analysis identified *60S RP L12* and *40S RP S17* as the most stable genes across all samples tested (Table 2

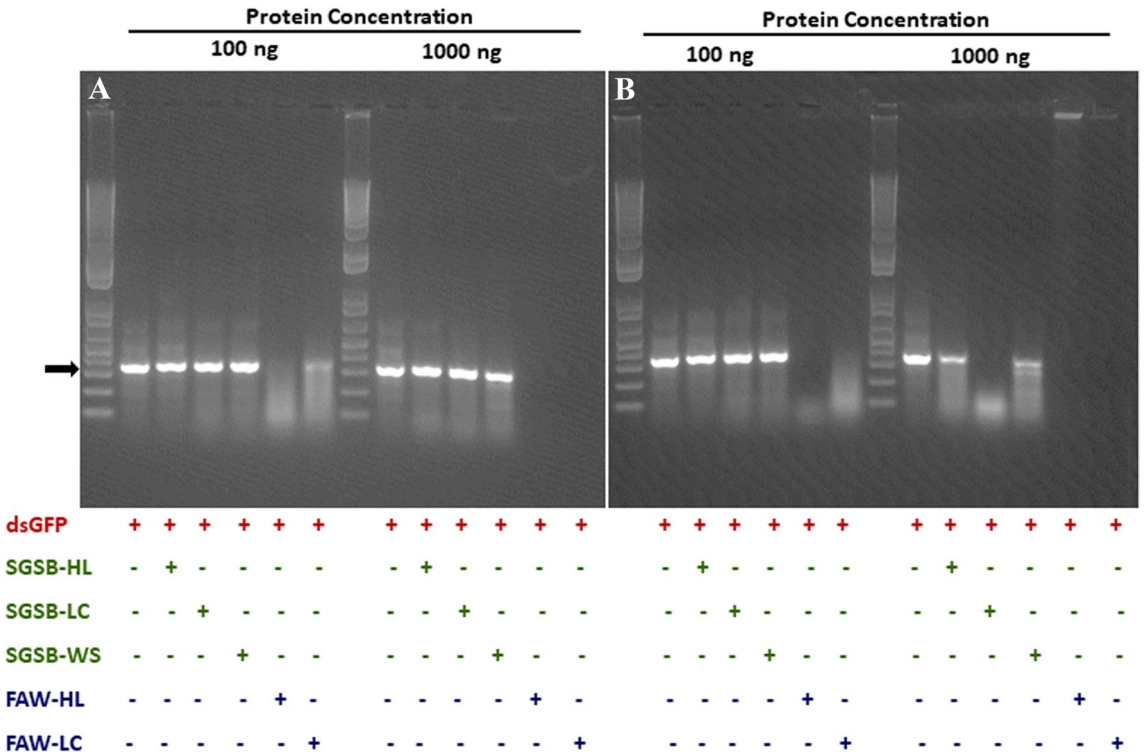

Fig. 1 Comparison of dsRNase activity in the hemolymph, lumen content and watery saliva collected from the southern green stink bug nymphs and fall armyworm larva. dsRNA was incubated with the hemolymph, lumen content and watery saliva collected from the southern green stink bug nymphs and fall armyworm larva for 12 (**a**) and 24 (**b**) hr at room temperature. The mixtures were collected and analyzed by 1% agarose gel electrophoresis, and the gels were stained and photographed using an Alpha Imager™ Gel Imaging System. Representative images from three independent experiments are shown. The arrow points to the dsRNA band. dsGFP, double-stranded green fluorescent protein; HL, hemolymph; LC, lumen contents; and WS, watery saliva

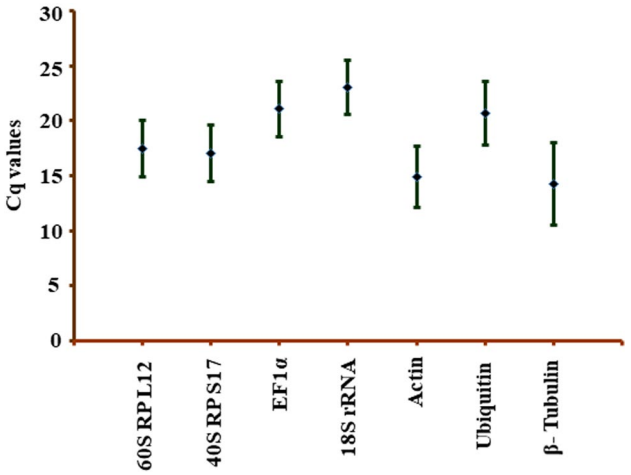

Fig. 2 Identification of stable reference genes in the southern green stink bug nymphs and adults. Variability of Cq values for seven reference genes in the nymph developmental stages, adults and their gut, fat body and epidermis tissues, dsRNA fed 4th instars and injected adults and their gut, fat body and epidermis tissues of *N. viridula* were tested. Total RNA was isolated from different developmental stages and tissues. The total RNA was converted to cDNA, and gene-specific primers were used in RT-qPCR to determine Cq values. Mean ± SD of Cq values are shown

and Fig. S3). The standard deviation (SD) values computed by the BestKeeper algorithm varied from 1.54 to 1.71, and the coefficient of variation (CV) values varied from 6.68% for *18S rRNA* to 19.52% for *β-Tubulin* across developmental stages and dsRNA treatments (Table S4). Based on these analyses, *60S RP L12* and *40S RP S17* are considered the most stable reference genes across developmental stages and dsRNA treatments tested.

Screening target genes by dsRNA injection assay in *N. viridula* adults

Three out of the seven dsRNAs tested induced 67–100% mortality (Fig. 3a). The dsRNA targeting *SNF7* showed 100% mortality at 7 days post-injection followed by 70 and 67% mortality induced by *IAP5* and *PP1* at 13 days post-injection, respectively (Fig. 3a). In the gene knockdown study, *SNF7* (Fig. 3b, S4A), *PP1* (Fig. 3c, S4B) and *IAP2* (Fig. 3d, S4C) mRNA levels decreased by 93, 85 and 63% at 4th day after the injection, when compared to their levels in control insects injected with dsGFP, respectively. Further, the expression levels of *IAP1* (Fig. 3e, S4D), *IAP5* (Fig. 3f, S4E), *GPCR* (Fig. 3g, S4F) and *ATPase1* (Fig. 3h, S4G) showed 90, 60, 67 and 80% decrease at 13th days post-injection of respective dsRNAs, respectively. Interestingly, despite 90% knockdown of *ATPase1* gene expression detected, no significant mortality was observed even at 13 days after injection of dsRNA targeting this gene.

Oral delivery of dsRNA in *N. viridula* nymphs and adults

Feeding dsSNF7 induced 59% mortality in the *N. viridula* adults at thirteen days post-feeding (Fig. 4a). The knockdown efficiency studies showed 46 and 56% reduction in *SNF7* gene expression when normalized with *60S RP L12* (Fig. 4b) and *40S RP S17* (Fig. 4c) housekeeping gene, respectively, at five days post-feeding. Likewise, feeding dsSNF7 induced 60% mortality in the *N. viridula* 3rd instars at thirteen days post-feeding (Fig. 4d) and knockdown efficiency studies showed 43 and 40% reduction in *SNF7* gene expression when normalized with *60S RP L12* (Fig. 4e) and *40S RP S17* (Fig. 4f) housekeeping gene, respectively, at five days post-feeding.

Discussion

Nezara viridula is a polyphagous pest that attacks many important crops causing losses of millions of dollars to annual crop yields. Encouraged by recent reports on successful RNAi in stink bugs, we set out on an elaborate study to find out effective target genes for RNAi and housekeeping genes for RT-qPCR. Analysis of the transcriptome from the nymphs and adults of *N. viridula* showed a representation of 97% of the arthropod gene set. This transcriptome, along with the other RNA sequences reported (Lavore et al. 2018; Liu et al. 2018), would provide an excellent resource for future studies in *N. viridula*. We searched the *N. viridula* transcriptome for sequences of genes coding for proteins that are known to function in the RNAi pathway and identified the orthologs corresponding to *N. viridula*. These data agree with recent reports on the identification of orthologs of some RNAi genes in *N. viridula* (Davis-Vogel et al. 2018).

We also compared the stability of dsRNA exposed to nucleases present in the watery saliva, lumen contents and hemolymph collected from *N. viridula* with those in the fall armyworm lumen and hemolymph and found that the activity of dsRNases in *N. viridula* is lower when compared to that of the fall armyworm. The dsRNase presence in the fall armyworm lumen contents and hemolymph is higher when compared to other insect orders, as reported previously (Singh et al. 2017). These data suggest that RNAi efficiency may be higher in the southern green stink bug when compared to fall armyworm (Fig. 1). In a previous study, a lower dsRNase activity and higher RNAi efficiency was reported in the Brown marmorated stink bug when compared to that in *Heliothis virescens* (Boddie) (Mogilicherla et al. 2018). A recent study in *N. viridula*

Table 2 Ranking of the candidate housekeeping genes according to their stability value per indicated analysis method.

Gene Name	Analysis method									
	geNorm		NormFinder		BestKeeper		ΔCT		Comprehensive	
	M	R	SV	R	SD	R	SD	R	GM	R
Developmental stages (1st, 2nd, 3rd, 4th, 5th instar nymphs and adult (whole insects) and adult body tissues (gut, fat body and epidermis)										
16S Ribosomal Protein L12	0.184	1	0.092	1	1.083	3	0.66	1	1.565	2
40S Ribosomal Protein S17	0.184	1	0.092	1	1.058	2	0.663	2	1.414	1
EF1 alpha	0.342	2	0.406	2	1.14	5	0.769	3	3.409	3
18S rRNA	0.75	5	1.126	5	1.055	1	1.199	6	3.834	4
Actin	0.495	3	0.45	3	1.119	4	0.815	4	4	5
Ubiquitin	0.601	4	0.594	4	1.34	6	0.878	5	5.233	6
Beta-Tubulin	0.888	6	1.155	6	1.666	7	1.235	7	7	7
dsRNA treated samples (dsGFP injected adults and fed 4th instar nymphs (whole insects) and their body tissues (gut, fat body and epidermis)										
16S Ribosomal Protein L12	0.222	1	0.241	1	2.21	3	1.146	1	1.316	1
40S Ribosomal Protein S17	0.222	1	0.371	2	2.216	4	1.17	2	2	2
EF1 alpha	0.778	3	0.52	3	2.133	2	1.305	4	3.13	3
18S rRNA	1.061	4	1.799	7	2.006	1	1.959	6	3.807	5
Actin	0.598	2	0.538	4	2.453	5	1.3	3	3.663	4
Ubiquitin	1.366	5	1.666	5	2.918	6	1.934	5	5.477	6
Beat-Tubulin	1.543	6	1.737	6	3.47	7	1.983	7	6.733	7
Pooled data (both developmental and dsRNA treated insects and their tissues)										
16S Ribosomal Protein L12	0.224	1	0.115	1	1.707	3	0.96	1	1.32	1
40S Ribosomal Protein S17	0.224	1	0.256	2	1.682	2	0.98	2	1.68	2
EF1 alpha	0.665	3	0.465	3	1.714	4	1.1	3	3.464	3
18S rRNA	0.921	4	1.567	7	1.54	1	1.69	6	3.807	4
Actin	0.536	2	0.491	4	1.833	5	1.11	4	3.936	5
Ubiquitin	1.135	5	1.262	5	2.268	6	1.53	5	5.477	6
Beta-Tubulin	1.298	6	1.525	6	2.789	7	1.71	7	6.735	7

M, the gene expression stability measure; *SD*, standard deviation value; *SV*, stability value; *GM*, Geomean value and *R*, Ranking

detected RNase activity in the saliva, where dsRNA was degraded almost entirely after 10 min of incubation at 25 °C. In addition, assays on the degradation of dsRNA by enzymes present in the lumen of adults showed degradation after 1 h of incubation. However, dsRNA was intact after 10 and 30 min of incubation. The study concluded that reduced RNAi efficacy observed after oral delivery of dsRNA is accompanied by a rapid degradation of dsRNA by digestive secretions in the *N. viridula* (Sharma et al. 2020). Furthermore, higher dsRNase activity levels in the saliva and lower levels of activity in the salivary gland and gut of *N. viridula* have been detected (Lomate et al. 2016). These data suggest that dsRNases could be produced as zymogens and are activated upon release into the saliva. Also, saliva collected from the neotropical stink bug, *Euschistus heros,* degraded naked dsRNA but the formulation of dsRNA with lipids and other materials protected dsRNA from degradation, suggesting that formulating dsRNA may help to improve RNAi efficiency in stink bugs as compared to delivering naked dsRNA (Castellanos et al. 2019).

Reverse transcriptase quantitative PCR (RT-qPCR) has emerged as a powerful tool to measure gene expression and is highly reproducible and sensitive due to its ability to detect transcripts expressed at lower levels (Bustin et al. 2009). Many factors influence the accuracy and interpretation of RT-qPCR results. These include the quantity and quality of the starting material, RNA extraction method, cDNA synthesis and other laboratory procedures. To limit variability, data are normalized by comparing target gene mRNA levels with those of reference genes, also known as housekeeping genes, where mRNA levels are assumed to have stable expression across various biotic and abiotic stresses and treatments (Butte et al. 2001). In RNAi experiments, RT-qPCR is used to measure mRNA levels to determine knockdown efficiency. Although the previous studies (Liu et al. 2018) used a few housekeeping genes that were

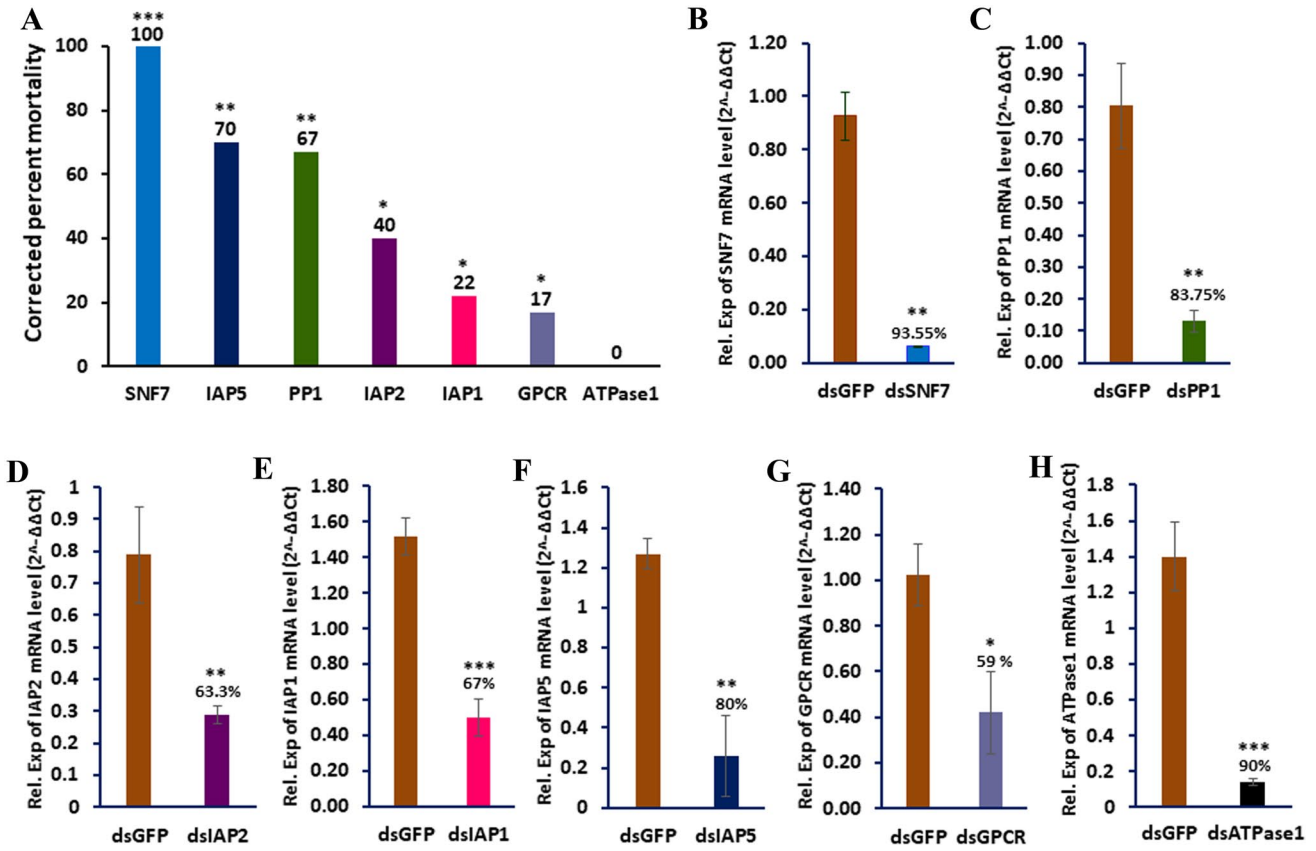

Fig. 3 **a** Mortality induced by dsRNA targeting select genes in the southern green stink bug, *Nezara viridula* (L.). One microgram of dsRNA targeting SNF7, IAP1, IAP2, IAP5, PP1, GPCR and ATPase1 was injected into each SGSB adult ($n=10$). The mortality was recorded from 7 to 13th day post-injection. DsRNA targeting gene coding for GFP was used as a control. The means of corrected percent mortality on the 13th day after injection was calculated based on Schneider-Orelli's formula are shown. The numbers shown on the top of the bars represent mean percent mortality. Asterisks represent Fisher exact test $***P<0.01$; $**P<0.05$; $*P<0.1$. (B-H) Knockdown efficiency of SNF7, PP1, IAP2, IAP1, IAP5, GPCR and ATPase1 on southern green stink bug, *Nezara viridula* determined by RT-qPCR. One µg of dsRNA targeting SNF7, PP1, IAP2, IAP1, IAP5, GPCR and ATPase1 or GFP (used as a control) was injected in to *N. viridula* adults. Total RNA was isolated on the 4th and 13th days after the injection of dsRNA targeting SNF7, PP1, IAP2, IAP1, IAP5, GPCR and ATPase1, respectively. The RNA was converted to cDNA, and the cDNA and gene-specific primers were used to quantify mRNA levels of SNF7, PP1, IAP2, IAP1, IAP5, GPCR and ATPase1 using RT-qPCR. The expression of *60S ribosomal protein* gene was used to normalize the expression of target genes. The mean ± SE ($n=5$ adults) in dsGFP and dsSNF7 (**b**), dsPP1 (**c**), dsIAP2 (**d**), dsIAP1 (**e**), dsIAP5 (**f**), dsGPCR (**g**) and dsATPase1 (**h**) injected insects are shown, and percent knockdown is shown on the top of the bars. Asterisks show the significance of the difference between control and treatment (*t* test, two-tailed $P<0.05$)

selected based on their use in other insects, no systemic studies have been performed to identify housekeeping genes for use in RT-qPCR experiments. Hence, based on our previous studies in brown marmorated stink bug (Mogilicherla et al. 2018) and reports on the southern green stink bug (Liu et al. 2018), we selected seven HKGs and determined their expression during developmental stages (1st–5th instar nymphs), adults and their body tissues, dsRNA injected adults and fed 4th instars and their body tissues. These experiments determined that the *60S ribosomal protein L12* and *40S ribosomal protein S17* reference genes exhibit the most stable expression across developmental stages and dsRNA treatments tested. In a previous study, *40S ribosomal protein S17* in southern green stink bug (Liu et al. 2018) and the *60S Ribosomal protein* and *GAPDH* in harlequin bug (Howell et al. 2020) was used as a reference gene for quantifying mRNA levels.

Out of the seven target genes tested, injection of dsSNF7 in southern green stink bug adults induced 100% mortality and feeding dsSNF7 in both nymphs and adults induced around 60% mortality suggesting that this gene may be a potential candidate for use in RNAi-mediated control of southern green stink bug. The *SNF7* gene encodes a class E vacuolar protein conserved in several organisms, such as in the fruit fly *Drosophila melanogaster* (Gao et al. 1999), nematode *Caenorhabditis elegans* (Kim et al. 2011) and plant *Arabidopsis thaliana* (Winter and Hauser 2006). This protein belongs to the ESCRT (endosomal sorting complex

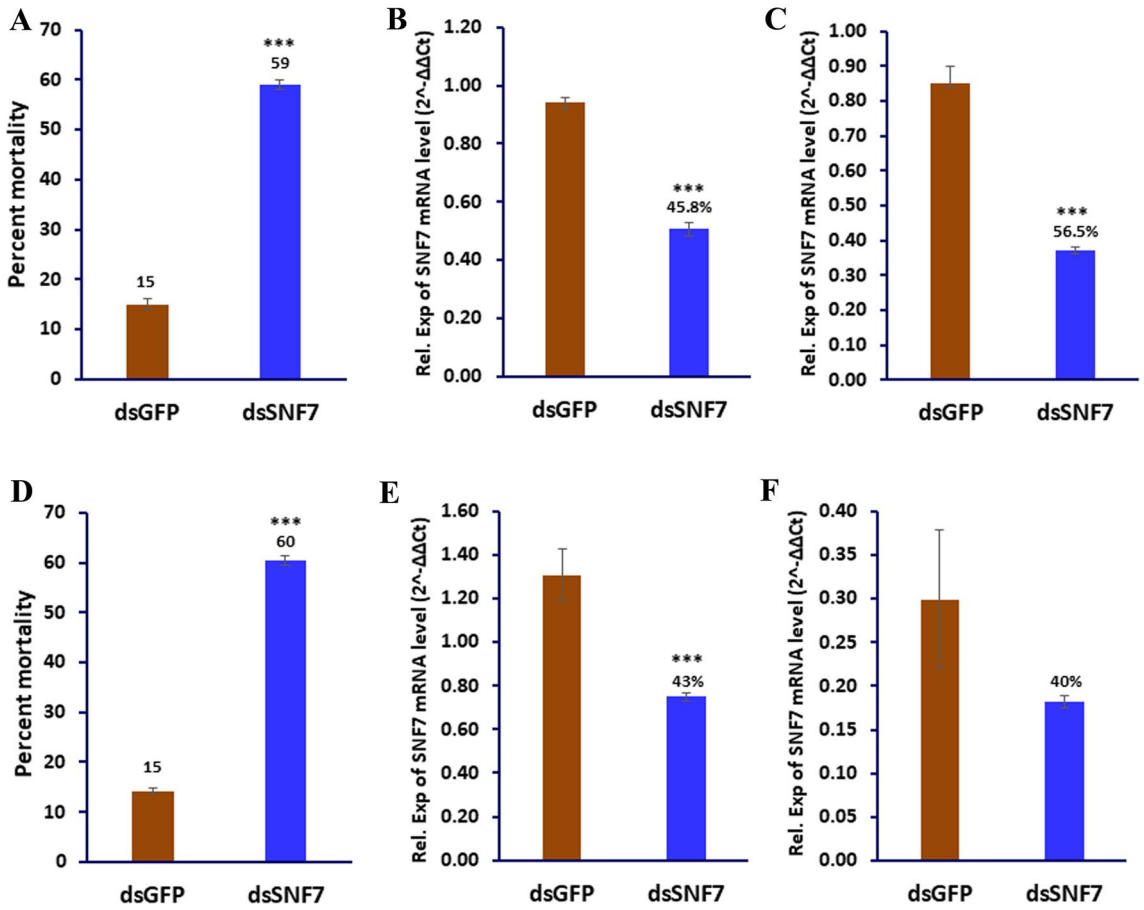

Fig. 4 Orally delivered SNF7 dsRNA induces knockdown of target gene and mortality in southern green stink bug, *Nezara viridula* adults and nymphs. **a–d** Five micrograms of dsRNA targeting SNF7 or GFP (used as a control) in 2% sucrose solution was fed to starved adults (**a**) and 3rd instar nymphs (**d**) of *N. viridula*. The mortality was recorded on 13th days after feeding. Mean + S.E ($n = 40$) are shown and the mean percent mortality is shown on top of the bars. Asterisks show the significance of the difference between control and treatment (t test, two-tailed $P < 0.05$). (**b–c and e–f**) For the knockdown study, the adults and nymphs were collected on the 5th day after feeding, and total RNA was isolated and used to quantify mRNA levels of SNF7 using RT-qPCR. The 60S RP L12 (**b–e**) and 40S RP S17 (**c–f**) mRNA levels were used for normalization of expression. The mean ± SE ($n = 6$) are shown. The percent knockdown is shown on the top of bars, and asterisks show the significance of the difference between control and treatment (t test, two-tailed $P < 0.05$)

required for transport) complex III and it is involved in the sorting of transmembrane proteins toward the lysosomal degradation pathway (Lee et al. 2007; Urquhart et al. 2015) and other multiple cellular processes (Vaccari et al. 2009; Sweeney et al. 2006). *Diabrotica virgifera* fed on dsRNA targeting SNF7 gene showed reduced expression of this gene in the carcass and midgut of the neonate larvae (Bolognesi et al. 2012) and midgut and fat body of 2nd instar larvae (Ramaseshadri et al. 2013).

Two recent studies explored the utility of RNA for control of *N. viridula.* Riga et al. (2020) showed that the RNAi works in this insect by injecting dsRNA targeting *Sex combs reduced, Actin, Chitin synthase* and *Acetyl-CoA carboxylase* into adults and nymphs. The authors suggested that RNAi could be used to validate insecticide target sites in *N. viridula.* Sharma et al. (2020) tested ten candidate genes by injection and showed that seven out of ten genes induced > 90%mortality. Feeding dsRNA targeting five of these genes induced 43–45% mortality. Further studies showed that the reduced RNAi efficacy after oral delivery of dsRNA is due to the rapid degradation of dsRNA by digestive secretions. In our studies reported here, we sequenced RNA isolated from *N. viridula* nymphs and adults and the assembled transcriptome that was used to identify orthologs of genes coding for proteins involved in the RNAi pathway. The transcriptome was also used to identify target genes for RNAi. Screening seven potential target genes, SNF7 was identified as the best target among those tested. Injection of dsRNA targeting *SNF7* into *N. viridula* adults caused 100% mortality while its feeding induced 60% mortality in both adults and nymphs. We also identified housekeeping genes for RT-qPCR quantification of mRNA levels in the *N.*

viridula developmental stages, adults and their body tissues, and dsRNA fed 4th instars or injected adults and their body tissues. Interestingly, the target genes screened for checking the functioning of RNAi in the two previous studies (Riga et al. 2020; Sharma et al. 2020) and our studies are different. Among the three reports, the 60% mortality induced by fed dsSNF7 is the best efficiency achieved so far for orally delivered dsRNA in this insect. These three reports demonstrate the potential of RNAi in *N. viridula* and show that it is possible to improve the efficiency of orally delivered dsRNA in this insect by identifying better target genes. Further research is needed to develop efficient delivery methods to realize the potential of RNAi for controlling *N. viridula*. Sprayable formulation of naked dsRNA, dsRNA expressed in bacteria, and other microorganisms or plants and nanoformulations of dsRNA are among potential methods that need to be explored in future studies.

Authors contribution

DG and SRP designed the experiments. DG, JH, SCRRC and KM performed the experiments. DG, JH, SCRRC and KM analyzed the data. DG, JH and SRP wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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