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ORIGINAL PAPER

Southern pine beetle-specific RNA interference exhibits no effect on model nontarget insects

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Abstract

The efficacy and high specificity of the RNA interference pathway has prompted its exploration as a potential molecular management tool for many insect pests, including the destructive southern pine beetle, *Dendroctonus frontalis* Zimmermann, in which gene knockdown and mortality via double-stranded RNAs (dsRNAs) have already been demonstrated in the laboratory. The nucleotide sequence of dsRNAs requires an exact match of at least 16 nucleotides with the targeted messenger RNA to trigger knockdown of that gene. This allows vital genes in a target pest to be silenced and mortality induced while reducing the probability of adverse effects in nontarget organisms. However, prior to utilization in forest ecosystems, demonstration of the specificity of dsRNAs through laboratory bioassays evaluating potential nontarget effects on model insects is required for proper risk assessment analyses. Consequently, we evaluated three SPB-specific dsRNAs for lethal effects, sublethal effects (larval growth rate, adult emergence or adult fecundity), and relative gene expression in three model nontarget insects representing key functional guilds, including a predator, herbivore, and pollinator. The SPB-specific dsRNAs had no effect on survival of our nontarget insects. Additionally, no sublethal effects were found and the gene expression analyses corroborated bioinformatic analyses in finding no gene knockdown. Our findings support the high specificity of RNAi technology and provide support for its development and deployment for protection of conifer forests against SPB with minimal nontarget concerns.

Keywords RNA interference · *Dendroctonus frontalis* · dsRNA · Specificity · Hazard analysis

Key message

- RNA interference (RNAi) is a highly specific, cellular viral immune response that can be manipulated through the introduction of carefully designed double-stranded RNA (dsRNA).
- RNAi can cause rapid insect mortality when essential genes are targeted; it is being developed as an innovative tool for pest suppression.
- RNAi works in southern pine beetle (SPB) and using oral ingestion of dsRNAs targeting essential genes, kills insects quickly.
- The specificity of selected SPB-specific dsRNAs was evaluated in feeding bioassays using model insects and evaluating lethal and sublethal effects, and when possible, gene expression and in silico analysis.
- No lethal, sublethal, or gene expression effects were found in the model insects evaluated.
- These findings corroborate the high specificity of RNAi technology and provide support for its development for protection of conifer forests against SPB with minimal nontarget concerns.

Introduction

Forests provide immeasurable benefits, both economic (Pye et al. 2011) and ecological (Tchakerian and Coulson 2011); implementing effective forest management strategies is essential to maintaining their function. In recent decades, temperate and boreal forests have experienced unprecedented pressure from bark beetle outbreaks (Coleoptera:

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Curculionidae, Scolytinae), reducing their economic value and threatening wildlife habitat, forest biodiversity, and their role in global carbon sequestration. Insecticides, while effective for bark beetle suppression, are impractical on a forest-wide scale (Grosman et al. 2009), and traditional bark beetle management has relied on silvicultural techniques to improve tree health and reduce susceptibility (Belanger et al. 1993; Nebeker 2004). However, the current management approaches are proving increasingly inadequate against outbreaking bark beetle populations, prompting investigations into novel mitigation strategies. Gene silencing through manipulation of the cellular RNA interference (RNAi) pathway is one such innovative approach.

RNAi-induced gene silencing takes advantage of an organism's endogenous defensive response to viral double-stranded RNA (dsRNA), triggering degradation of targeted genes and preventing production of corresponding proteins (Fire et al. 1998; Cerutti and Casas-Mollano 2006; Huvenne and Smagghe 2010). Introducing dsRNA targeting essential genes can cause mortality (Zotti and Smagghe 2015) and because the dsRNA must match at least a 16-nucleotide region of the target mRNA (Chen et al. 2021), this technology is highly specific (Agrawal et al. 2003; Whyard et al. 2009; Bachman et al. 2016). Manipulation of the RNAi pathway and its high specificity to target provides tremendous potential for insect pest management (Huvenne and Smagghe 2010; Zhang et al. 2013).

Many insects, especially coleopterans, are highly susceptible to RNAi (Palli 2014; Smagghe and Swevers 2014; Yoon et al. 2018), which has already been deployed for pest suppression in some agricultural (Zhang et al. 2017) and horticultural systems (Hunter et al. 2012). Genes that serve an integral function in western corn rootworm (*Diabrotica virgifera*) can be silenced using RNAi, causing larval mortality (Bolognesi et al. 2012). Development of effective delivery methods and demonstration of minimal nontarget effects have allowed this technology to move to the deployment stage (Bachman et al. 2013), and RNAi is now utilized as an additional tool in integrated management of western corn rootworm (Fishilevich et al. 2016). Efficacy of the RNAi pathway has been demonstrated in a number of tree-killing insect pests, including the emerald ash borer (*Agrilus planipennis*) (Zhao et al. 2015; Rodrigues et al. 2017b), Asian longhorned beetle (*Anoplophora glabripennis*) (Rodrigues et al. 2017a), and southern and mountain pine beetles (*Dendroctonus frontalis* and *D. ponderosae*) (Kyre et al. 2019, 2020).

The native southern pine beetle (SPB) is historically the most destructive forest insect pest in the southeastern USA (Nowak et al. 2008). SPB feed within the vascular cambium, causing tree mortality (Hain et al. 2011; Dodds et al. 2018). At innocuous levels, the oligophagous SPB target damaged or dying pines (*Pinus* spp.), but when populations reach outbreak levels, healthy trees are attacked, host preferences broaden, and widescale conifer mortality occurs leading to economic and ecological losses (Nebeker et al. 1992; Sullivan 2011). In recent years, the increasing severity of outbreaks, and unprecedented northward expansion of SPB's geographic range in response to warming temperatures, have prompted calls for more innovative, proactive management (Ungerer et al. 1999; Williams and Liebhold 2002; Dodds et al. 2018). The efficacy of RNAi in SPB (Kyre et al. 2019) opens up possibilities for its incorporation into integrated pest management programs, but developing viable delivery methods (Pampolini et al. 2020) and demonstrating specificity is essential to moving this technology to the deployment stage (Lundgren and Duan 2013).

Evaluating pest-specific dsRNAs for environment risks and potential negative effects on nontarget organisms is required for product registration and eventual commercialization (US Environmental Protection Agency 2014; Vélez et al. 2016; Christiaens et al. 2018; Haller et al. 2019; Mendelsohn et al. 2020; Romeis and Widmer 2020), and utilizing model organisms representing important functional guilds for nontarget assessments is an effective approach (Romeis et al. 2008; Whyard et al. 2009; US Environmental Protection Agency 2013). We evaluated lethal and sublethal effects, and gene expression, of dsRNAs designed to induce the RNAi pathway to kill SPB (Kyre et al. 2019) on model insects representing three functional guilds, including a predator, an herbivore, and a pollinator. Our goal is to demonstrate the specificity of the dsRNAs developed for gene silencing in SPB, to help advance this technology toward the deployment stage.

Materials and methods

Nontarget species selection

Nontarget insects were selected based on their significance as common representatives of important functional guilds (Bachman et al. 2013; Pampolini and Rieske 2020), and on the availability of published genome sequences to allow for bioinformatic analyses of potential homologous nucleotide sequences. Our model predator is the pink spotted lady beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae), our model herbivore the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), and our model pollinator the European honey bee, *Apis mellifera* (Hymenoptera: Apidae).

Table 1 Target genes, dsRNA (including the T7 polymerase promoter sequences, in bold) and RT-qPCR primer sequences

Gene	Primer	Primer sequence (5′–3′)	Amplicon size (bp)
shi—shibire	dsRNA-SHI F	**TAATACGACTCACTATAGGG**AGTTCGCCGTTGATGAAATC	370
	dsRNA-SHI R	**TAATACGACTCACTATAGGG**TCGAGCAGGGCTTTATGTCT	
	qRNA-SHI F	TAGATCGGTGTCAGTTCCCC	86
	qRNA-SHI R	GCGAGCGCGTTTTCTATTAC	
hsp—heat shock protein	dsRNA-HSP F	**TAATACGACTCACTATAGGG**ACACGCACACTCGTTCTCAC	351
	dsRNA-HSP R	**TAATACGACTCACTATAGGG**TACGCGTACTCGCTGAAGAA	
	qRNA-HSP F	TGCAGCAACTGGTCAAAGA	139
	qRNA-HSP R	TCTTTGGTCATGGGACGTT	
iap—inhibitor of apoptosis	dsRNA-IAP F	**TAATACGACTCACTATAGGG**TTTCGTTTGATGCTCGACTG	379
	dsRNA-IAP R	**TAATACGACTCACTATAGGG**TCTTCGCCTGTCCTGTCTTT	
	qRNA-IAP F	GTCCCGCTCATCCAGATAAA	109
	qRNA-IAP R	TTTTGCCTCTTTCGCACTTT	

Synthesis of SPB-specific dsRNAs and control dsRNAs

The efficacy of RNAi in SPB has previously been demonstrated; dsRNAs targeting three genes (*shi, hsp,* and *iap*) were evaluated, two of which were silenced (*shi* and *hsp*) and induced SPB mortality (Kyre et al. 2019) and all three of which were silenced and mortality induced in the congeneric mountain pine beetle (Kyre et al. 2020). To assess the potential effects of silencing, these three target genes on selected nontarget insects and any associated lethal and sublethal effects, dsRNA for each gene were synthesized *in vitro* according to published protocols for use in feeding bioassays for the specified nontarget insects (Table 1).

SPB adults were reared from loblolly pine (*P. taeda*) bark collected from outbreak areas in the southeastern USA. RNA extracted from SPB adults was used to synthesize complementary DNA (cDNA) which was then used in a polymerase chain reaction (PCR) to amplify DNA using the above dsRNA primer sequences. The PCR thermocycler was set to 94 °C for 4 min to denature the cDNA, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s to anneal primers to target DNA, and finishing at 72 °C for 10 min to allow DNA polymerase to extend the copied strands of nucleic acids. The resulting PCR product was purified with a PCR purification kit (Qiagen Inc., Valencia, CA) and used as the template to synthesize dsRNA using the MEGAscript RNAi Kit (Ambion Inc., Foster City, CA). The reaction was placed in a dry bath at 37 °C for 14 h and then provided Turbo DNAse for another 15 min at 37 °C. The solution was then precipitated using sodium acetate at 0.1 × per volume of reaction and 100% ethanol at 2.5 × per volume of reaction. The precipitated solution was kept at − 20 °C for a minimum of 2 h and then centrifuged at 14,000 rpm for 30 min at 4 °C. Supernatant was removed, and dsRNA pellet was then washed with 75% ethanol and centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatant was again removed, and dsRNA pellet was allowed to dry completely before being resuspended in deionized nuclease-free water. The dsRNA solution was checked for purity using gel electrophoresis and a spectrophotometer (Nanodrop Technologies, Wilmington, DE). The dsRNA was then diluted to concentrations specific to each nontarget insect.

Bioassays

Model insects were fed an SPB-specific dsRNA targeting the genes *shi, hsp,* or *iap*, or a negative control (*gfp*) for three days using protocols adapted from Pampolini and Rieske (2020). The *gfp* gene, which encodes for production of green fluorescent protein, does not exist in the genomes of insects and cannot be silenced and should thus demonstrate no effect on insects. For a positive control, either a species-specific dsRNA documented to cause mortality or a chemical control (potassium arsenate) was used (Romeis et al. 2011). Each insect received 10 µg of dsRNA each day for three days and was evaluated for survival. The predator and herbivore were also assessed for relative growth, adult emergence, or fecundity. Each assay was replicated three times for each model nontarget species. After three days of dsRNA exposure, a subsample of three insects from each treatment was collected for gene expression analysis.

Model predator—pink spotted lady beetle

Spotted lady beetle second instar larvae obtained commercially (Insect Lore, Shafter, CA) were divided into five

treatments, including SPB-specific dsRNAs (dsSHI, dsHSP, and dsIAP), the negative control (dsGFP), and a dsRNA positive control (dsVATP) (Yang et al. 2015). Silencing the *v-atpase* gene causes significant mortality in related lady beetle species *Adalia bipunctata* and *Coccinella septempunctata* at concentrations of 2 µg/µl (Haller et al. 2019). SPB-specific dsRNA and control treatments were administered at a concentration of 2 µg/µl, with 5 µl per larva daily for 3 days pipetted onto small balls of autoclaved cotton (~ 1 mm diameter). There were 24 larvae per treatment, for a total of $N = 120$ larvae per replicate for 3 replicates. Larvae were placed in 0.5 ml microcentrifuge tubes with the treated cotton; treatments were replenished daily for 3 days. Larvae were then transferred to 35 mm × 12 mm petri dishes and fed commercially obtained Lepidoptera eggs (*Ephestia kuehniella*; Beneficial Insectary, Guelph, ON, Canada), replenished as needed. Assays were held at 23 °C, 60–65% relative humidity, and 16:8 L:D. Mortality was evaluated daily, and a subset ($N = 6$ per treatment) were weighed, and their weight gain and relative growth rate ($RGR = \frac{((M_2-M_1)/M_1)}{t_2-t_1}$; where M = mass; t = time) were calculated (McEwan et al. 2009).

Following larval development and pupation, emerging adults were sexed (Nichols and Neel 1974) and grouped together by treatment into 90 mm × 15 mm petri dishes with Lepidoptera eggs ad libitum and oviposition substrate. Lady beetle eggs were collected from adult dishes daily, counted, and hatch rate (%) per day was calculated. Fecundity was expressed by calculating average hatch rate per female. The bioassay was terminated after 100 days or when all females had died.

Model herbivore—Colorado potato beetle

Eggs were collected from laboratory-reared CPB, reared to second instar, and weighed prior to use in bioassays evaluating SPB-specific dsRNAs (dsSHI, dsHSP, and dsIAP). The negative control was dsGFP, and the dsRNA positive control was dsACT, which causes significant second instar CPB mortality (Zhu et al. 2011). The dsRNAs were prepared at a concentration of 5 µg/µl with 2 µl administered to each CPB larvae daily for the initial three days of the bioassay. To prepare feeding assays, leaf discs were cut from terminal leaves of greenhouse grown potato using a 12 mm diameter punch and treated with 1 µl of 0.001% Triton (Sigma-Aldrich Co., St. Louis, MO), spread evenly across each leaf disc (Pampolini and Rieske 2020). Once dry (~ 10 min), 2 µl of dsRNA solution was administered to each leaf disc and allowed to dry before the leaf discs were placed in individual petri dishes (50 mm × 15 mm dishes).

Second instar larvae ($N = 20$ per treatment per replicate for 3 replicates) of approximately the same initial weight (5.1 ± 0.14 mg) were placed into individual petri dishes with a treated leaf disc. Treated leaf discs were replenished daily for the first three days and then replaced with untreated excised leaves thereafter. Assays were held at 23˚C at 60–65% relative humidity and 16:8 L:D. Mortality was evaluated daily. A subset of larvae ($N = 6$ per treatment) were weighed immediately after molting to the third instar and again following the molt to the fourth instar; weight gain and RGR were calculated as described above.

Following the molt to fourth instar, larvae were monitored daily until they entered the pre-pupal stage and ceased feeding (Wraight and Ramos 2015), at which point they were moved to individual ~ 166 ml cups with autoclaved cotton to provide a substrate in which to pupate. Moisture was maintained by misting the cotton lightly with distilled water every other day. Adult emergence was assessed daily, and the bioassay concluded when adult emergence ceased (~ 35 days following initiation of bioassay), and any remaining larvae or pupae were considered nonviable (~ 4–7 per treatment per replicate) and were treated as such in the statistical analyses.

Model pollinator—European honey bee

Newly emerged (< 24 h) adult European honey bees were collected directly from hive frames for use in assays (Tan et al. 2016). Assay dishes consisted of 100 mm × 20 mm petri dishes with a 1 cm diameter access hole in the side to administer the treatments and feed the bees, and with several ventilation holes in the lid (Pampolini and Rieske 2020). Treatments were administered from a modified 1.5 ml microcentrifuge tube containing 3 small holes near the tip, inserted through the hole in the side of the petri dish (Vélez et al. 2016).

Treatments included the SPB-specific dsRNAs (dsSHI, dsHSP, and dsIAP), the negative control (dsGFP), and a chemical positive control (0.5% potassium arsenate). For modified microcentrifuge tubes containing the SPB-specific dsRNAs, 100 µg of dsRNA was added as 50 µl of treatment at a concentration of 10 µg/µl. Additionally 500 µl of 50% sucrose solution was added to each tube. For the positive control, 50 µl of 0.5% potassium arsenate was added to 500 µl of 50% sucrose solution per microcentrifuge tube.

Each petri dish contained 10 adult bees, with 5 dishes per treatment per replicate ($N = 50$ per treatment per replicate and 3 replicates). Dishes were placed in a growth chamber in complete darkness at 34 °C and approximately 75% humidity. Following three days of dsRNA exposure, all dishes were maintained on 50% sucrose solution until assay termination. Mortality was monitored daily until all bees had died (~ 30 days). Dead honey bees were removed daily, and sucrose solution was replenished as needed (approximately every 2 days).

Table 2 RT-qPCR primer sequences used in gene expression analysis for model insects

Model	Gene	Primer Sequence (5′–3′)	Reference
Predator (pink spotted lady beetle)	*vATPase*	F—AGATCTCTTTTCCCATGT R—AGAGCATCTCGGCCAGAC	Yang et al. (2015)
	hsp	F—GCCGATGCGGAGAAGTATAAAG R—CGGCTTGCTTGAGTTGGAATA	Yang et al. (2015)
	16 s	F—TTGAAGGGCCGCAGTATTT R—AAGAAAGTCGTTCCCTCATCAA	Yang et al. (2015)
	18 s	F—AAGACGGACAGAAGCGAAAG R—GGTTAGAACTAGGGCGGTATCT	Yang et al. (2015)
Herbivore (Colorado potato beetle)	*B-act*	F—GCACGAGGTTTTTCTGTCTAGTG R—ATGTCATCCCAGTTGGTGATG	Zhu et al. (2011)
	shi	F—AACATTTCAGACCCGACGAC R—AGCCGGAACCTTCTATCGTT	Pampolini and Rieske (2020)
	hsp	F—GCTCCTGATGGACGAATTGATA R—CATGTCAGAGGGAGCAACAA	Pampolini and Rieske (2020)
	iap	F—CAATGGCAGACCATCGAGAA R—GCCGTACAGTCCACAAGTATC	
	rp4	F—AAAGAAACGAGCATTGCCCTTCCG R—TTGTCGCTGACACTGTAGGGTTGA	Zhu et al. (2011)
	rp18	F—TAGAATCCTCAAAGCAGGTGGCGA R—AGCTGGACCAAAGTGTTTCACTGC	Zhu et al. (2011)
Pollinator (European honey bee)	*shi*	F—AGGAGGAGGAGGAGGAAACA R—CTCGTCCGTCCATCATTTCT	Pampolini and Rieske (2020)
	hsp	F—TGAAGGTGAACGTCCAATGA R—GTGGTATTCCTCGTGGTGCT	Pampolini and Rieske (2020)
	iap	F—CCTGCATTTCCTAGTTCTTCCT R—GATGTTCCGTTTGGCGTTTC	Not applicable
	act	F- TGCCAACACTGTCCTTTCTG R—AGAATTGACCCACCAATCCA	
	rp49	F—GAGGCTATATTGGCGTTGGA R—CTGCGCGTTCTCTTCTTCTT	

Gene expression analysis

Gene expression was analyzed for a subset of insects ($n = 5$ per treatment) using RT-qPCR. RNA was extracted following three days of exposure and used to produce cDNA for each sample. Three replicates of each sample were utilized, and samples were assessed based on the mean Cq value. Using reference genes, values were normalized and relative gene expression could be assessed with the $2^{-\Delta\Delta Ct}$ outlined in Livak and Schmittgen (2001). The genes were analyzed based on sequences available and the ability to construct effective RT-qPCR primer sequences for the genes evaluated in each model insect (Table 2).

Bioinformatic analyses

To assess potential sequence ove2rlap of the SPB-specific dsRNAs and gene sequences of our model nontarget insects, bioinformatic analyses were conducted utilizing published sequences from NCBI (Bachman et al. 2013). For each gene targeted by the SPB-specific dsRNAs, orthologs in each of the model nontargets were assessed for alignments using the nucleotide BLAST (BLASTn) function from NCBI using a sequence match threshold based on Chen et al. (2021), which requires exact matches of ≥ 16 nucleotides or only one to two mismatches in a ≥ 26 nucleotide sequence. Complete coding sequences (cds) were used when available or were substituted by predicted sequences based on annotations from genes of closely related species when unavailable.

Statistical analyses

All analyses were performed using R statistical software (RStudio version 1.4.1106). For mortality data, logistic regressions were conducted using generalized linear models assuming binomial distributions and evaluated with a χ^2 test to identify any significant differences among treatments, replicates, or treatment and replicate interactions. For significant results, a Tukey's Least Significant Difference post hoc test was conducted to identify the predictor that was significant. For larval weight gain of the model

Table 3 Number of coding sequence (cds) matches of SPB-targeted genes to model nontarget sequences

SPB gene	Nontarget insect	Data source	Number of matches
shi	*C. maculata*	Not available	–
	L. decemlineata	cds	1
	A. mellifera	predicted	1
hsp	*C. maculata*	cds (*H. axyridis*)	2
	L. decemlineata	cds	10
	A. mellifera	cds	4
iap	*C. maculata*	partial cds (Coccinellidae)	1
	L. decemlineata	predicted	0
	A. mellifera	predicted	1

herbivore and predator, evaluated as the relative growth rate (RGR), data were assessed for homogeneity of variances using Levene's Test and for normality using the Shapiro–Wilk Test; if data met the requirements ($p < 0.05$) for both tests, RGR data were assessed using a two-tailed analysis of variance (ANOVA) and any significant findings were further evaluated using Student's t-tests. Data that did not meet the assumptions required for an ANOVA were assessed using the nonparametric Kruskal–Wallis Test, and significant results were further analyzed with a Wilcoxon Rank Sum Test. Adult fecundity data were assessed using a two-tailed ANOVA after evaluating for homogeneity of variances and normality. Adult emergence was evaluated using logistic regressions with generalized linear models assuming binomial distributions and also evaluated with a χ^2 test to assess any significant difference between treatments or replicates. Relative gene expression was analyzed for a subset of insects from each bioassay using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and assessed for differences in relative expression with a two-tailed Student's t-test to compare means between each gene of interest and the negative control, dsGFP.

Results

Bioinformatic analysis

The nucleotide BLAST results demonstrate that, of the available sequences, there is only one match per available nontarget sequence when the SPB genes *shi* and *iap* are evaluated in each of our nontarget species (Table 3). For the SPB gene *hsp,* matches are present in all three of the nontargets, and the number of matches ranges from 2 to 10 for the evaluated sequences.

Model predator—pink spotted lady beetle

Lethal effects—Larval survival

Second instar larvae exposed to SPB-specific dsRNAs exhibited > 80% survival until the prepupal stage (~ 12 days) for all treatments with no difference between treatments and the negative control ($\chi^2_{3,226} = 2.73$, $P = 0.44$). The positive control demonstrating ingestion of the dsRNAs, dsVATP, exhibited < 35% survival to the prepupal stage, which was significantly lower than all other treatments ($\chi^2_{4,255} = 41.63$, $P < 0.001$) (Fig. 1).

Sublethal effects—Larval growth

Second instar lady beetle larvae exposed to all treatments, including dsVATP, exhibited an average RGR of ~ 38% of initial weight per day over the course of the third instar,

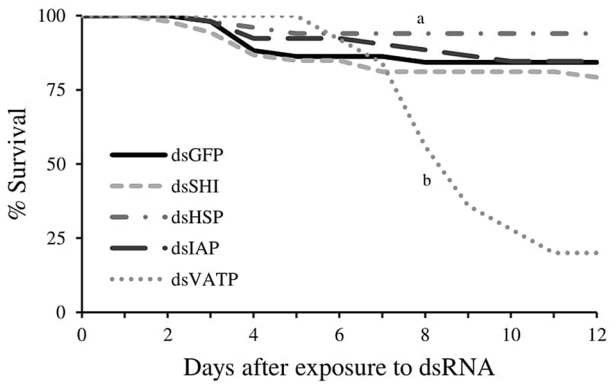

Fig. 1 Second instar lady beetle survival after ingestion of 10 µg/d for 3 d of SPB-specific dsRNAs (dsSHI, dsHSP, or dsIAP), a negative control (dsGFP), or a positive control (dsVATP)

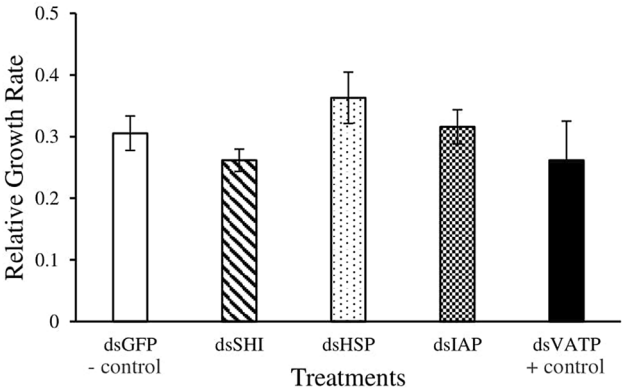

Fig. 2 Relative growth rate (RGR) of larval lady beetles fed on SPB-specific dsRNAs (dsSHI, dsHSP, or dsIAP), negative control (dsGFP), or positive control (dsVATP) shows no differences among dsRNA treatments

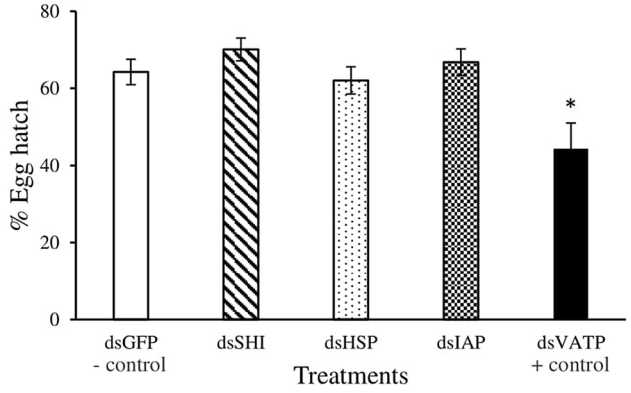

Fig. 3 Egg hatch for lady beetles fed SPB-specific dsRNAs (dsSHI, dsHSP, or dsIAP), negative control (dsGFP), or positive control (dsVATP) shows no differences among dsRNA treatments

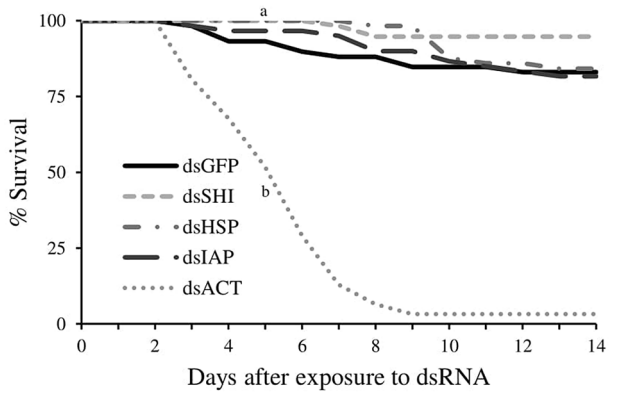

Fig. 5 Second instar CPB survival after ingestion of 10 µg/day for 3 days of SPB-specific dsRNAs (dsSHI, dsHSP, and dsIAP), negative control (dsGFP), or positive control (dsACT)

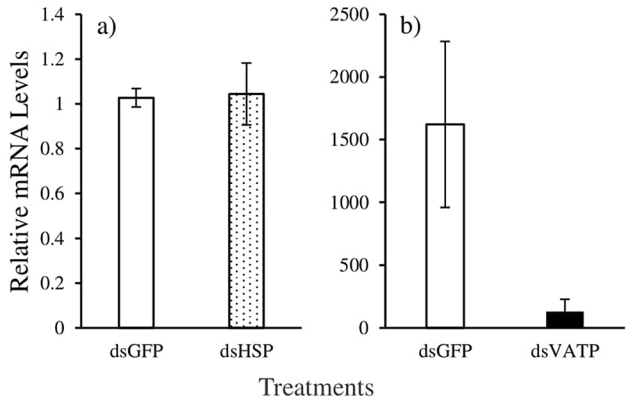

Fig. 4 Relative expression of the genes **a** *hsp* and **b** the positive control (*vATPase*) relative to the negative control (dsGFP treatment) in second instar lady beetle larvae fed 10 µg/d of dsRNA treatments for 3 days shows no differences among dsRNA treatments; expression assessed with *vATPase* primers showed high standard error, obscuring potential differences

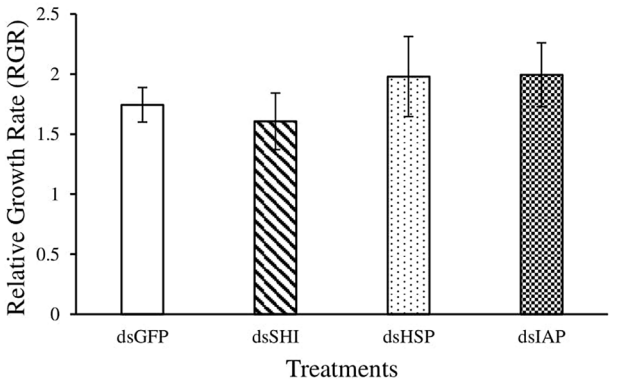

Fig. 6 Relative growth rate (RGR) of larval Colorado potato beetles fed SPB-specific dsRNAs (dsSHI, dsHSP, or dsIAP) or negative control (dsGFP) shows no differences among dsRNA treatments. The positive dsACT control was excluded from this analysis

with time spent in that stage ranging from 3 to 6 days. For larval weight gain, expressed as RGR, there is no effect of the SPB-specific dsRNAs on development ($F_{4,39}$= 1.53; P=0.21) (Fig. 2).

Sublethal effects—Adult fecundity

Following adult emergence, beetles were assessed for egg production and hatch rate for 100 d. Egg hatch per clutch averaged 60% across all treatments, and there was no effect of the SPB-specific dsRNAs on fecundity ($F_{3,350}$= 2.16; P=0.09); the only significant reduction in egg hatch occurred for the positive dsRNA control, dsVATP ($F_{4,383}$= 3.88; P=0.004) (Fig. 3).

Gene expression

Gene expression analyses, normalized with reference genes *16 s* and *18 s* (Yang et al. 2015), revealed no significant difference in relative expression of *hsp* (P=0.47) in second instar lady beetle larvae fed on the SPB-specific dsRNA (Fig. 4), corroborating the bioassay results evaluating lethal and sublethal effects. Although the positive control (dsVATP) was assessed for relative gene expression (P=0.11), the primers used for this analysis (Yang et al. 2015) yielded high standard error among both the negative control (dsGFP treated larvae) and the dsVATP treated larvae, leading to inconclusive results for relative expression of the positive control.

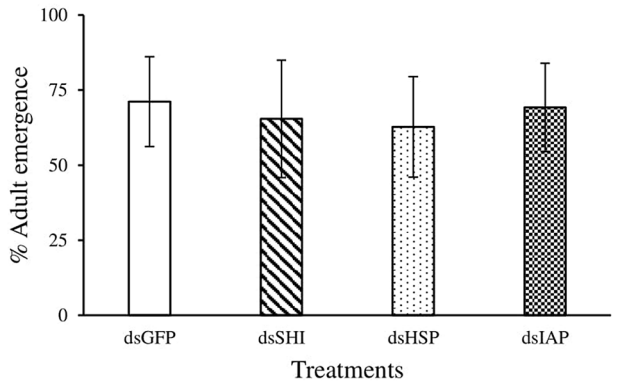

Fig. 7 Adult emergence of CPB that were fed SPB-specific dsRNAs (dsSHI, dsHSP, or dsIAP) or negative control (dsGFP) as second instar larvae and reared to adulthood on untreated potato leaves shows no differences among dsRNA treatments

Model herbivore—Colorado potato beetle

Lethal effects—Larval survival

For second instar larvae exposed to SPB-specific dsRNAs, survival to pupation (~ 14 days) was > 80% for all treatments except the positive control, and there was no difference among treatments and the negative control ($\chi^2_{3,229}$= 5.07, P = 0.16). The positive dsACT control showed significant decline in survival ($\chi^2_{4,259}$= 106.16, P < 0.001), with < 5% of larvae reaching pupation (Fig. 5).

Sublethal effects—Larval growth

Data for larval growth of CPB had a non-normal distribution (P = 0.04) and were therefore analyzed with a nonparametric test. The subset of larvae evaluated from all treatments exhibited a RGR of ~ 183% per day over the course of the third instar, requiring 2–5 days. Among the SPB-specific dsRNAs and the negative control (dsGFP), there is no difference between treatments (P = 0.59) (Fig. 6). None of the subset of larvae from the positive control treatment (dsACT) survived through the third instar, so no RGR was calculated for the positive control.

Sublethal effects—Adult emergence

Of the individuals exposed to SPB-specific dsRNAs that survived to pupation, > 55% successfully emerged as adults (Fig. 7), and there was no significant difference between SPB-specific dsRNAs and the negative control ($\chi^2_{3,202}$ = 5.81, P = 0.12). The positive control, dsACT, had no adult emergence; only three experimental larvae survived to the prepupal stage and none of those eclosed as adults.

Gene expression

There were no differences in relative expression of *shi* (P = 0.22), *hsp* (P = 0.37), or *iap* (P = 0.39) in second instar CPB larvae exposed to SPB-specific dsRNAs for 3 days when normalized with reference genes *rp4* and *rp18* (Zhu et al. 2011) (Fig. 8). As expected, the positive dsACT control caused significant knockdown of *act* when evaluated

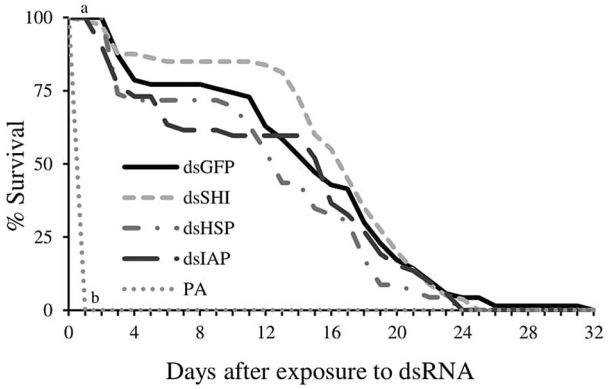

Fig. 9 Honey bee worker survival after ingestion of SPB-specific dsRNAs (dsSHI, dsHSP, and dsIAP), negative control (dsGFP), or positive potassium arsenate control (PA)

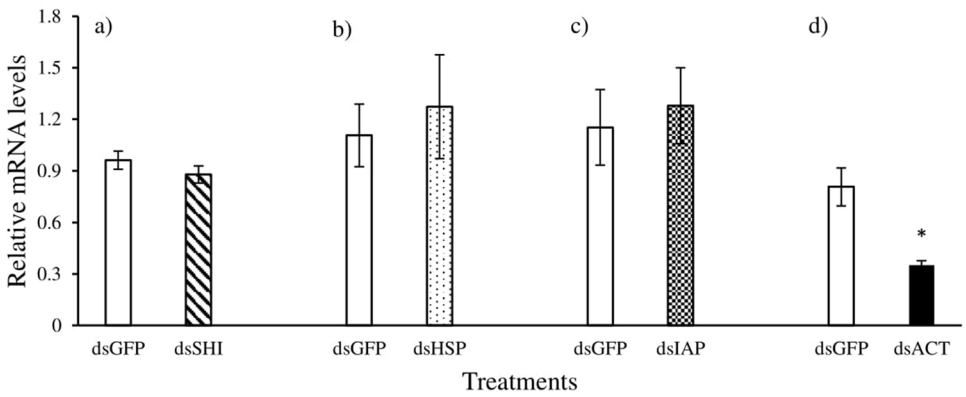

Fig. 8 Relative mRNA levels of **a** *shi,* **b** *hsp,* and **c** *iap* genes showed no significant difference in second instar CPB larvae fed SPB-specific dsRNAs for 3 days, with gene knockdown only demonstrated with the d) *act* gene for larvae that received the positive dsRNA control (dsACT)

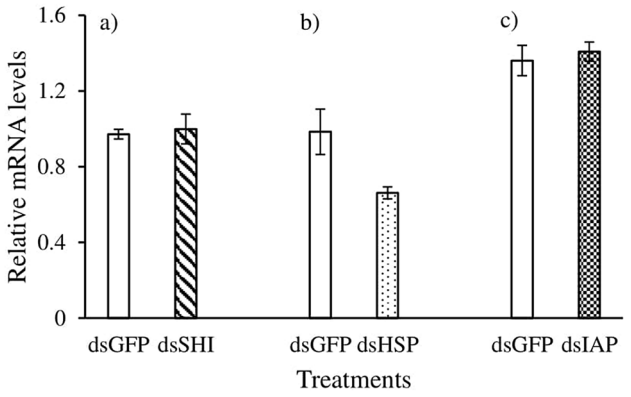

Fig. 10 Relative mRNA levels of **a** *shi,* **b** *hsp,* and **c** *iap* genes showed no significant difference from the negative dsRNA control in honey bee workers fed SPB-specific dsRNAs

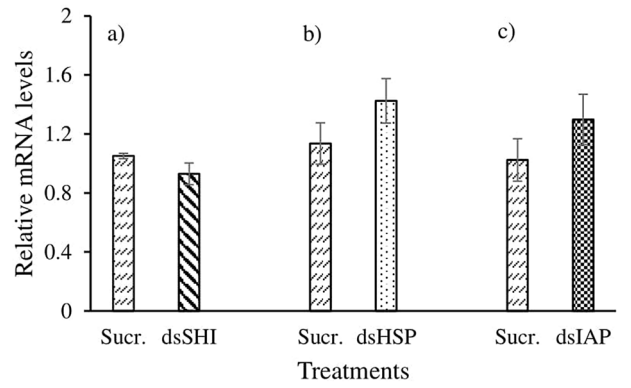

Fig. 11 Relative mRNA levels of **a** *shi,* **b** *hsp,* and **c** *iap* genes showed no significant difference from the negative sucrose control in honey bee workers fed SPB-specific dsRNAs

for relative gene expression ($P=0.01$) (Zhu et al. 2011). Although the bioinformatic analysis showed some potential matches in nucleotide sequence for *hsp* in CPB, the results of our gene expression analysis demonstrate no change in expression for *hsp*, or for any of the SPB-specific treatments, further supporting the findings of our bioassays.

Model pollinator—European honey bee

Lethal effects—Honey bee worker survival

Newly emerged adult workers exposed to SPB-specific dsRNAs were evaluated for 32 d, until all bees had died. There was no difference in survival between the dsRNA treatments and the negative control ($\chi^2_{3,280}=3.75$, $P=0.29$), whereas the positive potassium arsenate control caused significant mortality ($\chi^2_{4,248}=58.6$, $P<0.001$), confirming that workers were ingesting the treatments (Fig. 9).

Gene expression

Relative gene expression, normalized with reference genes *rp49* and *act* (Lourenco et al. 2008), demonstrated no differences in relative expression of *shi* ($P=0.41$), *hsp* ($P=0.06$), or *iap* ($P=0.38$) when compared to the negative dsRNA control (dsGFP) in adult honey bee workers (Fig. 10). Additionally, because honey bees have been noted to exhibit a generalized immune response to dsRNAs (Yang et al. 2018), relative expression was also assessed in relation to the sucrose negative control (Fig. 11); this also indicated no significant difference in relative gene expression for *shi* ($P=0.14$), *hsp* ($P=0.16$), and *iap* ($P=0.17$). These findings corroborate those of the bioassay, demonstrating no effect of the SPB-specific dsRNAs on this vital model pollinator.

Discussion

With the advent of new molecular management techniques, research into RNA interference and the high sequence specificity required to induce this pathway has allowed development of both effective and selective control of insect pests. Already this novel molecular approach has offered hope for managing populations of agricultural pests (Fishilevich et al. 2016) and been demonstrated as efficacious against the southern pine beetle (Kyre et al. 2019) and several other forest pests (Rodrigues et al. 2017a, 2018; Kyre et al. 2020). While laboratory assays have demonstrated the efficacy of oral ingestion of dsRNAs in SPB (Kyre et al. 2019), demonstrating its specificity and developing feasible methods of delivery are key steps to move this technology to the deployment stage (Bachman et al. 2013; Lundgren and Duan 2013; Romeis and Widmer 2020; Silver et al. 2021).

Here, we definitively demonstrate the specificity of dsRNAs targeting the genes *shi*, *hsp* and *iap* in southern pine beetle utilizing model insects from different functional guilds of ecological importance; this is a recommended approach for evaluating the specificity of dsRNAs (Environmental Protection Agency 2013) and offers broad insights into potential nontarget effects of this emerging technology (Lundgren and Duan 2013). To construct the most stringent analysis possible and because coleopterans are noted for their sensitivity to dsRNAs (Smagghe and Swevers 2014), we included two phylogenetically distant coleopterans in our evaluations: our model herbivore, a Chrysomelidae and model predator, a Coccinellidae. Of these, our model herbivore, CPB, is phylogenetically closer to SPB than our model predator, as the Chrysomeloidea superfamily is more closely related to the Curculionoidea superfamily to which SPB belongs (McKenna et al. 2019). Our initial bioinformatic analysis, using a conservative threshold established by Chen et al. (2021), found minimum overlap of

nucleotide sequences between the nontarget insects and the SPB-specific sequences, with the greatest overlap in the *hsp* gene of the more closely related CPB. While bioinformatic analysis is insightful for generalized evaluations of potential nontarget effects, bioassays evaluating activity of dsRNA treatments on live insects are imperative to demonstrate specificity (Environmental Protection Agency 2013) and to confirm the lack of non-sequence dependent effects of dsRNA (Christiaen et al. 2018). The greater relatedness of CPB to SPB may have contributed to a greater overlap of nucleotides in the *hsp* gene, but this overlap did not correlate with any lethal, sublethal, or gene expression differences for CPB.

Similarly, we found no lethal or sublethal effects of the SPB dsRNAs on our model predator, but the results of our gene expression analysis for the spotted lady beetle were less definitive. Although the positive control, dsVATP, was assessed for relative gene expression and showed a substantial reduction in relative mRNA levels, the primers we used (Yang et al. 2015) yielded high standard error among both the negative control (dsGFP-treated larvae) and the dsVATP-treated larvae, leading to a substantial but non-significant ($P = 0.11$) reduction in relative expression of the positive control. Limitations in insect availability prevented our ability to analyze the more ecological relevant *Thanasimus* spp., a primary predator in the SPB–pine system, but our data suggest that SPB-specific dsRNAs may not affect these essential predators.

Although phylogenetically distant, our model pollinator, the European honey bee, represents an imperiled species of enormous economic and ecological value (van Engelsdorp et al. 2009). While several studies have documented dsRNA sensitivity in honey bees, suggestive of a generalized immune response (Nunes et al. 2013; Vélez et al. 2016; Pampolini and Rieske 2020), we found no lethal effects or gene knockdown from our SPB-specific dsRNAs on our model pollinator. Further studies evaluating immunological responses in honey bees are warranted and should offer additional insights.

For toxicity studies with non-target arthropods, dsRNA exposures should exceed the maximum amount projected to be encountered in the environment (EFSA 2014). SPB experiences significant gene knockdown and mortality following a single exposure to dsRNA solutions of 2.5 µg (Kyre, unpublished data). The experimental diets we used incorporated dsRNAs at quantities ~ 12 × higher than that causing mortality in SPB, over the course of three days of treatment (5 µl at 2 µg/µl for 3 days for both the spotted lady beetle and Colorado potato beetle, and 10 µg for 3 days per honeybee). Regardless of concentration, we found no lethal or sublethal effects of SPB-specific dsRNA ingestion in our model nontarget insects.

Related studies evaluating specificity of dsRNAs have also found a generalized lack of nontarget effects on model insects, including those developed for western corn rootworm (Bachman et al. 2013) and emerald ash borer (Pampolini and Rieske 2020). While utilizing model insects may allow laboratory evaluations of dsRNA specificity on a range of functional guilds from various habitats, these findings also have implications for practical field applications of RNAi technology. Our model predator, the pink spotted lady beetle, could co-occur in southern pine ecosystems where SPB is common; laboratory evaluations of co-occurring species can grant insight into potential effects on organisms that might interact with dsRNA treatments under field conditions. Importantly, the lack of negative effects on our model predator suggests that predators in pine ecosystems are also unlikely to be affected by SPB-specific dsRNAs. As natural enemies play an integral role in regulating SPB populations at endemic levels, the lack of nontarget effects suggests that RNAi management strategies will not conflict with the natural enemy complex already functioning in pine ecosystems. The complementarity of RNAi technology to existing biological control efforts is an important consideration in evaluating its feasibility as a pest management strategy in other tree-killing pests (Pampolini and Rieske 2020). Further studies evaluating co-occurring pine-associates will add to the body of evidence demonstrating the specificity of SPB-targeted dsRNAs and its compatibility with pre-existing SPB population regulators.

Our findings in this study will help validate the feasibility of RNAi as an additional tool for suppression of southern pine beetle in pine ecosystems by ensuring the safety of this emerging technology for nontarget insects. Once the challenges associated with practical delivery are overcome, the efficacy and high specificity of RNAi to target could provide a management option that reduces the impact on beneficial or other nontarget organisms and complements the natural enemy complex already in the environment. The potential for use of RNAi in conjunction with current pest management techniques could allow a sustainable multi-faceted management approach utilizing silvicultural, biological, and molecular techniques that suppress pest populations while preserving beneficial species.

Author contributions

LKR and HH conceived the experiments; HH conducted the experiments; HH analyzed the results; HH and LKR prepared the manuscript. Both authors provided input and approved the manuscript.

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Availability of data and material Data will be made available in a free and publicly available depository.

Declarations

Conflict of interest The authors declare no conflict or competing interests.

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