Received: 12 January 2020 | Revised: 19 March 2020 | Accepted: 2 April 2020

DOI: 10.1002/arch.21683

RESEARCH ARTICLE

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

Double-stranded RNAs targeting inhibitor of apoptosis gene show no significant cross-species activity

Shankar C. R. R. Chereddy | **Dhandapani Gurusamy** | **Jeffrey L. Howell** | **Subba R. Palli**

[1]Department of Entomology, University of Kentucky, Lexington, Kentucky

Correspondence
Subba R. Palli, Department of Entomology, University of Kentucky, Lexington, KY 40546.
Email: rpalli@uky.edu

Present address
Dhandapani Gurusamy, Department of Botany, Kongunadu Arts and Science College, Bharathiar University, Coimbatore Tamil Nadu, 641029, India.

Funding information
National Institutes of Health, Grant/Award Numbers: GM070559-14, 1R21AI131427; USDA, HATCH, Grant/Award Number: 2353057000; USDA, Agriculture and Food Research Initiative Competitive, Grant/Award Number: 2019-67013-29351

Abstract

RNA interference (RNAi) has become an integral part of mainstream research due to its versatility and ease of use. However, the potential nontarget effects associated with double-stranded RNAs (dsRNA) are poorly understood. To explore this, we used dsRNAs targeting the inhibitor of apoptosis (*iap*) gene from nine insect species and assayed their possible nontarget effects. For each assay, we used a control (dsRNA targeting the gene coding for green fluorescent protein, GFP) and a species-specific dsRNA targeting nine *iap* genes in insect species to evaluate target gene knockdown efficiency, apoptosis phenotype in cells and mortality in insects. Our results revealed that dsIAP efficiently knocks down *iap* gene expression and induces apoptosis phenotype and mortality in target insect species. In contrast, no significant knockdown of the *iap* gene expression, apoptosis phenotypes, or mortality were detected in cell lines developed from nontarget insects or nontarget insects treated with dsIAPs. Interestingly, even among closely related insects such as stink bugs, *Nezara viridula*, *Halyomorpha halys*, and *Murgantia histrionica*, with substantial sequence similarity among *iap* genes from these insects, no significant nontarget effects of dsIAP were observed under the

[†]Shankar C. R. R. Chereddy and Dhandapani Gurusamy contributed equally to this study.

conditions tested. These data demonstrate no significant nontarget effects for dsIAPs and suggest that the threat of nontarget effects of RNAi technology may not be substantial.

KEYWORDS

Inhibitor of apoptosis, *Leptinotarsa decemlineata*, *Nezara viridula*, nontarget effects, *Tribolium castaneum*

1 | INTRODUCTION

The use of double-stranded RNA (dsRNA) to induce knockdown of a target gene through the RNA interference (RNAi) pathway has become a valuable tool in understanding the functions of a plethora of genes (Fjose, Ellingsen, Wargelius, & Seo, 2001). Since its discovery in the nematode, *Caenorhabditis elegans*, significant progress has been made in integrating the inner workings of the RNAi pathway into mainstream research and has contributed to advances in science (Fire et al., 1998). RNAi has also become the subject of widespread interest in the agrochemical industry due to its potential as a pest control method. The growing need for an alternative to chemical pesticides due to resistance development in pests and their nontarget effects on other organisms in the ecosystem has increased interest toward RNAi-based pest control methods (Baum et al., 2007; Burand & Hunter, 2013; Huvenne & Smagghe, 2010; Zhu & Palli, 2020). Furthermore, studies have shown that the dsRNA molecules have a short half-life, which further reduces their environmental impact (Dubelman et al., 2014).

The major targets for designing dsRNAs for pest management are lethal genes that are highly conserved in pathways across taxa (Yu et al., 2013). The apoptosis pathway, which is highly conserved across species, is a good target for RNAi-based control of pests (Crawford et al., 2012). The process of apoptosis occurs naturally during development to maintain homeostasis and also drives immune responses in the case of cell damage (Norbury & Hickson, 2001). Apoptosis in cells is caused by caspases, which were discovered in 1993 in *C. elegans* (Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). Caspases function by cleaving nuclear, mitochondrial and plasma membrane proteins to activate them, ultimately leading to cell death (Martinez, Reif, & Pappas, 2010). Inhibitors of apoptosis proteins (IAP) bind and inactivate caspases, thereby preventing cell death (Deveraux & Reed, 1999; Kobayashi, Hatano, Otaki, Ogasawara, & Tokuhisa, 1999). Multiple IAP proteins containing baculoviral IAP repeat (BIR) domains have been identified in insects (Birnbaum, Clem, & Miller, 1994; Crook, Clem, & Miller, 1993; Hay, Wassarman, & Rubin, 1995) and shown to inhibit caspase activity (Shi, 2002). As many as five different *iap* genes have been identified in insect genomes (Srinivasula & Ashwell, 2008; Verhagen, Coulson, & Vaux, 2001; Yoon, Koo, George, & Palli, 2020).

The effectiveness of dsIAP in inducing knockdown of the *iap* gene, apoptosis, and mortality in several insect species has been reported recently (Cao, Gatehouse, & Fitches, 2018; Dhandapani, Gurusamy, Howell, & Palli, 2019; Igaki, Yamamoto-Goto, Tokushige, Kanda, & Miura, 2002; Mogilicherla, Howell, & Palli, 2018; Powell, Bradish, Gatehouse, & Fitches, 2017; Rodrigues, Dhandapani, Duan, & Palli, 2017; Yoon, Shukla, Gong, Mogilicherla, & Palli, 2016; Yoon et al., 2018). However, the cross-species activity of dsIAP is not studied extensively. Injection of dsRNA targeting the *Drosophila melanogaster* iap1 gene into two nontarget dipteran pests, *Musca domestica* and *Delia radicum* induced knockdown of iap1 orthologs and mortality in these insects despite the absence of 21 bp conserved regions (Powell et al., 2017). In this study, we looked at the degree of nontarget effects caused by dsRNAs targeting *iap* genes from nine insect species: *Spodoptera frugiperda* (*Sf*), *Leptinotarsa decemlineata* (*Ld*), *Tribolium castaneum* (*Tc*), *Anoplophora glabripennis* (*Ag*), *Lasioderma serricorne* (*Ls*), *Aedes*

aegypti (*Aa*), *Halyomorpha halys* (*Hh*), *Murgantia histrionica* (*Mh*) and *Nezara viridula* (*Nv*) from four insect orders. We also compared dsRNA targeting regions of *iap* genes across the nine species tested to determine the degree of sequence similarity. We used in vitro, in vivo, and computational methods to study potential nontarget effects of nine *iap* genes in four cell lines and three insect species. Interestingly, no significant nontarget effects were detected even among closely related species, such as the three stink bug species under these testing conditions.

2 | MATERIALS AND METHODS

2.1 | Transcriptome analysis

The transcriptome for *A. aegypti* was obtained from Vectorbase; the version of the transcriptome used for the analysis was L5.2 (Giraldo-Calderón et al., 2014). Transcriptomes for *H. halys*, *T. castaneum*, *L. decemlineata*, and *A. glabripennis* were obtained from the NCBI database. As the curated databases for *S. frugiperda*, *L. serricorne*, *M. histrionica*, and *N. viridula* are not available, we assembled transcriptomes for these insects using the TRINITY transcriptome assembler.

2.2 | Small interfering RNA (siRNA) fragment analysis

To analyze the similarity of siRNA produced by small interference *iap* gene fragments (siIAP) across species, we created local blast directories of transcriptomes for all nine species using CLC Bio. All possible siIAPs produced by the dsIAPs were obtained using a python script. These siIAPs were blasted against the transcriptomes of all species (Camacho et al., 2009).

2.3 | IAP domain analysis

To perform the domain analysis, we used the nucleotide sequences of nine *iap* gene sequences. We obtained the open reading frames for these nucleotide sequences using the ExPASY (Gasteiger et al., 2003). After obtaining the open reading frames, an NCBI Conserved domain search was performed to identify conserved domains and their locations within each *iap* gene. The MUSCLE alignment program was used to align *iap* gene sequences (Edgar, 2004).

2.4 | Target gene amplification, polymerase chain reaction (PCR) purification, and dsRNA synthesis

The dsRNA targeting the *iap* gene in each of the nine insects tested was designed at different times for experiments in our laboratory. The dsRNA targeting sequences (see Supplementary Information) and primer sequences (Supplementary Information Table S1) for all species tested are included. Species-specific complementary DNA (cDNA) was used as a template to amplify fragments of *iap* genes from nine insects using gene-specific primers (Supplementary Information Table S1) as described previously (Yoon et al., 2016). dsRNAs targeting the gene coding for green fluorescence protein (GFP, control) and *iap* genes were synthesized using a MEGAscript® T7 RNAi kit (Ambion). The integrity of the dsRNA was analyzed on 1% agarose gels, and the concentration was determined by using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific).

2.5 | Insect cell culture, apoptosis phenotypes, and gene knockdown study

The *L. decemlineata* cell line (Lepd-SL1 cells) and *T. castaneum* cells, BCIRL-TcA-CLG1 were received from Dr. Cynthia Goodman from Biological Control of Insect's Research Laboratory (USDA-ARS, Columbia, MO) and cultured, as described previously (George, Gaddelapati, & Palli, 2019; Roy & Palli, 2018; Shukla et al., 2016; Yoon et al., 2016). The *A. aegypti*, Aag-2 cells (Cui, Sui, Xu, Zhu, & Palli, 2014) were cultured in Schneider's insect medium with L-glutamine (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Seradigm FBS, VWR® Life Science). Sf9 cells were derived from IPLB-Sf21-AE, an established cell line originally isolated from *S. frugiperda* ovaries. The plasmids containing the genes, *Luciferase* and *SID-1* (dsRNA transporter channel) from *C. elegans* (Xu et al., 2013) were stably expressed in Sf9 cells and named *Sf9_LUC_CeSID-1* and maintained in Sf-900™ II SFM (Gibco™, Thermo Fisher Scientific). All the cell lines were maintained in a 27–28°C incubator. For apoptosis phenotypes and gene knockdown studies, cells were seeded in 96- and 24-well plates in 100 and 250 µl culture medium, respectively and exposed to dsGFP or dsmalE as control and dsIAP targeting the *iap* genes in nine insect species.

2.6 | In vivo assays

To study the effect of nontarget dsRNAs in insects, we selected three insect species: the Colorado potato beetle (CPB), the Red flour beetle (RFB; Coleopterans) and the Southern Green Stink Big (SGSB; Hemipteran). Four micrograms of dsGFP or dsIAP genes were spread on washed and TritonX-100 treated eggplant leaf discs (7 mm diameter). The treated leaf discs were fed to third instar CPB larvae that were starved overnight. Four micrograms dsRNA/larva/day was fed over a 3-day period to a total of 12 µg dsRNA/insect. The mortality was recorded on the fifth-day post-feeding. For SGSB feeding, the green been mediated dsRNA delivery method (Ghosh, Hunter, Park, & Gundersen-Rindal, 2017; Ghosh, Hunter, Park, & Gundersen-Rindal, 2018) was used. Certified organic green beans were washed and cut to a length of 5 cm, then immersed in capless 2 ml microcentrifuge tubes containing 300 µl nuclease-free water and 20 µg dsGFP or dsIAP. The beans, dsRNA, and water were replaced daily for 3 days. The tubes were sealed with parafilm to prevent evaporation of the solution and to prevent insects from entering the solution. Beans were immersed in water containing dsRNA 3 hr before feeding. The tubes were then placed in a solid platform to keep them upright and enclosed within magenta jars (Sigma-Aldrich). Second or third instar SGSB nymphs were starved overnight before feeding. Ten nymphs were released per magenta jar containing one green bean in a microcentrifuge tube containing dsRNA (20 µg daily for 3 days) solution. The mortality was recorded on the 14th day after the initiation of feeding. In the RFB assay, 1 µg dsRNA in 200 nl was injected into each larva. Afterward, the phenotypic changes and mortality were recorded daily until the 10th day after injection.

2.7 | Extraction of total RNA, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

For the gene knockdown study, total RNA was isolated from dsRNA-treated cells, injected *Tc* larvae and fed *Ld* larvae and *Nv* nymphs using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH). In the cell line, each well that was treated using dsRNA was considered as one replicate and RNA was extracted accordingly. We isolated RNA only from live insects and used one larva and nymph each in *Ld* and *Nv* and two larvae in *Tc* per replicate. The RNA was used for cDNA synthesis followed by a quantitative PCR assay. Two micrograms of total RNA were used to cDNA synthesize using the M-MLV reverse transcriptase (Invitrogen, USA). RT-qPCR was performed, as described previously (Mogilicherla et al., 2018). Relative mRNA levels of Ld_IAP1, Tc_IAP1, Aa_IAP1, Sf_IAP1, and Nv_IAP5 were determined by the $2^{-\Delta\Delta Ct}$ method using Rp4 (*Ld*), Rp49

(*Tc*), SRP7 (*Aa*), 28S rRNA (*Sf*), and 60S RP L12 (Nv) as reference genes. The primers used for dsRNA synthesis and RT-qPCR analysis are listed in Supporting Information Table S1.

2.8 | Statistical analysis

The CLC Bio tool was used to create local blast directories of transcriptomes for all nine species and a python script was used to obtain all possible siIAP produced from the dsIAP. The siIAPs were then blasted against the transcriptomes of all species (Camacho et al., 2009). The open reading frames for nine IAP nucleotide sequences were obtained by using the ExPASY (Gasteiger et al., 2003). An NCBI Conserved domain search was performed to identify conserved domains and their locations within each *iap* gene and the MUSCLE alignment program was used to align *iap* gene sequences (Edgar, 2004). The delta delta C_t ($\Delta\Delta Ct$) method was used for RT-qPCR data analysis and one-tailed *t* test was used to compare the mean of a single variable. The significance of the target gene knockdown in cells/tissues was tested by one-way analysis of variance (ANOVA) with Duncan multiple range test with the significance level set at $p < .05$ using IBM SPSS Statistics 20 software.

3 | RESULTS

3.1 | Transcriptomes analysis——Prediction of IAP domains and siRNA fragments

To identify conserved domains present in *iap* gene sequences from the nine insects selected, open reading frames of these genes were searched at the NCBI. The presence of conserved domains in nine *iap* genes tested are shown in Figure 1. The location of the dsRNA target region in each *iap* gene is also marked in Figure 1. The dsRNA targeting the *iap* gene in each of the nine insects tested was designed at various times for RNAi experiments in each insect (Supporting Information File S2). Therefore, the dsRNA targeting regions (Supporting Information File S2) are different in each *iap* gene tested. The nucleotide sequences of nine *iap* genes are aligned and shown in Supplementary information File S3. The dsRNA targeting regions are also marked in the aligned nucleotide sequences. To identify all possible siRNAs produced from each of the nine dsIAPs, a python code was used. The presence of complementary sequences for all identified siRNAs was checked by searching the transcriptomes of nine insects (Table 1). These searches revealed minimal similarity between siIAP and transcriptome sequences. For example, out of a total of 332 siIAPs produced from *A. aegypti* dsIAP, only four of them were perfectly matched in *A. glabripennis* transcriptome suggesting that region of the *iap* gene used has low nucleotide sequence conservation. In general, the siIAPs produced from each dsIAP tested showed less than 10 siRNAs that matched 100% with the transcriptome sequences searched (Table 1). The only exception to this general conclusion is the three stink bug species tested. Forty-three and 95 siRNAs produced from *H. halys* dsIAP2 (Hh_dsIAP2) matched 100% with the sequences in the transcriptomes of *M. histrionica* and *N. viridula*, respectively. Similarly, 118 and 79 siRNAs produced from *M. histrionica* dsIAP2 (Mh_dsIAP2) matched 100% with the sequences in the transcriptomes of *H. halys* and *N. viridula*, respectively. Also, 51 and 57 siRNAs produced from *N. viridula* dsIAP5 (Nv_IAP5) matched 100% with the sequences in the transcriptomes of *H. halys* and *M. histrionica*, respectively.

3.2 | dsIAPs induced knockdown of *iap* genes and apoptosis only in cell lines developed from target insects

Nine dsIAPs targeting *iap* genes from CPB, RFB, ALB, YFM, FAW, CB, BMSB, HB, and SGSB along with a control dsGFP/dsmalE were added to the culture medium of Lepd-SL1, TcA, Aag-2, and Sf9_LUC_CeSID-1 cells.

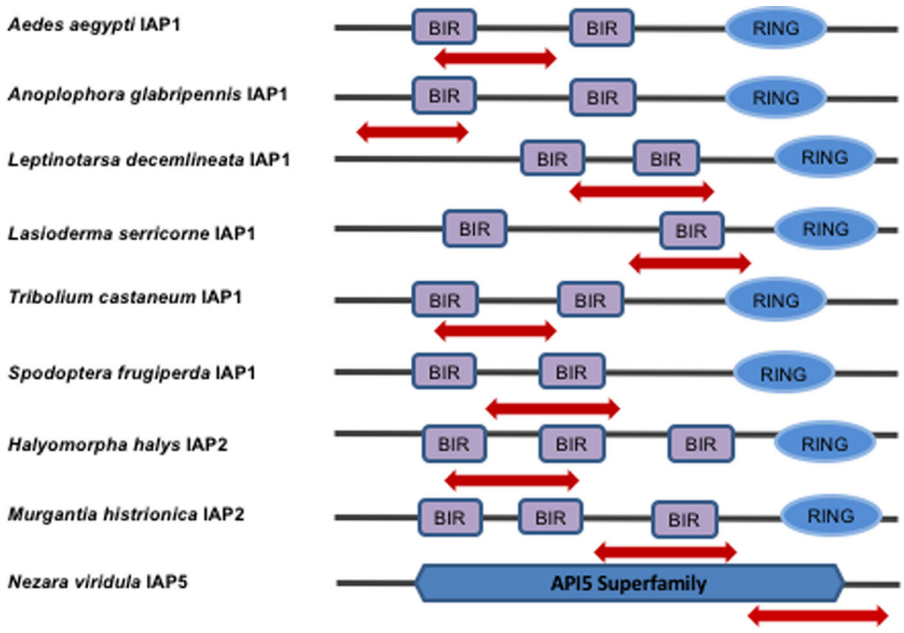

FIGURE 1 Analysis of domains present in the coding sequences of *iap* genes from nine insect species. The coding sequences of *iap* genes from *Spodoptera frugiperda* (*Sf*), *Leptinotarsa decemlineata* (*Ld*), *Tribolium castaneum* (*Tc*), *Anoplophora glabripennis* (*Ag*), *Lasioderma serricorne* (*Ls*), *Aedes aegypti* (*Aa*), *Halyomorpha halys* (*Hh*), *Murgantia histrionica* (*Mh*), and *Nezara viridula* (*Nv*) were selected and the presence of conserved domains were analyzed using the NCBI Conserved domain search tool. The boxes indicate the location of conserved domains and the red arrows indicate the position of the dsRNA target sequences. BIR, baculoviral IAP repeat

The CPB dsIAP knockeddown *iap* gene expression and induced apoptosis only in Lepd-SL1 cells developed from this insect (Figure 2a and Supporting Information Figure S1a). The RFB dsIAP induced knockdown of the *iap* gene and apoptosis only in TcA cells developed from this insect (Figure 2b and Supporting Information Figure S1b). The YFM dsIAP induced knockdown of *iap* gene and apoptosis only in Aag-2 cells developed from this insect (Figure 3a and Supporting Information Figure S2a). Also, the FAW dsIAP induced knockdown of the *iap* gene and apoptosis only in Sf9_LUC_CeSID-1 cells developed from this insect (Figure 3b and Supporting Information Figure S2b). Other than the target-specific dsIAPs, none of the other eight dsIAPs or control dsRNA induced significant knockdown of the *iap* gene or apoptosis in four cell lines tested.

3.3 | dsIAPs induce knockdown of *iap* genes and mortality only in target insects

The CPB larvae fed on nine dsIAPs and dsGFP showed knockdown of the *iap* gene and 100% mortality only in larvae fed on dsIAP targeting the CPB *iap* gene (Figure 4). Similarly, RFB larvae injected with nine dsIAPs and dsmalE showed knockdown of the *iap* gene and 100% mortality only in larvae injected with dsIAP targeting the RFB *iap* gene (Figure 5). Also, feeding nine dsIAPs and dsGFP to SGSB nymphs induced knockdown of the *iap* gene and 70% mortality only in nymphs fed on dsIAP targeting the SGSB *iap* gene (Figure 6). Except for the dsIAP produced from a fragment of the *iap* gene from the target species, none of the other eight dsIAPs or dsGFP induced significant knockdown of the *iap* gene or mortality.

TABLE 1 siRNA fragment analysis in transcriptomes of nine insect species

19-Mer IAP	Total siRNA fragments/complete siRNA match								
	Aa	*Ag*	*Ls*	*Ld*	*Sf*	*Tc*	*Hh*	*Mh*	*Nv*
Aedes aegypti IAP1	332/332	332/0	332/1	332/0	332/2	332/3	332/0	332/9	332/0
Anoplophora glabripennis IAP1	368/4	368/368	368/7	368/4	368/1	368/1	368/6	368/13	368/5
Lasioderma serricorne IAP1	323/3	323/3	323/323	323/5	323/0	323/0	323/4	323/5	323/3
Leptinotarsa decemlineata IAP1	385/4	385/5	385/11	385/385	385/1	385/4	385/0	385/7	385/8
Spodoptera frugiperda IAP1	317/1	317/0	317/0	317/1	317/317	317/2	317/0	317/0	317/0
Tribolium castaneum IAP1	415/5	415/2	415/2	415/1	415/4	415/415	415/0	415/1	415/1
Halyomorpha halys IAP2	325/1	325/1	325/3	325/1	325/3	325/3	325/325	325/43	325/95
Murgantia histrionica IAP2	363/3	363/3	363/3	363/13	363/8	363/9	363/118	363/363	363/79
Nezara viridula IAP5	392/3	392/9	392/5	392/3	392/8	392/7	392/51	392 /57	392/392

Abbreviations: IAP, inhibitor of apoptosis; siRNA, small interfering RNA.

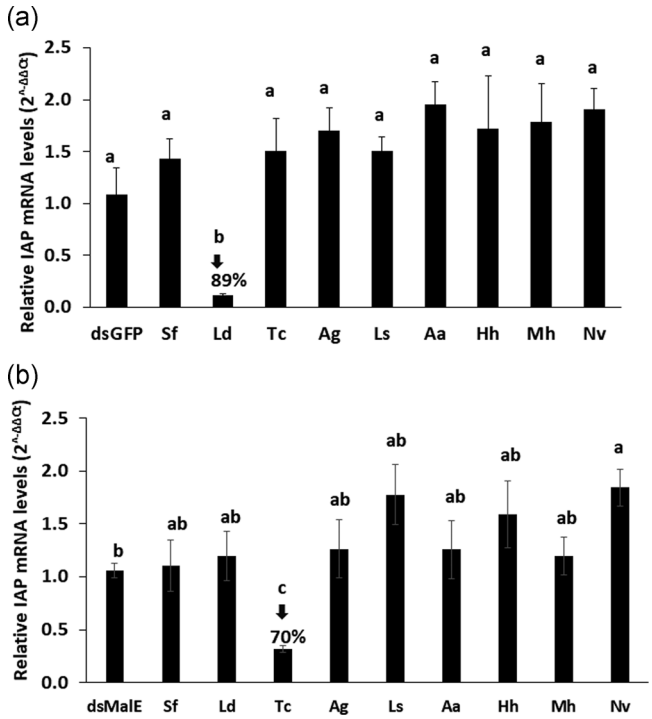

FIGURE 2 Target gene knockdown analysis in the Colorado potato beetle (*Leptinotarsa decemlineata*) Lepd-SL1 and red flour beetle (*Tribolium castaneum*) BCIRL-TcA-CLG1 (TcA) cell lines exposed to dsIAP. (a) 70,000 Lepd-SL1 cells per well seeded in 24-well plates in 250 µl EX-CELL® 420 containing 10% FBS were exposed to 100 ng of dsRNA targeting *iap* genes from nine insects or dsGFP as a control. At 48 hr after treatment, the total RNA was extracted and used to determine relative IAP mRNA levels gene by RT-qPCR normalized with RP4 as a reference gene. Mean + *SE* ($n = 4$) are shown. Data were analyzed by one-way ANOVA with Duncan multiple range test with the significance level set at $p < .05$ using IBM SPSS Statistics 20 software. The numbers and arrow toward down on the top of the bars show the percent decrease in the IAP mRNA levels in dsIAP-treated cells when compared to those in the control cells treated with dsGFP. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). (b) 70,000 TcA cells per well seeded in 24-well plates with 250 µl EX-CELL 420 containing 10% FBS and exposed to 10 µg of dsRNA targeting *iap* genes from nine insects or malE and the relative mRNA levels were determined by RT-qPCR normalized with RP49 as a reference gene as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). Aa, *Aedes aegypti*; *Ag*, *Anoplophora glabripennis*; ANOVA, analysis of variance; dsRNA, double-stranded RNA; FBS, fetal bovine serum; GFP, green fluorescence protein; *Hh*, *Halyomorpha halys*; IAP, inhibitor of apoptosis; *Ld*, *Leptinotarsa decemlineata*; *Ls*, *Lasioderma serricorne*; *Mh*, *Murgantia histrionica*; mRNA, messenger RNA; *Nv*, *Nezara viridula*; RT-qPCR, quantitative reverse transcription polymerase chain reaction; *Sf*, *Spodoptera frugiperda*; SE, standard error; *Tc*, *Tribolium castaneum*

4 | DISCUSSION

RNAi-based pest management methods are currently under development (Zhu & Palli, 2020). A product to control Western corn rootworm (WCRW) has recently been approved and several other products are under development. Potential nontarget effects of dsRNA used in pest control have been discussed since RNAi-based pest management was first proposed. Initial testing of dsRNA nontarget effects followed established protocols used for testing of nontarget pest control agents, especially biological insecticides such as *Bacillus thuringiensis* toxins (Bachman et al., 2013). The concept of designing dsRNAs that target a conserved or variable region of a target gene provides

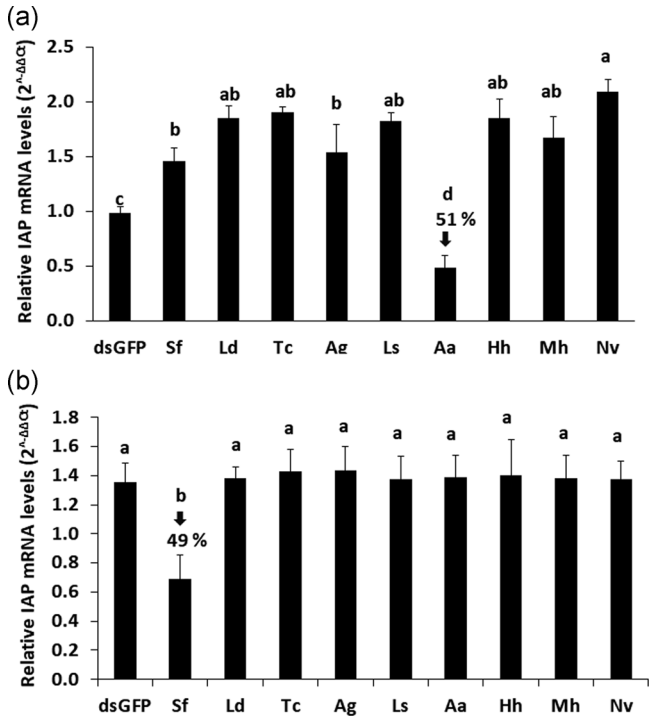

FIGURE 3 Target gene knockdown analysis in the yellow fever mosquito, *Aedes aegypti* Aag-2 cells and fall armyworm, *Spodoptera frugiperda* Sf9 stable cells expressing *Luciferase* and *CeSID-1* genes exposed to dsIAP. (a) 100,000 Aag-2 cells per well seeded in 24-well plates in Schneider's insect medium containing 10% FBS were exposed to 30 μg of dsRNAs (10 μg/day) targeting GFP or IAP genes from nine different insects. On the sixth day after treatment, total RNA was extracted and IAP mRNA levels were determined by RT-qPCR with *SRP7* as a reference gene as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). (b) 50,000 Sf9_LUC + CeSID-1 stable cells per well seeded in 24-well plates in 250 μl Sf-900™ II SFM and exposed to 2000 ng of dsRNAs targeting GFP or *iap* genes from nine insects. On the fifth day after treatment, total RNA was extracted and IAP mRNA levels were determined by RT-qPCR with *28S rRNA* as a reference gene as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). *Aa*, *Aedes aegypti*; *Ag*, *Anoplophora glabripennis*; dsRNA, double-stranded RNA; GFP, green fluorescence protein; *Hh*, *Halyomorpha halys*; IAP, inhibitor of apoptosis; *Ld*, *Leptinotarsa decemlineata*; *Ls*, *Lasioderma serricorne*; *Mh*, *Murgantia histrionica*; mRNA, messenger RNA; *Nv*, *Nezara viridula*; RT-qPCR, quantitative reverse transcription polymerase chain reaction; *Sf*, *Spodoptera frugiperda*; *Tc*, *Tribolium castaneum*

flexibility in targeting one or multiple related species of insect pests depending on the needs. However, the possibility of using a highly conserved region for designing the dsRNAs could lead to undesirable nontarget effects that can influence the populations of other beneficial insects (Lundgren & Duan, 2013; Roberts, Devos, Lemgo, & Zhou, 2015). To understand and evaluate the possibility of nontarget effects, there is a need to study the effects of multiple dsRNAs targeting genes in the conserved pathways across species. Multiple *iap* genes have been identified in several insects and the *iap* gene has been identified as an efficient target for RNAi in many insects (Cao et al., 2018; Dhandapani et al., 2019; Igaki et al., 2002; Mogilicherla et al., 2018; Powell et al., 2017; Rodrigues et al., 2017; Yoon et al., 2016, 2018). Here, we assayed nine dsRNAs targeting *iap* gene fragments spanning conserved domains in nine insects and found that the dsIAPs work well in target species, but did not show significant knockdown of *iap* gene expression and apoptosis in cells derived from nontarget insects or mortality in nontarget insects. This is, in contrast, to reports on the cross-species activity of dsRNA targeting the

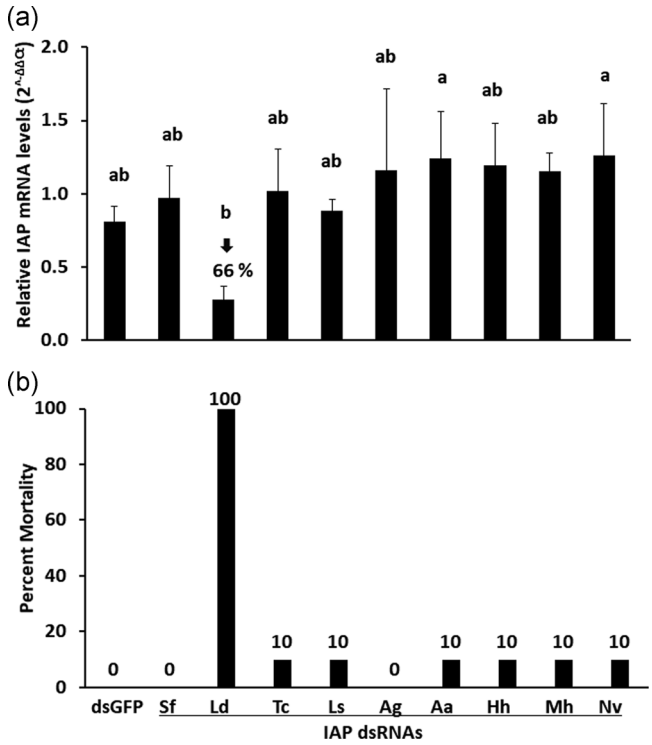

FIGURE 4 Knockdown of *iap* gene and mortality induced by dsIAP in the Colorado potato beetle, *Leptinotarsa decemlineata*, larvae that fed on dsRNA targeting the *iap* gene in nine insects or GFP. (a) Four micrograms of dsRNA targeting GFP or *iap* gene from nine insects was spread on washed and TritonX-100-treated eggplant leaf disc and fed to one each of overnight starved third instar larvae. On the third day after feeding dsRNA, total RNA was extracted and used to determined the IAP mRNA levels by RT-qPCR with *RP4* as a reference gene, as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). (b) Fresh leaf disc with dsRNA was replaced daily for 3 days and mortality recorded on the fifth day. Experiments were repeated twice with each experiment consisting of 10 larvae for each dsRNA tested. The numbers on the top of the bars show percent mortality induced by dsIAPs. *Aa*, *Aedes aegypti*; *Ag*, *Anoplophora glabripennis*; dsRNA, double-stranded RNA; GFP, green fluorescence protein; *Hh*, *Halyomorpha halys*; IAP, inhibitor of apoptosis; *Ld*, *Leptinotarsa decemlineata*; *Ls*, *Lasioderma serricorne*; *Mh*, *Murgantia histrionica*; mRNA, messenger RNA; *Nv*, *Nezara viridula*; RT-qPCR, quantitative reverse transcription polymerase chain reaction; *Sf*, *Spodoptera frugiperda*; *Tc*, *Tribolium castaneum*

D. melanogaster iap gene in two dipteran insects (Powell et al., 2017). Interestingly, among the three stink bug species, the targets of dsIAPs showed some sequence similarity but this did not result in a significant knockdown of the *iap* gene expression or mortality in nontarget stink bug species. Based on the number of siRNA matches with the *iap* gene fragments in nontarget stink bug species, we expected some knockdown of *iap* gene and mortality, but this was not the case. Further research is needed to understand the relationship between the sequence similarity of siRNA targets and knockdown efficiency and mortality. Most of the published studies on dsRNA in nontarget species, especially those that are phylogenetically distant from the target insect, reported no significant effects (Bachman et al., 2013; Pan et al., 2016, 2017). However, a few studies using highly conserved target genes such as the vATPaseA, ribosomal protein (rpl19) gene, and ecdysone receptor (EcR) reported some effect on target gene knockdown and mortality. For example, dsRNA targeting WCRW vATPaseA induced mortality in CPB at a concentration 10× higher than the LC50 in WCRW due to >80% sequence identity of vATPaseA gene between WCRW and CPB (Baum et al., 2007). Also, the same dsRNA adversely affected two species of ladybird beetles by inducing

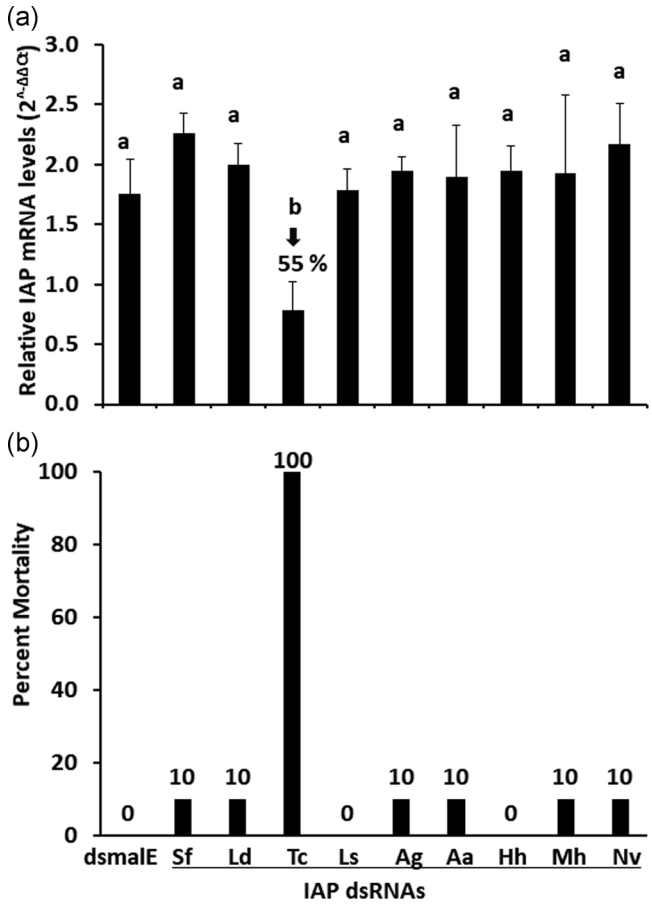

FIGURE 5 Knockdown of *iap* gene and mortality induced by dsIAP in the red flour beetle, *Tribolium castaneum* larvae injected with different insect IAP dsRNAs. (a) The dsRNA (1 μg/larva) targeting malE or *iap* gene from nine insects was injected into the last instar larvae. On the third day after treatment, the total RNA was extracted and IAP mRNA levels were determined by RT-qPCR with RP49 as a reference gene as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). (b) The dsRNA (1 μg/larva) targeting malE or *iap* gene from nine insects were injected into the last instar larvae and the rate of mortality recorded from the third to 10th day after treatment. Experiments were repeated twice and each experiment consisted of 12 larvae/each dsRNA used. The numbers on the top of the bars show percent mortality induced by dsIAPs. *Aa*, *Aedes aegypti*; *Ag*, *Anoplophora glabripennis*; dsRNA, double-stranded RNA; *Hh*, *Halyomorpha halys*; IAP, inhibitor of apoptosis; *Ld*, *Leptinotarsa decemlineata*; *Ls*, *Lasioderma serricorne*; *Mh*, *Murgantia histrionica*; mRNA, messenger RNA; *Nv*, *Nezara viridula*; RT-qPCR, quantitative reverse transcription polymerase chain reaction; *Sf*, *Spodoptera frugiperda*; *Tc*, *Tribolium castaneum*

knockdown of orthologs and affecting survival at dsRNA concentrations at orders of magnitude higher than expected to occur in the field (Haller, Widmer, Siegfried, Zhuo, & Romeis, 2019). The WCR vATPaseA dsRNA showed 34 and 6 siRNA matches, respectively, with those in *Coccinella septempunctata* and *Coccinella bipunctata* (Haller et al., 2019). Similarly, dsRNA targeting *Spodoptera exigua* EcR gene induced mortality in *Helicoverpa armigera* due to 89% sequence identify of EcR between these two insect species (Zhu et al., 2012). Also, dsRNA targeting the *Bactrocera minax rpl19* gene induced knockdown of this gene ortholog in a closely related species, *Bactrocera dorsalis* due to >90% sequence similarity between these genes in the two insect species tested (Chen, Zheng, Zheng, & Zhang, 2015). The dsIAP tested did not show high sequence similarity among the orthologs of

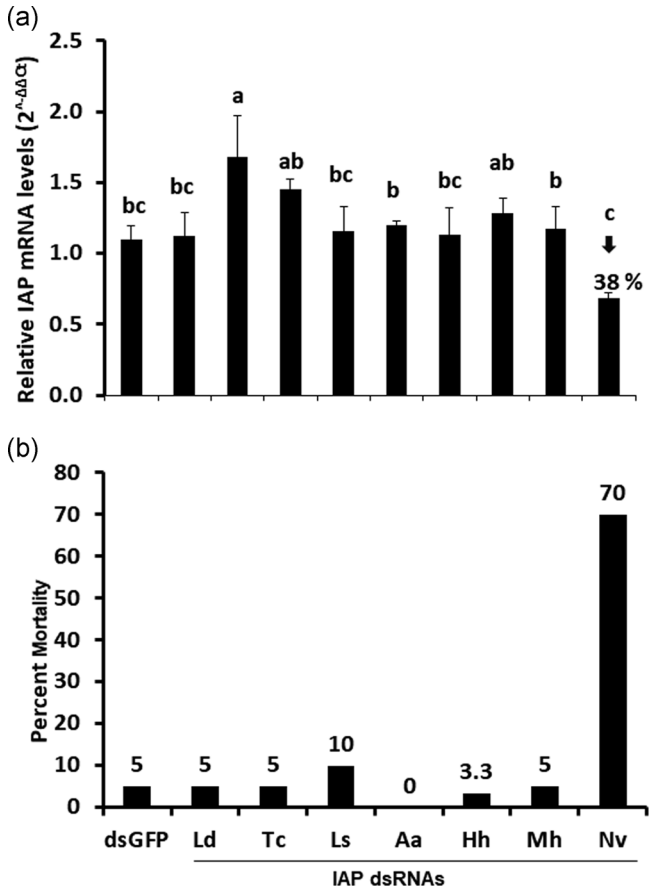

FIGURE 6 Knockdown of *iap* gene and mortality induced by dsIAP in the Southern Green Stink Bug, *Nezara viridula*, SGSB. (a) Certified organic beans were inserted in 2 ml tubes containing 300 µl of nuclease-free water and 20 µg of dsRNA targeting GFP or *iap* gene from nine insects were added to water and one 10 SGSB nymph were fed on each bean for 3 days. The dsRNA solution was replaced daily. Total RNA was isolated on the fifth day after initiation of feeding dsRNA and used to determine IAP mRNA levels by RT-qPCR using 60S RP L12 as a reference gene as described in Figure 2a legend. Means followed by the same letter are not significantly different (Duncan multiple range test ($p < .05$). (b) Certified organic beans were inserted in 2 ml tubes containing 300 µl of nuclease-free water and 20 µg of dsRNA targeting *iap* gene in nine insects (20 µg of dsRNA in water was added to the tube on the first, second, and third day). Ten second/third instar nymphs were fed on each bean and mortality was recorded on the 14th day after treatment. Mean + *SE* ($n = 20$) are shown. The numbers on the top of the bars show percent mortality induced by dsIAP. *Aa*, *Aedes aegypti*; *Ag*, *Anoplophora glabripennis*; dsRNA, double-stranded RNA; GFP, green fluorescence protein; *Hh*, *Halyomorpha halys*; IAP, inhibitor of apoptosis; *Ld*, *Leptinotarsa decemlineata*; *Ls*, *Lasioderma serricorne*; *Mh*, *Murgantia histrionica*; mRNA, messenger RNA; *Nv*, *Nezara viridula*; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SE, standard error; *Sf*, *Spodoptera frugiperda*; *Tc*, *Tribolium castaneum*

insect species used in our studies and this may be one of the reasons for no major nontarget effects detected. It would be interesting to test the cross-species effects of dsIAPs targeting regions of *iap* genes that are highly conserved (e.g., BIR domain) among the species tested. Work is in progress to test this possibility. The results reported here lay a foundation toward designing dsRNAs for controlling only one target species or multiple related species, but further work is needed to establish guidelines for designing dsRNAs for targeting single or multiple species of insect pests.

ACKNOWLEDGMENTS

Supported by grants from the National Institutes of Health (GM070559-14 and 1R21AI131427-02), the National Science Foundation (Industry/University Cooperative Research Centers, the Center for Arthropod Management Technologies under Grant IIP-1821936), the National Institute of Food and Agriculture, USDA, HATCH under 2353057000 and the Agriculture and Food Research Initiative Competitive Grant no. 2019-67013-29351. We thank Mrs. Smitha George from Palli lab for help with the injection of dsRNAs into RFB larvae.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Shankar C. R. R. Chereddy: Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-original draft (supporting). **Dhandapani Gurusamy:** Formal analysis (lead); Investigation (lead); Methodology (lead); Writing-original draft (lead); Writing-review & editing (supporting). **Jeffrey L. Howell:** Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). **Subba R. Palli:** Formal analysis (lead); Project administration (lead); Supervision (lead); Writing-original draft (supporting); Writing-review (lead).

ORCID

Jeffrey L. Howell http://orcid.org/0000-0002-0939-8647
Subba R. Palli http://orcid.org/0000-0002-0873-3247

REFERENCES

Bachman, P. M., Bolognesi, R., Moar, W. J., Mueller, G. M., Paradise, M. S., Ramaseshadri, P., … Levine, S. L. (2013). Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera* virgifera LeConte). *Transgenic Research*, *22*(6), 1207–1222. https://doi.org/10.1007/s11248-013-9716-5

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., … Pleau, M. (2007). Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, *25*(11), 1322–1326.

Birnbaum, M. J., Clem, R. J., & Miller, L. K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *Journal of Virology*, *68*(4), 2521–2528.

Burand, J. P., & Hunter, W. B. (2013). RNAi: Future in insect management. *Journal of Invertebrate Pathology*, *112*, S68–S74.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*(1), 421.

Cao, M., Gatehouse, J. A., & Fitches, E. C. (2018). A systematic study of RNAi effects and dsRNA stability in *Tribolium castaneum* and *Acyrthosiphon pisum*, following injection and ingestion of analogous dsRNAs. *International Journal of Molecular Sciences*, *19*(4), 1079.

Chen, A., Zheng, W., Zheng, W., & Zhang, H. (2015). The effects of RNA interference targeting *Bactrocera dorsalis* ds-Bdrpl19 on the gene expression of rpl19 in non-target insects. *Ecotoxicology*, *24*(3), 595–603. https://doi.org/10.1007/s10646-014-1407-3

Crawford, E., Seaman, J., Barber, A., II, David, D., Babbitt, P., Burlingame, A., & Wells, J. (2012). Conservation of caspase substrates across metazoans suggests hierarchical importance of signaling pathways over specific targets and cleavage site motifs in apoptosis. *Cell Death and Differentiation*, *19*(12), 2040–2048.

Crook, N. E., Clem, R. J., & Miller, L. K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *Journal of Virology*, *67*(4), 2168–2174.

Cui, Y., Sui, Y., Xu, J., Zhu, F., & Palli, S. R. (2014). Juvenile hormone regulates *Aedes aegypti* Krüppel homolog 1 through a conserved E box motif. *Insect Biochemistry and Molecular Biology*, *52*, 23–32.

Deveraux, Q. L., & Reed, J. C. (1999). IAP family proteins—Suppressors of apoptosis. *Genes & Development*, *13*(3), 239–252.

Dhandapani, R. K., Gurusamy, D., Howell, J. L., & Palli, S. R. (2019). Development of CS-TPP-dsRNA nanoparticles to enhance RNAi efficiency in the yellow fever mosquito, *Aedes aegypti*. *Scentific Reports*, *9*(1), 8775. https://doi.org/10.1038/s41598-019-45019-z

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., … Carson, D. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLOS One*, *9*(3):e93155.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, *391*(6669), 806–811.

Fjose, A., Ellingsen, S., Wargelius, A., & Seo, H. -C. (2001). RNA interference: Mechanisms and applications. *Biotechnology Annual Review*, *7*, 31–57.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., & Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, *31*(13), 3784–3788.

George, S., Gaddelapati, S. C., & Palli, S. R. (2019). Histone deacetylase 1 suppresses Krüppel homolog 1 gene expression and influences juvenile hormone action in *Tribolium castaneum*. *Proceedings of the National Academy of Sciences*, *116*(36), 17759–17764.

Ghosh, S. K. B., Hunter, W. B., Park, A. L., & Gundersen-Rindal, D. E. (2017). Double strand RNA delivery system for plant-sap-feeding insects. *PLOS One*, *12*(2):e0171861.

Ghosh, S. K. B., Hunter, W. B., Park, A. L., & Gundersen-Rindal, D. E. (2018). Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *JoVE (Journal of Visualized Experiments)*, *135*, e57390.

Giraldo-Calderón, G. I., Emrich, S. J., MacCallum, R. M., Maslen, G., Dialynas, E., Topalis, P., … Madey, G. (2014). VectorBase: An updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. *Nucleic Acids Research*, *43*(D1), D707–D713.

Haller, S., Widmer, F., Siegfried, B. D., Zhuo, X., & Romeis, J. (2019). Responses of two ladybird beetle species (Coleoptera: Coccinellidae) to dietary RNAi. *Pest Management Science*, *75*(10), 2652–2662. https://doi.org/10.1002/ps.5370

Hay, B. A., Wassarman, D. A., & Rubin, G. M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell*, *83*(7), 1253–1262. https://doi.org/10.1016/0092-8674(95)90150-7

Huvenne, H., & Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *Journal of Insect Physiology*, *56*(3), 227–235.

Igaki, T., Yamamoto-Goto, Y., Tokushige, N., Kanda, H., & Miura, M. (2002). Down-regulation of DIAP1 triggers a novel Drosophila cell death pathway mediated by Dark and DRONC. *Journal of Biological Chemistry*, *277*(26), 23103–23106. https://doi.org/10.1074/jbc.C200222200

Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T., & Tokuhisa, T. (1999). Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proceedings of the National Academy of Sciences*, *96*(4), 1457–1462.

Lundgren, J. G., & Duan, J. J. (2013). RNAi-based insecticidal crops: Potential effects on non-target species. *BioScience*, *63*(8), 657–665.

Martinez, M. M., Reif, R. D., & Pappas, D. (2010). Detection of apoptosis: A review of conventional and novel techniques. *Analytical Methods*, *2*(8), 996–1004.

Mogilicherla, K., Howell, J. L., & Palli, S. R. (2018). Improving RNAi in the Brown Marmorated Stink Bug: Identification of target genes and reference genes for RT-qPCR. *Scientific Reports*, *8*(1), 3720. https://doi.org/10.1038/s41598-018-22035-z

Norbury, C. J., & Hickson, I. D. (2001). Cellular responses to DNA damage. *Annual Review of Pharmacology and Toxicology*, *41*(1), 367–401.

Pan, H., Xu, L., Noland, J. E., Li, H., Siegfried, B. D., & Zhou, X. (2016). Assessment of potential risks of dietary RNAi to a soil micro-arthropod, *Sinella curviseta* Brook (Collembola: Entomobryidae). *Frontiers in Plant Science*, *7*, 1028. https://doi.org/10.3389/fpls.2016.01028. -1028.

Pan, H., Yang, X., Bidne, K., Hellmich, R. L., Siegfried, B. D., & Zhou, X. (2017). Dietary risk assessment of v-ATPase A dsRNAs on Monarch butterfly larvae. *Frontiers in Plant Science*, *8*, 242. https://doi.org/10.3389/fpls.2017.00242. -242.

Powell, M. E., Bradish, H. M., Gatehouse, J. A., & Fitches, E. C. (2017). Systemic RNAi in the small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae), a serious pest of the European honey bee *Apis mellifera*. *Pest Management Science*, *73*(1), 53–63.

Roberts, A. F., Devos, Y., Lemgo, G. N., & Zhou, X. (2015). Biosafety research for non-target organism risk assessment of RNAi-based GE plants. *Frontiers in Plant Science*, *6*, 958.

Rodrigues, T. B., Dhandapani, R. K., Duan, J. J., & Palli, S. R. (2017). RNA interference in the Asian Longhorned Beetle: Identification of key RNAi genes and reference genes for RT-qPCR. *Scientific Reports*, *7*(1), 8913. https://doi.org/10.1038/s41598-017-08813-1

Roy, A., & Palli, S. R. (2018). Epigenetic modifications acetylation and deacetylation play important roles in juvenile hormone action. *BMC Genomics*, *19*(1), 934.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Molecular Cell*, *9*(3), 459–470. https://doi.org/10.1016/s1097-2765(02)00482-3

Shukla, J. N., Kalsi, M., Sethi, A., Narva, K. E., Fishilevich, E., Singh, S., … Palli, S. R. (2016). Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biology*, *13*(7), 656–669.

Srinivasula, S. M., & Ashwell, J. D. (2008). IAPs: What's in a name?*Molecular Cell*, *30*(2), 123–135. https://doi.org/10.1016/j.molcel.2008.03.008

Verhagen, A. M., Coulson, E. J., & Vaux, D. L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biology*, *2*(7), reviews3009. https://doi.org/10.1186/gb-2001-2-7-reviews3009

Xu, J., Nagata, Y., Mon, H., Li, Z., Zhu, L., Iiyama, K., … Lee, J. M. (2013). Soaking RNAi-mediated modification of Sf9 cells for baculovirus expression system by ectopic expression of *Caenorhabditis elegans* SID-1. *Applied Microbiology and Biotechnology*, *97*(13), 5921–5931.

Yoon, J.-S., Koo, J., George, S., & Palli, S. R. (2020). Evaluation of inhibitor of apoptosis genes as targets for RNAi-mediated control of insect pests. *Archives of Insect Biochemistry and Physiology*. Manuscript submitted for publication. https://doi.org/10.1002/arch.21685

Yoon, J.-S., Mogilicherla, K., Gurusamy, D., Chen, X., Chereddy, S. C., & Palli, S. R. (2018). Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects. *Proceedings of the National Academy of Sciences*, *115*(33), 8334–8339.

Yoon, J.-S., Shukla, J. N., Gong, Z. J., Mogilicherla, K., & Palli, S. R. (2016). RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors. *Insect Biochemistry and Molecular Biology*, *78*, 78–88.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., … Smagghe, G. (2013). Delivery of dsRNA for RNAi in insects: An overview and future directions. *Insect Science*, *20*(1), 4–14.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., & Horvitz, H. R. (1993). The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell*, *75*(4), 641–652.

Zhu, J. Q., Liu, S. M., Ma, Y., Zhang, J. Q., Qi, H. S., Wei, Z. J., … Li, S. (2012). Improvement of pest resistance in transgenic tobacco plants expressing dsrna of an insect-associated gene EcR. *PLOS One*, *7*(6):e38572. doi:ARTN e3857210.1371/journal.pone.0038572.

Zhu, K. Y., & Palli, S. R. (2020). Mechanisms, applications, and challenges of insect RNA interference. *Annual Review of Entomology*, *65*, 293–311. https://doi.org/10.1146/annurev-ento-011019-025224

SUPPORTING INFORMATION

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

How to cite this article: Chereddy SCRR, Gurusamy D, Howell JL, Palli SR. Double-stranded RNAs targeting inhibitor of apoptosis gene show no significant cross-species activity. *Arch Insect Biochem Physiol*. 2020;e21683. https://doi.org/10.1002/arch.21683