RESEARCH ARTICLE

Development of RNAi methods to control the harlequin bug, *Murgantia histrionica*

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Abstract

The harlequin bug (HB), *Murgantia histrionica*, is a major pest of cabbage family plants throughout its range in the United States. RNA interference (RNAi) is a post-transcriptional gene silencing mechanism that is showing promise as a biopesticide due to the ability to target species-specific genes necessary for growth and/or survival with synthetic double-stranded RNA (dsRNA). In the present study, dsRNA stability assays revealed that nucleases present in the saliva of harlequin bugs did not rapidly degrade dsRNA. We tracked the movement and localization of radioactively labeled dsRNA in both mustard plant seedlings and harlequin bug nymphs that fed on treated host plants. Movement of ^{32}P-labeled-dsRNA from soil to plant and plant to insect was detected. The efficacy of RNAi in inducing mortality in harlequin bug adults and nymphs injected or fed with dsRNA targeting *inhibitor of apoptosis* (*IAP*), *ATPase N2B* (*ATPase*), *serine/threonine-protein phosphatase PP1-β catalytic subunit* (*PP1*), *signal recognition particle 54 kDa protein* (*SRP*), and *G protein-coupled receptor 161-like* (*GPCR*) genes was evaluated. Injection of dsRNA targeting candidate genes into adults caused between 40% and 75% mortality and induced significant knockdown of target gene expression. 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. These findings suggest that RNAi may be a viable approach for managing this pest.

KEYWORDS

dsRNA, IAP, pest control, RNAi, stink bug

1 | INTRODUCTION

The harlequin bug (HB), *Murgantia histrionica* (Hahn; Hemiptera: Pentatomidae), is a serious pest of cabbage and related brassicaceous crops that has spread across much of the United States from its native range in Mexico and Central America (Aldrich et al., 1996; Wallingford, Kuhar, Schultz, & Freeman, 2011). This pest is most widely encountered throughout the southern United States, but damaging population blooms can occur in northern contiguous states if conditions are mild enough to facilitate overwintering survival (DiMeglio, Wallingford, Weber, Kuhar, & Mullins, 2016; Koch, Pezzini, Michel, & Hunt, 2017; Wallingford et al., 2011). Like other stink bugs, the HB damages plant tissues by piercing and sucking feeding activity, which induces scarring damage that can render plants unmarketable, and eventually causes wilting, browning, and death. Current management strategies include hand-picking, trap cropping (using turnip, kale, or mustard), and insecticidal sprays; however, infestations can quickly overwhelm and decimate entire fields due to limited natural enemies and development of insecticide resistance (Capinera, 2008; McPherson, 2018). Thus, there is an urgent need to develop alternative control methods for this invasive stink bug pest. The development of effective, species-specific, and environmentally friendly molecular biopesticides offer a possible alternative (or complement) to chemical pesticides. RNA interference (RNAi) involves the targeting of specific host genes using double-stranded RNA (dsRNA), thereby causing knockdown of target gene expression and associated gene function (Fire et al., 1998). The availability of high quality and annotated HB transcriptome (Sparks et al., 2017) facilitates the identification of target genes for RNAi-based control.

The ability to silence target genes using in vitro synthesized dsRNA has been a powerful research tool for functional genomics, and more recently, as an avenue to control pests. Characteristics such as target specificity and lack of environmental persistence make RNAi approaches desirable for crop protection against many insect pests (Baum et al., 2007; Palli, 2014; F. Zhu, Xu, Palli, Ferguson, & Palli, 2011). The RNAi event transpires when dsRNA is taken up by cells and then subsequently processed by the cellular core RNAi machinery, thus triggering a silencing effect of the target gene (Fire et al., 1998). These steps are regulated by many key proteins that play central roles in determining the efficacy of RNAi and can result in significant differences in RNAi efficiency among different insect species (K. Y. Zhu & Palli, 2020). For example, while numerous successful RNAi experiments have been reported in Coleoptera (Baum et al., 2007; Palli, 2014; F. Zhu et al., 2011), RNAi experiments in Lepidoptera are known to be challenging due to variability and low efficiency (Shukla et al., 2016; Terenius et al., 2011; Yoon, Gurusamy, & Palli, 2017). Several factors play a role in this observed differential RNAi efficiency. The presence of dsRNA degrading enzymes in the hemolymph and lumen (Arimatsu, Kotani, Sugimura, & Furusawa, 2007; Christiaens, Swevers, & Smagghe, 2014; Song et al., 2018; Wynant et al., 2014), inefficient intracellular transport of dsRNA including entrapment in the endosomes (Shukla et al., 2016; Yoon et al., 2017), and missing some core components of the RNAi machinery (Yoon et al., 2018) are shown to contribute to variable and inefficient RNAi in insects outside the order Coleoptera. Thus, it is prudent to assess available sequence information of each species for the presence of RNAi machinery genes and potential target genes, and to conduct bioassays to evaluate dsRNA degradation, knockdown, and mortality.

Previous studies on RNAi in other stink bugs suggest RNAi is efficient in this group of insects (Ghosh, Hunter, Park, & Gundersen-Rindal, 2017; Mogilicherla, Howell, & Palli, 2018). If harlequin bugs show evidence of effective RNAi, the next major hurdle is the development of a practical delivery method in the field. Recently, a plant-mediated delivery system for sap-sucking insects that uses the plant's vascular system to pull dsRNA from a solution and deliver it to a feeding insect using green beans or leaf stem cuttings has been reported (Ghosh et al., 2017). In the present study, we analyzed five target genes in harlequin bugs by injection, plant-mediated or droplet feeding for RNAi efficiency. We also conducted feeding assays with intact plant seedlings to examine systemic dsRNA degradation and used ^{32}P-UTP labeled dsRNA to track its movement from the soil, into the plant, and into the target insect after feeding to verify proof of concept for root delivery methods in the field.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and plant assay material

The HBs used in this study were collected from the University of Kentucky's South Farm and reared in an incubator at 25°C under a 16L:8D photoperiod. The insects were maintained on a diet of organic collard greens, mustard greens, Brussel's sprouts, raw carrots, and blanched peanuts. The mustard plants (*Brassica juncea*) used in this study were started from seeds and maintained in a greenhouse with supplemental artificial light (16L:8D photoperiod).

2.2 | Synthesis of ^{32}P-UTP labeled dsGFP

^{32}P-UTP labeled dsRNA of green fluorescent protein (dsGFP) was synthesized as described previously (Shukla et al., 2016). The radioactivity of the labeled dsGFP was measured using a scintillation counter.

2.3 | Tracking of dsRNA in mustard seedlings and HB nymphs

Two-week-old mustard seedlings were inserted into a 2 ml Eppendorf tube containing 50 μl of Murashige and Skoog (MS) liquid media with 0.4×10^6 counts per minute (CPM) dsGFP (Figure S1). After 4 hours, the leaf, stem, and root parts were carefully separated and collected into 1.5 ml Eppendorf tubes. The tissues were washed three times with 1 × phosphate-buffered saline, total RNA was isolated and the activity of labeled dsRNA was measured by scintillation. 2,000 CPM dsRNA was resolved on 8 M urea-16% acrylamide:bis-acrylamide gel. The gels were dried and exposed to a phosphorImager screen overnight and the image was scanned using a phosphorImager.

2.4 | Identification and phylogenetic analysis of RNAi genes

Core RNAi genes coding for proteins involved in microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) pathways, including vesicle-mediated transport, proton transport, intracellular transport, lipid metabolism, and dsRNA uptake were identified in the HB. The nucleotide sequences of the genes were obtained from NCBI *M. histrionica* Transcriptome Shotgun Assembly (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) based on sequence homology searches by running BLASTX using the NCBI BLAST service (http://www.ncbi.nlm.nih.gov/). These searches used *Tribolium*

castaneum sequences as the query. Hits obtained from these searches were used to verify their sequence similarity, identity, and detection of functional domains.

2.5 | Selection of reference genes

In HBs, *M. histrionica*, 10 stable reference genes were identified based on previous studies in other insects (Mogilicherla et al., 2018; Rodrigues, Dhandapani, Duan, & Palli, 2017; S. Singh et al., 2018). *60S ribosomal protein* (60S RP), *glyceraldehyde-3-phosphate dehydrogenase 2-like* (GAPDH), *eukaryotic translation initiation factor 6* (ETIF), *ubiquitin* (Ubi), *arginine kinase* (AK), *succinate dehydrogenase cytochrome B subunit* (SDCB), *elongation factor 1α* (EF1α), *18S ribosomal RNA* (18S rRNA), *actin* (Act), and *β-Tubulin* (Tub) were selected (Table S1) for evaluation. The sequences of these genes were retrieved from *M. histrionica* transcriptome sequence data (Sparks et al., 2017). BLASTX search was performed for each sequence in the NCBI database for reconfirmation of their annotation.

2.6 | Synthesis of dsRNA

The synthesis of dsRNAs was performed using the MEGAscript T7 RNAi kit (Thermo-Fisher Scientific) according to the manufacturer's instructions. The template DNA for dsRNA production was generated by polymerase chain reaction (PCR) amplification using gene-specific primers containing T7 promoter sequences tailed at the 5′ end of each primer. All primer sequences are listed in Table S2. A dsRNA targeting the gene coding for GFP was used as a negative control in the experiments. After synthesis, dsRNA concentrations were measured using a NanoDrop™ ND2000 spectrophotometer (Thermo-Fisher Scientific, Waltham) and analyzed by agarose gel electrophoresis. The sequences of dsRNA regions used in this study are included in the Supporting information file.

2.7 | Injection and feeding bioassays

To assess gene silencing in HB adults, 1 µg of dsRNA targeting the five selected genes was injected. Droplet feeding of dsRNA to HB nymphs was carried out by adding 5 µg of dsIAP or dsGFP (used as control) mixed with a 2.5% sucrose solution (5 µl total volume) containing food coloring dye. Drops of this solution were placed in a small Petri dish. About 24–36 hr starved last instar nymphs or adults were released into Petri dishes containing the dsRNA-sucrose mixture. After the complete consumption of the droplet, the nymphs were returned to a container with normal food materials. The plant-feeding bioassay we conducted was based on methods described previously (Ghosh et al., 2017) to deliver dsRNA through mustard seedlings. Briefly, seedlings were collected from potted plants and washed by submersion in autoclaved or filtered water. The seedlings were inserted in a 2 ml tube containing 0.3 ml water and 20 µg of dsRNA; then the tube top was wrapped with Parafilm™ (American National Can™, Neenah, WI 54) and placed under artificial lighting to stimulate the absorption of the dsRNA solution. After 3 hr, the tubes were then transferred to magenta jars with four 2nd instar nymphs (Figure S1). Treated mustard seedlings were replaced daily for 3 days then insects were returned to normal diet and rearing conditions. Each treatment of HB-dsRNA and dsGFP was performed in triplicate and experiments were repeated three times. The nymphs and adults were collected for the knockdown study on the 3rd or 4th day after treatment and mortality was scored daily for 10 days. Data for total mortality were analyzed with analysis of variance and *t* test, with $p < .05$ of probability.

2.8 | RNA extraction, primer design, and RT-qPCR

To examine the RNAi efficiency by quantitative reverse transcription-PCR (RT-qPCR), specific primers for each of the target genes were designed using IDT PrimerQuest software and shown in Table S3. RNA was extracted from dsRNA injected adults on the 3rd day and fed nymphs on 4th day after treatment, and treated with DNase I (Ambion) to remove any genomic DNA contamination. The complementary DNA (cDNA) synthesized with random and oligo dT primers was used as a template for RT-qPCR (ABI 7300). For each reaction, 0.4 µl of each primer (10 µM), 2 µl of cDNA (1:2 dilutions), and 5 µl of SYBR mix were added into a total volume of 10 µl. The RT-qPCR was performed under the following conditions: an initial denaturation step for 30 s at 95°C, followed by 40 cycles of amplification with 5 s of denaturation at 95°C, 30 s of annealing, and extension at 60°C. The melt curve was obtained by heating the amplicon at 95°C for 15 s, followed by +0.3°C increment from 60°C for 1 min to 95°C for 15 s. A nontemplate control (NTC) was also included in each run for each gene. Relative messenger RNA (mRNA) levels were calculated by the $2^{-\Delta\Delta Ct}$ method using the GADPH and 60S RP as reference genes.

2.9 | Expression stability analysis of candidate genes

The candidate reference gene expression levels were determined based on the quantitation cycle (Cq) or threshold cycle (Ct), defined as the cycle at which the fluorescence from amplification exceeds that coming from the background. The Cq values were determined using 7500 software version 2.0.5 (Applied Biosystems) and box plot diagrams were made using Microsoft Excel 2017 to illustrate levels and variations in the expression of each tested reference gene.

2.10 | Identification of reference genes

The expression data of candidate genes were analyzed using the RefFinder tool as described previously (Mogilicherla et al., 2018).

2.11 | Collection and dsRNA stability of watery saliva from HB

The HB watery saliva was collected in a fashion similar that described previously (Peiffer & Felton, 2014) and protein concentrations were determined by the Bradford method (Bradford, 1976). Various concentrations (4.125–1.031 mg/ml) of watery saliva protein were exposed to 250 ng GFP dsRNA and incubated at room temperature for an hour. The mixtures were analyzed by 1% agarose gel electrophoresis, and the gels were stained with ethidium bromide and photographed.

3 | RESULTS

3.1 | Identification of *M. histrionica* RNAi core genes

In *M. histrionica*, a total of 38 RNAi machinery genes coding for proteins known to be involved in siRNA (*Dicer 2*, *R2D2/TARBP2*, and *Ago 2*), miRNA (*Dicer 1*, *Drosha*, *Loqs*, *Pasha*, and *Ago 1*), and piRNA (*Piwi/Aubergine* and *Ago 3*) pathways were identified (Table S4). The Dicer proteins contained two RNase III, helicase, Dicer, Paz, and dsRNA binding domains. Argonaute proteins contained PAZ and PiWi domains and Drosha contained two RNase III

domains. Loquacious, R2D2, and Pasha contained three, two, and one dsRNA binding domains, respectively. The presence of genes coding for proteins involved in the RNAi pathway suggests that RNAi may work well in this insect.

3.2 | dsRNA stability assay in *M. histrionica* saliva and plant seedlings

To analyze the degradation of dsRNA by the nucleases present in *M. histrionica* saliva, the dsRNA was exposed to different concentrations of saliva and the products were run on agarose gels. The results showed that no dsRNA degradation occurred at the highest concentration (4.1 mg/ml) tested after incubation with saliva for 1 hr (Figure 1). We used ^{32}P-labeled dsRNA to test the stability of dsRNA in plant seedlings to determine whether or not dsRNA is degraded by plant nucleases. Plant seedlings were inserted in Eppendorf tubes containing ^{32}P-labeled dsRNA (Figure S2). Leaf samples were collected at 3, 6, 12, and 24 hr after treatment. Total RNA was isolated and resolved on 8 M urea-16% acrylamide:bis-acrylamide gels. dsRNA reached the top leaves of plants in ~12 hr (Figure 2a). Intact dsRNA was detected in leaves, stems, and roots collected at 24 hr after treatment (Figure 2b). Also, HB and brown marmorated stink bug (BMSB) fed on plants treated with dsRNA showed the presence of intact dsRNA at 48 hr after treatment (Figure 2c). These results suggest that seedlings take up dsRNA from soil and water, then insects acquire the dsRNA after feeding on them. Therefore, seedling-mediated delivery can potentially be used to deliver dsRNA for insect biocontrol.
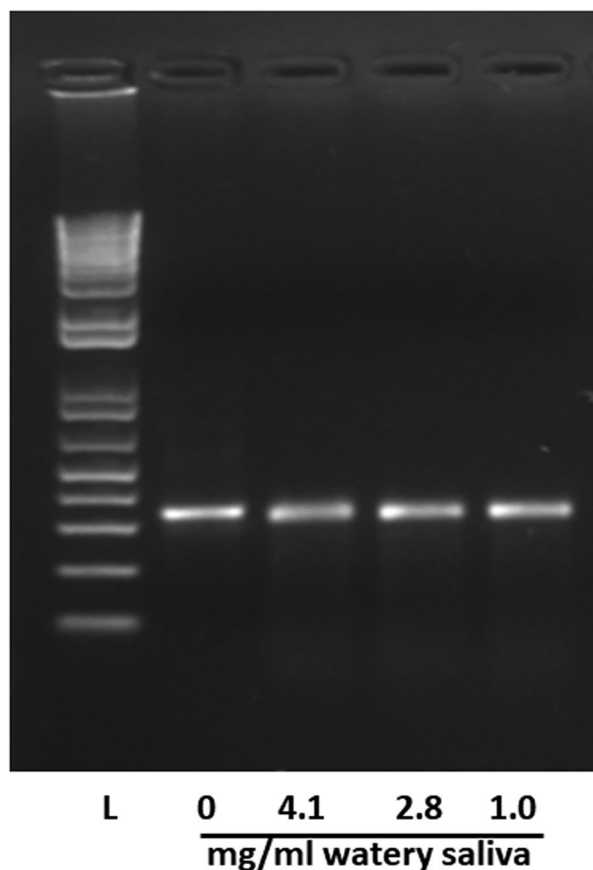

FIGURE 1 Analysis of dsRNA stability in harlequin bug (*Murgantia histrionica*) watery saliva. The HB watery saliva was collected and protein concentration was determined. Various concentrations (4.125–1.031 mg/ml) of watery saliva protein were exposed to 250 ng GFP dsRNA and incubated at room temperature for an hour. The mixtures were analyzed by 1% agarose gel electrophoresis, and the gel was stained with ethidium bromide and photographed. L, 1 kb plus DNA ladder; 0, 250 ng dsGFP alone; 4.1, 4.1 mg/ml watery saliva exposed to 250 ng dsGFP; 2.8, 2.8 mg/ml watery saliva exposed to 250 ng dsGFP; and 1.0, 1.0 mg/ml watery saliva exposed to 250 ng dsGFP. The arrow indicates the dsRNA band. dsRNA, double-stranded RNA; HB, harlequin bug; GFP, green fluorescent protein
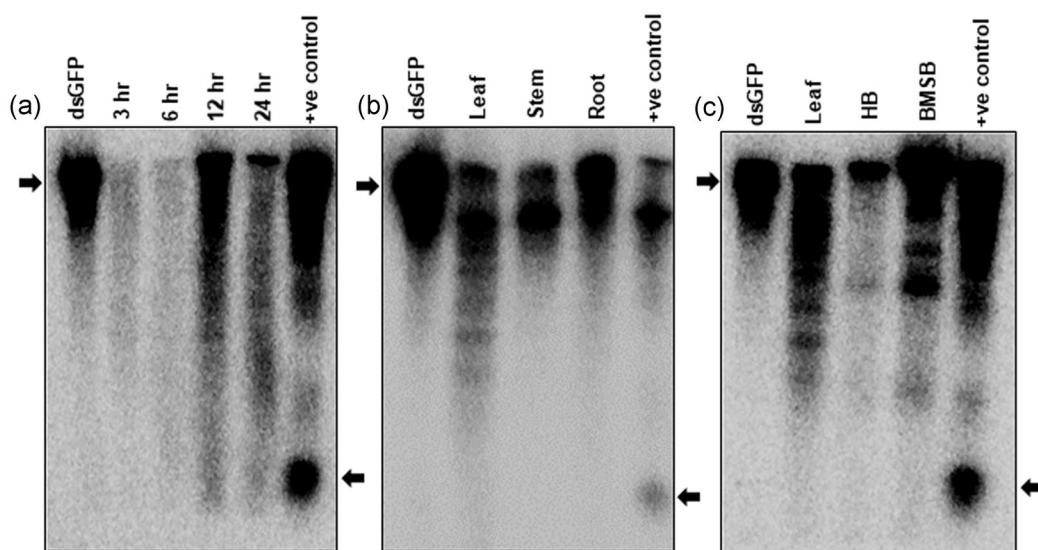
FIGURE 2 Tracking dsRNA in the mustard plant seedling tissues and insects. (a) Two-week-old mustard seedlings were inserted into 2.0 ml Eppendorf tubes containing 60 mg soil and 10×10^6 CPM ^{32}P-labeled dsGFP. The leaf samples were collected at 3, 6, 12, and 24 hr after treatment. (b) Seedlings were placed in 50 µl MS liquid media containing 0.4×10^6 CPM dsGFP. The leaf, stem, and root tissues were collected at 4 hr after treatment. (c) Seedlings were placed in 50 µl MS liquid media containing 0.4×10^6 CPM, HB and BMSB nymphs on the plants and the insects were collected at 48 hr after treatment. All samples were washed three times with $1 \times$ PBS and total RNA was extracted. The activity of labeled dsRNA was measured in scintillation counter and 2,000 CPM RNA was resolved on 8 M urea-16% acrylamide:bis-acrylamide gels. The gels were dried and exposed to a phosphorImager screen overnight and the image was scanned using a phosphorImager. The top arrows point to dsRNA and the bottom arrows point to siRNA bands. BMSB, brown marmorated stink bug; CPM, counts per minute; dsRNA, double-stranded RNA; GFP, green fluorescent protein; HB, harlequin bug; PBS, phosphate-buffered saline

3.3 | Identification and validation of reference genes

A total of 10 reference genes were selected from previous studies, homologs were identified in the *M. histrionica* transcriptome, and RT-qPCR primers were designed. The primer specificity was confirmed by a single peak in the melt curve analysis during PCR amplification (Figure S3). The gene expression levels and their variations were analyzed using RNA isolated from dsRNA injected and fed animals (Figure 3; Table S5). The expression levels of 10 potential reference genes showed a wide range of Ct values (16.9–30.35). *EF1α* showed the lowest expression levels with an average Ct value equivalent to 30.35 cycles. *60S RP* showed the highest expression levels with an average value of 16.90 cycles. The highest expression variation among all tested samples was observed for *β-Tubulin* and *60S RP* genes (ΔCq = 4.87 and 3.62, respectively), and the lowest variation was detected for *18S rRNA* and *GAPDH* genes (ΔCq = 1.47 and 2.38, respectively). ΔCq represents the variation between the maximum and minimum Ct for each gene.

3.4 | Expression stability of candidate reference genes

In the stability analysis of candidate reference gene expression in insects fed or injected with dsRNA (Table S5), the M values calculated by geNorm software were lower than the stability cutoff value (1.5). *GAPDH* and *60S RP* in dsRNA injected insects (M = 0.415), *ETIF* and *SDCB* in dsRNA fed insects (M = 0.471), and *ETIF* and *Ubi* in combined samples (M = 0.571) were identified as the most stable genes. NormFinder identified *GAPDH* (SV = 0.523) in dsRNA
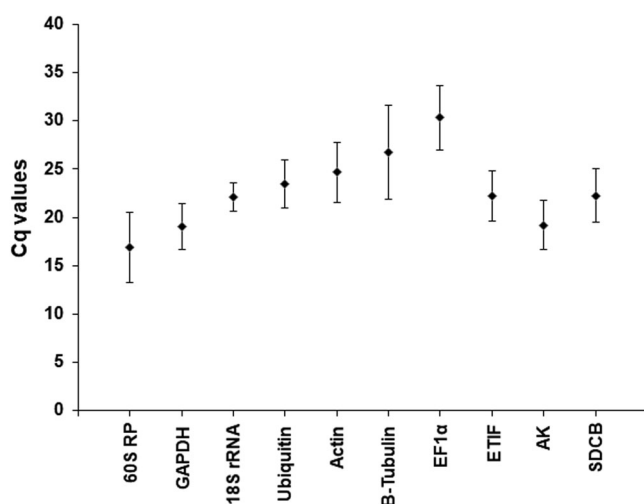
FIGURE 3 Identification of stable reference genes for RT-qPCR study in the harlequin bug, *M. histrionica*. Variability of the *Cq* values for 10 reference genes in HB nymphs and adults fed and injected with dsRNA, respectively. Total RNA was isolated, converted to cDNA, and the cDNA and gene-specific primers were used in RT-qPCR to determine *Cq* values. Mean ± *SD* of *Cq* values are shown. cDNA, complementary DNA; *Cq*, quantitation cycle; dsRNA, double-stranded RNA; HB, harlequin bug; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; *SD*, standard deviation

injected insects, *18S rRNA* (*SV* = 0.569) in dsRNA fed insects, and *AK* (*SV* = 0.792) in combined samples as the most stable reference genes. BestKeeper identified the *GAPDH* gene as the most stable gene (standard deviation [*SD*] = 0.71) in dsRNA injected insects, *18S rRNA* in dsRNA fed insects and combined samples (*SD* = 0.97) as the most stable genes. The Delta-*Ct* method identified GAPDH in dsRNA injected insects, *ETIF* and *60S RP* in dsRNA fed insects and *ETIF* for combined analysis as the most stable genes, with stability values equal to 1.4, 2.09, and 2.2, respectively. The combined ranking revealed that the most stable reference gene is *GAPDH* (1) followed by *60S RP* (1), and *Ubi* (1), while *β-Tubulin* and *Actin* were the least stable genes (10).

3.5 | Screening of target genes and induction of RNAi through injection and feeding dsRNA

Based on our previous study (Mogilicherla et al., 2018), we selected five genes that are known to cause mortality in BMSB after their knockdown by RNAi. About 1.0 μg of dsRNA targeting each of the selected genes or GFP as control were injected into each HB adult. Ten days after injection, the mortality was recorded. Two out of the five dsRNAs tested caused more than 50% mortality by 10 days after injection (Figure 4a). Quantification of mRNA levels of five genes (*serine/threonine-protein phosphatase PP1-β catalytic subunit* [*PP1*]; *inhibitor of apoptosis* [*IAP*]; *signal recognition particle 54 kDa protein* [*SRP*]; *ATPase N2B* [*ATPase*]; *G protein-coupled receptor 161-like* [*GPCR*]) using RT-qPCR showed a 40–90% knockdown in the expression of these target genes in insects injected with dsRNA targeting each of these genes (Figure 4b–f). A considerable difference in the efficacy of the five dsRNAs in causing mortality of HBs was observed. This may be due to the differences in the function of these target genes. Also, differences in knockdown efficiency of the target genes by the dsRNAs used could account for some of the variation in the efficacy of dsRNAs tested.

HBs predominantly feed on *Brassica* plants and related cole crops in agricultural systems using their needle-like stylets by alternate salivation and ingestion. Two-week-old mustard seedlings were washed with water and immersed into dsRNA solution (0.066 μg/μl) in a 2 ml microcentrifuge tube. Each seedling was placed in a magenta vessel, and four nymphs were released per vessel. Second instar nymphs were starved overnight before exposure of dsRNA. Five dsRNAs targeting *IAP*, *ATPase*, *SRP*, *GPCR*, and *PP1* were tested in the feeding RNAi bioassay. dsRNA targeting the *IAP* gene caused around 60% mortality, whereas the other four genes induced between 40% and 50% mortality (Figure 5a). The RT-qPCR analysis showed 70%, 95%, and 46% significant knockdown in the expression of *IAP*, *ATPase*, and *PP1* genes in HB nymphs fed on dsRNA targeting each of these genes, respectively (Figure 5b–d).
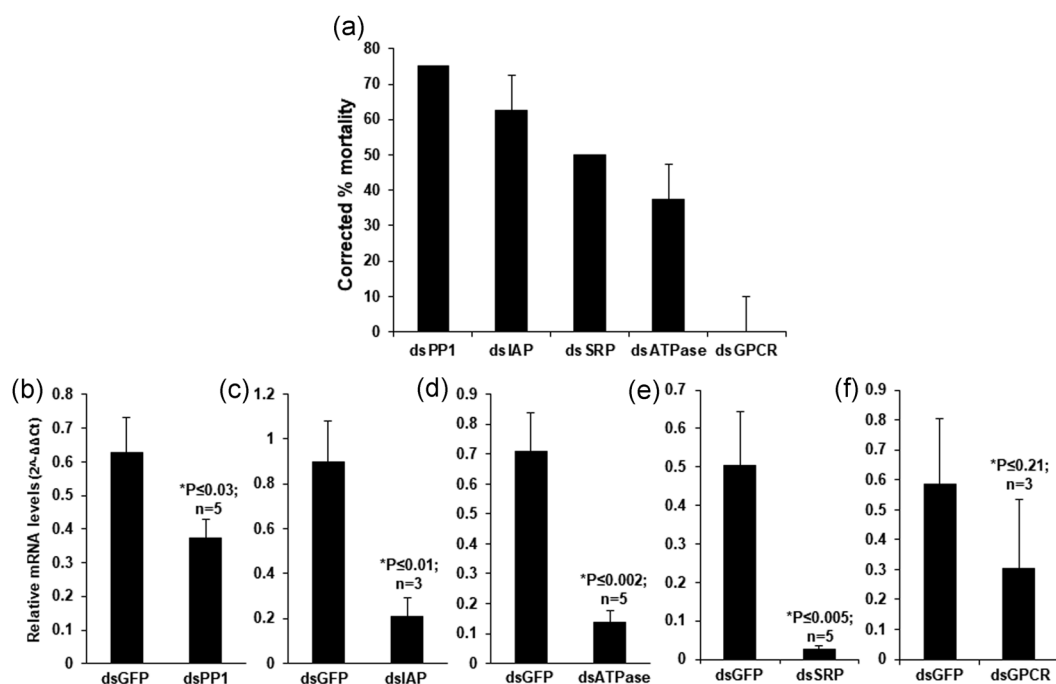
FIGURE 4 (a) Screening of RNAi target genes in HB adults by injection of gene-specific dsRNA. One microgram of dsRNA targeting *ATPase* (putative ATPase N2B, Acc. no: gb|GECQ01263448.1), *PP1* (serine/threonine-protein phosphatase PP1-beta catalytic subunit, Acc. no: gb|GECQ01499168.1), *GPCR* (G protein-coupled receptor 161-like, Acc. no. gb|GECQ01392602.1), *IAP* (baculoviral IAP repeat-containing protein 7-B-like, Acc. no: gb|GECQ01089940.1), and *SRP* (signal recognition particle 54 kDa protein, Acc. no: gb|GECQ01513793.1) was injected into HB adults. The mortality was recorded on the 10th day after injection. dsRNA targeting the gene coding for GFP was used as a control. (b–f) The knockdown efficiency of target genes was determined by RT-qPCR. One microgram of dsRNA targeting *PP1*, *IAP*, *ATPase*, *SRP*, and *GPCR* or GFP (control) was injected in HB adults. Total RNA was isolated on the 3rd day after the injection of dsRNA. The RNA was converted to cDNA, and the cDNA and gene-specific primers were used to quantify mRNA levels of *PP1*, *IAP*, *ATPase*, *SRP*, and *GPCR* using RT-qPCR. The *GAPDH* gene was used to normalize expression. The mean of relative mRNA levels and *SE* (*n* = 3–5) in dsGFP and dsPP1 (b), dsIAP (c), dsATPase (d), dsSRP (e), and dsGPCR (f) injected insects are shown. A one-tailed *t* test was used to compare the means of a single variable. cDNA, complementary DNA; dsRNA, double-stranded RNA; GFP, green fluorescent protein; HB, harlequin bug; mRNA, messenger RNA; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; RNAi, RNA interference; *SE*, standard error

These data suggest that feeding dsRNA through plant-mediated delivery causes significant knockdown of target gene and mortality in HBs nymphs. In addition, a sucrose based droplet feeding assay was performed by feeding 5 µg of dsRNA targeting the *IAP* gene in 24–36 hr starved 2nd–3rd instar HB nymphs. The results showed 55% mortality at 13 days postfeeding (Figure 6a) and relative *IAP* mRNA levels were significantly reduced (85%) at 5 days postfeeding (Figure 6b,c). These data also validate that feeding dsRNA causes significant mortality and induced higher gene knockdown with a known concentration of dsRNA administrated.

4 | DISCUSSION

RNAi is a promising technology to control insect pests. RNAi works well in coleopteran insects and a product to control western corn rootworm based on this technology has recently been approved by the US Environmental
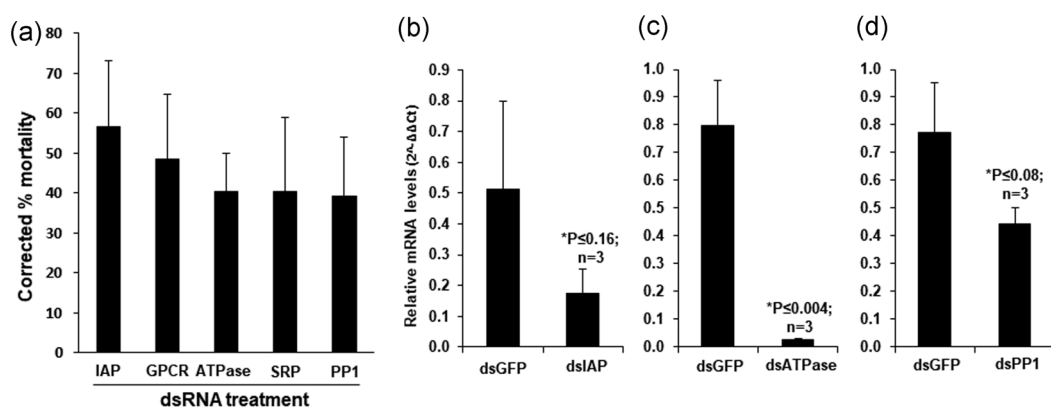
FIGURE 5 Feeding dsRNA causes mortality and induces gene knockdown of target genes in HB. (a) Greenhouse-grown mustard seedlings were inserted into 2 ml tubes containing 300 μl of nuclease-free water and 20 μg of dsRNA. Four 2nd instar nymphs fed on each dsRNA treated seedling, and the treated seedlings were replaced daily for 3 days before returning the insects to normal diet and rearing conditions. The mortality was recorded on the 10th day, and the corrected percent mortality was calculated using Schneider–Orelli's formula. The asterisk reflects significant differences in mortality rate (ANOVA, Student–Newman–Keuls method, and $p < .05$). (b–d) Knockdown efficiency determined by RT-qPCR in dsRNA fed HB. Twenty micrograms of dsRNA targeting *IAP*, *ATPase*, *PP1*, or *GFP* (control) were fed to HB nymphs on each day for 3 days. Total RNA was isolated on the 4th day after the initiation of feeding dsRNA. The RNA was converted to cDNA, and the cDNA and gene-specific primers were used to quantify mRNA levels of *IAP*, *ATPase*, and *PP1* using RT-qPCR. The 60S ribosomal protein mRNA levels were used to normalize the expression of target genes. The mean of relative mRNA levels and *SE* ($n = 3$) in dsGFP and dsIAP (b), dsATPase (c), or dsPP1 (d) fed nymphs are shown. A one-tailed t test was used to compare the mean of a single variable. ANOVA, analysis of variance; cDNA, complementary DNA; dsRNA, double-stranded RNA; GFP, green fluorescent protein; HB, harlequin bug; mRNA, messenger RNA; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; *SE*, standard error

Protection Agency. Other products to control coleopteran pests such as the Colorado potato beetle and the canola flea beetle are under development. Application of this technology in other insect pests is lagging mainly due to issues associated with dsRNA delivery and RNAi efficiency.

Orally fed dsRNAs travel through the alimentary canal, where they encounter dsRNases that may degrade them. Our previous studies showed that midgut lumen contents and hemolymph collected from lepidopteran insects showed higher dsRNase activity than that in coleopterans and the stink bugs (Mogilicherla et al., 2018; Shukla et al., 2016; I. K. Singh, S. Singh, Mogilicherla, Shukla, & Palli, 2017). In harlequin bugs, dsGFP incubated with serial dilutions of watery saliva for an hour at room temperature did not completely degrade the dsRNA even at high concentrations (Figure 1), suggesting that degradation by nucleases may not be a problem in this insect. dsRNA degradation by dsRNases has been suggested as one of the major factors contributing to inefficient RNAi in the hemipteran insect, the pea aphid (Christiaens et al., 2014). It appears that this is not a major issue in stink bugs. The functioning of RNAi has been demonstrated in the BMSB adults by injecting catalase dsRNA (Bansal et al., 2016). In another study, collard leaf and green bean-based feeding methods were employed to show effective gene knockdown in HBs and BMSB, respectively (Ghosh et al., 2017). Injected ^{32}P-labeled dsRNA into BMSB and HBs was successfully processed to siRNA (I. K. Singh et al., 2017). The results from the previous reports and our finding of lower levels of dsRNase activity in the saliva of HBs suggest that the RNAi could work well in HBs and may be a viable option for controlling this invasive pest.

For successful RNAi, the presence of genes/proteins involved in dsRNA transport into the insect and spread throughout the organism is very important. Systemic RNA interference deficient (SID) proteins are important in mediating uptake and systemic spread of RNAi in *Caenorhabditis elegans* (Winston, Molodowitch, & Hunter, 2002).
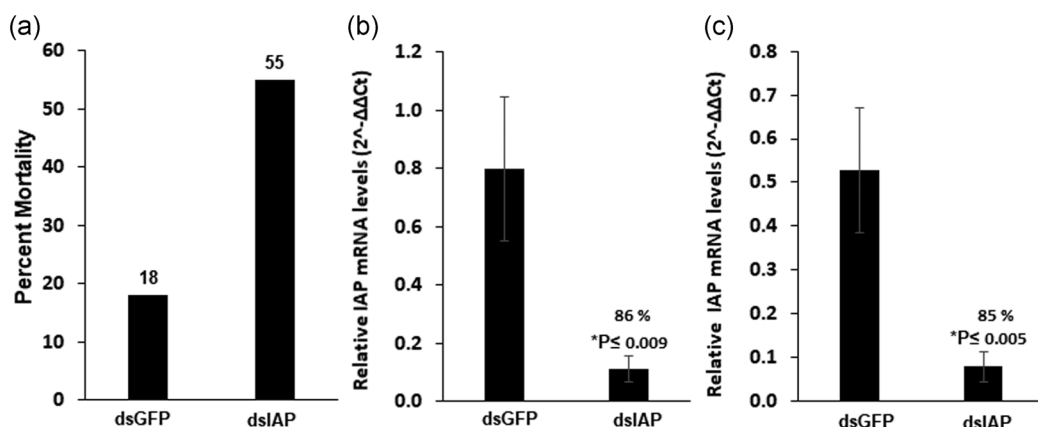
FIGURE 6 Mortality and knockdown efficiency analysis of HB nymphs fed with dsIAP by droplet method. (a) Five micrograms of dsRNA targeting *IAP* or *GFP* (used as a control) was fed to starved 2nd or 3rd instar HB nymphs by 2.5% sucrose based droplet method. The mortality rate was recorded between 10 and 13 days after feeding. (b and c) For the knockdown study, the nymphs were collected on the 5th day after treatment. The RNA was isolated, converted to cDNA, and the cDNA and gene-specific RT-qPCR primers were used to quantify mRNA levels of *IAP* using RT-qPCR. The 60S Ribosomal protein (b) and GAPDH (c) mRNA levels were used to normalize the expression. The mean of relative mRNA levels and *SE* ($n = 4$–5) in dsGFP and dsIAP (b and c) fed nymphs are shown. A one-tailed *t* test was used to compare the mean of a single variable. cDNA, complementary DNA; dsRNA, double-stranded RNA; GFP, green fluorescent protein; HB, harlequin bug; mRNA, messenger RNA; RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction; *SE*, standard error

Although we did not find SID protein homologs in the HB, we identified homologs of HB genes coding for proteins known to function in dsRNA uptake (FBX011, Scavenger receptor), vesicle-mediated transport (Arf72A, AP50, Chc, and Rab7), and proton transport (Vha16, VhaSFD, Light, and NinaC). Some of these proteins could act as dsRNA receptors and transporters that facilitate uptake (Table S4).

After dsRNA administration, the knockdown efficiency of target gene mRNA or protein levels needs to be quantified. For most of the genes, antibodies are not available and mRNA level quantification is the most commonly used method. A reliable reference gene is required to quantify mRNA levels by RT-qPCR. No reports exist for reference gene identification and standardization in HBs, therefore, we conducted experiments to identify reference genes. To identify suitable reference genes, 10 genes (*Ubi*, *elongation factor*, *ETIF*, *SDCB*, *AK*, *GAPDH*, *60S RP*, *Actin*, *18S rRNA*, and *β-Tubulin*) were selected based on previous studies in BMSB and other insects (Mogilicherla et al., 2018).

RT-qPCR is a powerful technique to study gene expression due to its high sensitivity, accuracy, specificity, and reproducibility. During RNA isolation, cDNA conversion and reaction assembly variability could be introduced. This could be countered using appropriate reference genes. Also, the use of multiple reference genes is important because using a single reference gene may not be sufficient to control variability across all treatments. In the present study, 10 reference genes were selected and validated in adult and nymphal stages treated with different dsRNAs by injection and feeding and the data were analyzed by RefFinder containing geNorm, NormFinder, BestKeeper, and delta-*Ct* statistical algorithms. The results showed that *18S rRNA*, *60S RP*, and *ETIF* are the most stable reference genes in dsRNA fed HB nymphs; *18S rRNA* and *60S RP* in dsRNA injected adults and when both dsRNA treated nymphs and adults were combined, the *18S rRNA* and *60S RP* were identified as the most stable genes. In a recent study, 10 housekeeping genes were evaluated for their stability across various treatments in BMSB and found that *ARP8* and *Ubi E4A* were the most stable genes across tissues and developmental stages tested (Bansal et al., 2016). *Ubi* is the common gene identified in both these studies and maybe the most stable gene

to target across developmental stages and treatments. These studies also confirm the previous finding that the same housekeeping gene may not work well for all stages and treatments. Therefore, one needs to identify one or more reference genes for the specific treatments compared by RT-qPCR.

The candidate genes for RNAi in HB were identified by injecting 1 μg of dsRNA targeting five selected endogenous genes in HB adults. The dsRNA targeting the *PP1* gene showed higher percent mortality (75%) followed by *IAP* (65%), *SRP* (50%), and *ATPase* (35%), whereas dsRNA targeting *GPCR* did not show significant mortality even despite knockdown of mRNA levels. Nymphal feeding assays induced significant knockdown and mortality when groups of insects fed on young seedlings over consecutive days after the roots were exposed to a 0.066 μg/μl dsRNA solution. In feeding dsRNA through plant-mediated delivery, higher percent mortality (58%) was induced with *IAP* dsRNA and 40–49% with other dsRNAs tested. The present study showed that *PP1* and *IAP* cause higher mortality by injection or feeding dsRNA to HB adults and nymphs, respectively. Furthermore, *IAP* dsRNA was evaluated with a known concentration of dsRNA delivered to individual nymphs by the droplet feeding method and results showed that a single feeding event of 5 μg of dsIAP induced 55% mortality and showed *IAP* gene knockdown. Previous studies have successfully demonstrated that injection and feeding dsIAP cause significant mortality and gene knockdown in BMSB (Mogilicherla et al., 2018), neotropical stink bug (Castellanos, Smagghe, Sharma, Oliveira, & Christiaens, 2019), and southern green stink bug (Riga et al., 2020). In conclusion, *IAP* is an effective candidate gene to control the HB by RNAi methods. Spraying or watering crops with naked or formulated *IAP* dsRNA in the field and/or stably expressing long *IAP* dsRNA in *Brassicas* or other suitable crop plants may be effective methods to manage this pest in the field. However, further research is needed to identify target genes and optimal dsRNA target sites within those genes to reduce the amount of dsRNA needed to trigger an efficient knockdown of the target gene and mortality in HB but not in the other organisms in the ecosystem. Research on the development of formulations for efficient delivery of dsRNA also needs to be conducted.

AUTHOR CONTIBUTIONS

Jeffrey L. Howell: Conceptualization (Supporting); Data curation (Lead); Formal analysis (Lead); Methodology (Lead); Resources (Supporting); Writing-original draft (Supporting); Writing-review & editing (Lead). **Kanakachari Mogilicherla:** Conceptualization (Supporting); Formal analysis (Supporting); Investigation (Supporting); Methodology (Supporting); Writing-review & editing (Supporting). **Dhandapani Gurusamy:** Conceptualization (Supporting); Data curation (Supporting); Formal analysis (Supporting); Investigation (Supporting); Methodology (Supporting); Writing-review & editing (Supporting). **Subba Reddy Palli:** Conceptualization (Lead); Formal analysis (Supporting); Funding acquisition (Lead); Investigation (Supporting); Resources (Lead); Supervision (Lead); Writing-original draft (Supporting); Writing-review & editing (Lead).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.