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Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects

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ABSTRACT

RNA interference (RNAi) has become a widely used reverse genetic tool to study gene function in eukaryotic organisms and is being developed as a technology for insect pest management. The efficiency of RNAi varies among organisms. Insects from different orders also display differential efficiency of RNAi, ranging from highly efficient (coleopterans) to very low efficient (lepidopterans). We investigated the reasons for varying RNAi efficiency between lepidopteran and coleopteran cell lines and also between the Colorado potato beetle, *Leptinotarsa decemlineata* and tobacco budworm, *Heliothis virescens*. The dsRNA either injected or fed was degraded faster in *H. virescens* than in *L. decemlineata*. Both lepidopteran and coleopteran cell lines and tissues efficiently took up the dsRNA. Interestingly, the dsRNA administered to coleopteran cell lines and tissues was taken up and processed to siRNA whereas the dsRNA was taken up by lepidopteran cell lines and tissues but no siRNA was detected in the total RNA isolated from these cell lines and tissues. The data included in this paper showed that the degradation and intracellular transport of dsRNA are the major factors responsible for reduced RNAi efficiency in lepidopteran insects.

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Introduction

RNA interference (RNAi) is a posttranscriptional gene-silencing mechanism where exogenous double-stranded RNAs (dsRNA) knockdown genes by triggering degradation of target mRNAs in a cell.^{1,2} It is a widely conserved cellular mechanism displayed in eukaryotic organisms.^{3–6} In the first step of RNAi pathway, long dsRNA is cleaved into small RNA molecules of 21–23 base pairs (bp) short interfering RNAs (siRNAs) by RNase III type of proteins called Dicers.^{7,8} The second step involves loading of one of two strands of siRNA onto a multi-protein complex called “RNA-induced silencing complex” (RISC).⁹ siRNA binds to the Argonaute protein (an RNase H class of enzyme which is the main component of RISC) and guides the RISC complex to the cognate mRNA resulting in its cleavage and degradation.^{5,10} RNAi has recently become a powerful reverse genetic tool for functional characterization of genes in different model and non-model organisms. Further, technologies are being developed to apply this tool in crop improvement, pest management and therapeutics.^{11–18} However, the potential use of RNAi in various applications is restricted due to variability in RNAi efficacy (the extent to which dsRNA silences a gene) in different organisms.¹⁹ For example, RNAi efficiency varies among insect species ranging from highly efficient (in coleopterans, *i.e.*, beetles) to relatively inefficient (in lepidopterans, *i.e.* moths and butterflies).^{15,20–22}

RNAi is said to be ‘systemic’ when the silencing effect is propagated in cells and tissues of the organism.^{4,15,23} Most of the coleopterans studied thus far display a systemic RNAi response, except for the differences observed due to the different dsRNA delivery methods used.^{12,24–26} In contrast, RNAi is non-systemic and less efficient in lepidopteran insects displaying varying effects in different species (reviewed in²⁷). Further, a relatively large amount of dsRNA is required to trigger RNAi response in lepidopteran insects compared to coleopteran insects.²⁷ Several mechanisms have been proposed for the RNAi insensitivity in insects^{16,28} and many have been experimentally demonstrated. dsRNA degradation in the hemolymph,^{29,30} reduced uptake of dsRNA by the cells,³¹ reduced induction of RNAi components upon exposure to dsRNA^{32,33} and/or missing domains in the RNAi components (<http://docs.lib.purdue.edu/dissertations/AAI1529807/>) have been studied.

Although the amount of the information about the insensitivity of lepidopterans to oral RNAi has increased during the recent years, the only limited effort has been made to understand why coleopteran and lepidopteran insects differ in terms of their RNAi response. Here, we compared the RNAi efficiency between cell lines of representative insects of Coleoptera [TcA (*Tribolium castaneum*) and Lepd-SL1 (*Leptinotarsa decemlineata*)] and Lepidoptera [Sf9 (*Spodoptera frugiperda*) and Hv-E6 (*Heliothis virescens*)]. A strong knockdown in the expression

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of three housekeeping genes (*Actin*, *Sec23-B* and *vATPase-B*) was observed in coleopteran cells (*TcA* and *Lepd-SL1*) whereas no reduction in the expression of these target genes was detected in lepidopteran cells (*Sf9* and *Hv-E6*) upon incubation of these cells with the respective dsRNAs. Interestingly, no differences in the uptake of dsRNA into coleopteran (*TcA* and *Lepd-SL1*) and lepidopteran cells (*Sf9* and *Hv-E6*) were observed. Further, we compared the fate of injected or fed labeled dsRNA between *L. decemlineata* and *H. virescens* larvae. Degraded dsRNA was recovered from *H. virescens* hemolymph whereas intact dsRNA was recovered from *L. decemlineata* hemolymph. Also, a siRNA band was detected in the total RNA isolated from *L. decemlineata* tissues whereas no such band was detected in the RNA isolated from *H. virescens* tissues. Our results suggest that multiple factors including the degradation of dsRNA and trapping of dsRNA in acidic bodies within the cell and hence the absence of processing of long dsRNA into siRNAs, are responsible for the poor RNAi response observed in lepidopteran insects.

Materials and methods

Knockdown experiments in cell lines

Knockdown efficiency of three housekeeping genes, *actin*, *Sec23* and *V-ATPase-B* was determined in coleopteran (*Tc* and *Lepd-SL1*) cells, from Dr. Goodman lab at USDA ARS, Columbia, MO) and lepidopteran cell lines (*Sf9* and *Hv-E6*, from Dr. Goodman lab at USDA ARS, Columbia, MO). One day prior to the treatment, 10^6 cells/well were seeded in a six-well plate. Cells were starved in the serum free medium (1 ml) for one hour (hr) prior to exposure to 40 μ g of *actin*, *Sec23* and *V-ATPase-B* or *GFP* (control) dsRNA. After 5 hr of incubation, 1 ml of medium containing 20% serum was added to each well. Cells were then cultured for 48hr, after which total RNA was isolated, followed by washing the cells with 1XPBS. DNase-treated total RNA was denatured at 75°C for 5 min and immediately chilled on ice. First strand cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, USA) and 17-mer polyT primer, according to the manufacturer's instructions. The cDNAs were used in qRT-PCR using the SYBR Green kit (Roche, USA) and primers (Table S1) to determine relative mRNA levels of *actin*, *Sec23*, and *V-ATPase-B*. Three independent biological replicates were included for each treatment. Ribosomal protein (RP) genes were used as an endogenous control to normalize the expression data and the gene expression levels were analyzed by $2^{-\Delta\Delta Ct}$ method.³⁴ Primers used in qRT-PCR were designed based on *actin*, *V-ATPase-B*, *Sec23* and *ribosomal protein* gene sequences from *L. decemlineata*, *T. castaneum*, *S. frugiperda* and *H. virescens* (Table S1).

³²P UTP and fluorescent labeling of dsRNA (dsGFP)

The MEGascript T7 kit (Ambion, USA) and α -³²P UTP were used to label dsRNA. 2.0 μ l each of 10X Buffer, ATP, GTP and CTP, 0.1 μ l of UTP, 8.4 μ l of ³²P UTP and 1.5 μ l template (248 bp GFP amplicon-250 ng/ μ l) and 2.0 μ l T7 enzyme were mixed and incubated at 37°C for 16 hrs. The input DNA was digested using Turbo DNase (Ambion, USA) and the dsRNA

was purified using PCR purification columns (Qiagen, USA). The dsRNA was eluted in RNase-free water and stored at -20°C. One microliter of the product was used to measure radioactivity using scintillation counter.

Fluorescent dsRNA was synthesized using Fluorescein RNA labeling Mix (Roche Diagnostics, USA) according to the manufacturer's protocol. DNase-treated dsRNA was purified using Qiagen PCR purification columns and dsRNA concentration was measured using Nanodrop 2000 (Thermo Scientific, USA).

Aminoallyl dsRNA was synthesized by *in vitro* transcription using MEGascript T7 kit (Ambion, USA) according to the manufacturer's instructions except that UTP was replaced with Amino-allyl UTP (Thermo Fisher Scientific, USA). dsRNA was purified using Qiagen PCR purification columns and the purified dsRNA was conjugated with a pH-sensitive cyanine dye, CypHer5E mono-NHS ester (GE Healthcare, UK).³⁵ One vial of CypHer-5E dye (1 mg) was dissolved in 40 μ l of DMSO and divided into aliquots of 2 μ l in Eppendorf tubes. The dye was dried using freeze dryer (Labconco Freezone 6) and stored at 4°C until use. For conjugation of dye to dsRNA, one vial of dye (~50 μ g) was re-suspended in a mixture containing 2 μ g of aminoallyl UTP-labeled dsRNA (in 3.33 μ l H₂O), 5 μ l of DMSO and 1.66 ml of 0.3 M sodium bicarbonate buffer, pH 9.0. The conjugation reaction was carried in the dark for one hour at room temperature. The conjugated dsRNA was re-purified using Qiagen PCR purification columns and eluted in DEPC-treated water.

dsRNA uptake/processing experiments in cell lines

We selected two coleopteran cell lines (*Lepd-SL1* and *TcA*), in which soaking RNAi is known to work well³⁶ and two lepidopteran cell lines (*Sf9* and *Hv-E6*), in which soaking RNAi does not work well³⁷ to perform dsRNA uptake and processing studies. One day prior to the experiment, 1×10^6 cells/well were seeded in a six-well plate for the processing experiment (incubation with radiolabeled dsRNA) and 1×10^5 cells/well were seeded in chamber slides (Nunc™ Lab-Tek™ II, Thermo Scientific) for uptake experiments (incubation with fluorescently labeled dsRNA). Cells were starved in serum-free medium for one hour prior to the exposure to ³²P (2×10^6 counts) or fluorescently labeled (10 ng/ml or 20 ng/ml or 40 ng/ml final concentration) GFP dsRNA in serum-free medium. Five hours after incubation, 1 ml medium containing 20% serum was added to each well. Total incubation time varied for different experiments. A small section of midgut tissue from *S. frugiperda* larvae (final instar) was dissected and washed with 1X PBS. Midgut tissue was incubated (at 26°C) with CypHer5E conjugated dsRNA in Sf900 medium for 1hr. For dye only experiment, one vial of dried dye (~50 μ g) was re-suspended in 1 ml medium and 5 μ l of diluted dyes was added to each well of chamber slide containing 100 μ l medium and 10,000 cells.

To standardize the minimum incubation time needed by cells to take up the dsRNA from the medium, cells were incubated (at 26°C) with Fluorescein-labeled dsRNA for different time periods (30 min, 1, 3, 5 and 7 hrs). To check the transport of dsRNA, midgut tissue and cells were washed (multiple times with 1x PBS), fixed with 4% paraformaldehyde solution, mounted in a medium

containing glycerol and PBS and finally covered with a coverslip. Cells were examined under fluorescence/confocal microscope (excitation filters, 488 nm for Fluorescein and 635 nm for CypHer5E). In a different experiment, Sf9 cells were incubated (at 26°C) with unlabeled or fluorescein-labeled dsRNA (2 µg in 100 µl medium) for 1 hr. After 1 hr, the medium from each well was collected in 1.5 ml tubes. Cells were supplied with fresh medium with or without 1 µg of RNase A (Qiagen, USA). An equal amount of RNase was added to the media collected from each well. Cells and media were incubated (at 26°C) for an additional hour. Cells were washed, fixed and visualized under a fluorescence microscope. Media (50 µl) samples were run on 1.2% agarose gel to check the dsRNA integrity.

To check the fate of dsRNA, total RNA was isolated from the cells that were exposed to ³²P labeled dsRNA. Total RNA samples were run on 20% polyacrylamide-8M urea gels using 1X TBE buffer.³⁸ Gels were washed, fixed (10% methanol and ethanol), and dried in a gel drier. Dried gels were exposed overnight on a phosphor-Imager screen, and the screen was scanned in a phosphor-Imager (Typhoon 9500, GE Healthcare Life Sciences, USA).

dsRNA processing experiments in coleopteran and lepidopteran larvae

Newly-molted final instar larvae were starved for 2 – 3 hr and chilled on ice for 5 min prior to dsRNA injection. dsRNA containing about 9×10^6 counts per minute (CPM) in 5 µl was injected into hemolymph of two larvae each of *L. decemlineata* and *H. virescens* using an insulin syringe. After injection, the larvae were reared on their respective diet in plastic cups. For feeding radiolabeled dsRNA, larvae were starved overnight and dsRNA (9×10^6 CPM) was applied onto excised potato leaf for *L. decemlineata* larvae or was mixed with diet for *H. virescens* larvae. Different tissues (gut, fat body and epidermis) and hemolymph were collected from each injected larva at different time points after feeding dsRNA. Total RNA was isolated from these tissues after extensive washing with 1X PBS. Then, hemolymph and RNA samples were run on 20% polyacrylamide-8M Urea gels and the gels were processed as described in the previous section.

dsRNA degradation studies in hemolymph and midgut lumen contents

L. decemlineata and *H. virescens* were reared on potato plants and artificial diet as described previously.^{39,21} Hemolymph (30 µl) from *L. decemlineata* and *H. virescens* larvae (four of each) was collected through an incision made on one of the legs, at room temperature and immediately chilled on ice. Phenylthiourea (6 mg/30 µl of hemolymph) was added to the collection tubes in order to avoid melanization. The hemolymph was centrifuged at 13,000 rpm for 7 min and diluted in PBS. Midguts from 5th instar larvae of *H. virescens* and *L. decemlineata* were dissected in 1XPBS solution. A single larva was used to dissect midgut, added into 100 µl PBS and slightly pressed using a pestle to release the midgut lumen contents. The contents were centrifuged at 8000 rpm for 7 min at 4°C. The supernatant was transferred to a new tube and was used immediately to perform the dsRNA digestion assay. Protein concentration was estimated using Bradford's assay.⁴⁰ For each

assay, serial dilutions of lumen contents were prepared to which 1 µg of dsRNA was added. The mixture was incubated for 1.5 hr at room temperature and then analyzed by gel electrophoresis (1% agarose gel) using 10 µl of the mixture.

Results

Differences in knockdown efficiency between coleopteran and lepidopteran cells

Coleopteran cell lines (TcA and Lepd-SL1) showed significant ($P < 0.05$) knockdown with a two to eight-fold reduction in mRNA levels of all the three genes *actin*, *Sec23*, or *V-ATPase* after incubation with respective dsRNAs (Fig. 1). In contrast, both lepidopteran cell lines (Sf9 and Hv-E6) showed no significant reduction in the mRNA levels of target genes (Fig. 1). These data showed that exposure of lepidopteran cells to gene-specific dsRNA does not cause efficient knockdown in the expression of target genes.

dsRNA uptake by cells

An incubation time as short as 30 min was enough for the cells to take up fluorescein-labeled 248 bp GFP dsRNA from the medium (Fig. S1). No difference in the fluorescence in the cells was observed upon incubation of cells with higher concentrations of dsRNA (Fig. S1). Fluorescence was detected in both lepidopteran and coleopteran cells upon incubation of cells with 20 ng/µl dsRNA for 2 hr (Fig. 2). These data suggest that dsRNAs are taken-up by both coleopteran and lepidopteran cells.

To determine whether or not the fluorescent dsRNA signal detected in lepidopteran cells is due to the labeled dsRNA adsorbed to the plasma membrane, pH sensitive CypHer5E-labeled dsRNA was used. The fluorescence emitted by CypHer5E is lower at neutral or alkaline pH conditions and the fluorescence signal increases significantly at acidic conditions. Increased acidification of materials internalized by cells takes place when these materials are taken up by endocytosis (resulting into formation of early endosomes, pH range –6.1–6.8) and the endosomes mature (late endosome, pH range –4.8–6) to fuse with lysosomes (pH ~4.5).⁴¹ Punctate pattern of fluorescence was observed when Sf9 cells were incubated for 30 min or 60 min with CypHer5E-labeled dsRNA (Fig. 3A & B). Similar results were obtained when freshly dissected *S. frugiperda* midgut tissue was incubated with CypHer5E-labeled dsRNA (Fig. 3C). No fluorescence was detected in Lepd-SL1 cells upon incubation of these cells with CypHer5E labeled dsRNA (Fig. 3D). Also, no punctate fluorescence signals were detected in Sf9 cells incubated with CypHer-5E dye alone (Fig. S2). These data suggest that lepidopteran cells take up dsRNA but the dsRNAs are accumulated within endocytic compartments in these cells.

To confirm results that fluorescent dsRNA signals detected in Sf9 cells is inside cells, we used RNase III to digest dsRNA present outside the cells perhaps adsorbed to the plasma membrane. dsRNA was completely degraded by addition of RNase III to the medium (Fig. S3). However, fluorescence was detected in Sf9 cells incubated with fluorescein-labeled dsRNA even after

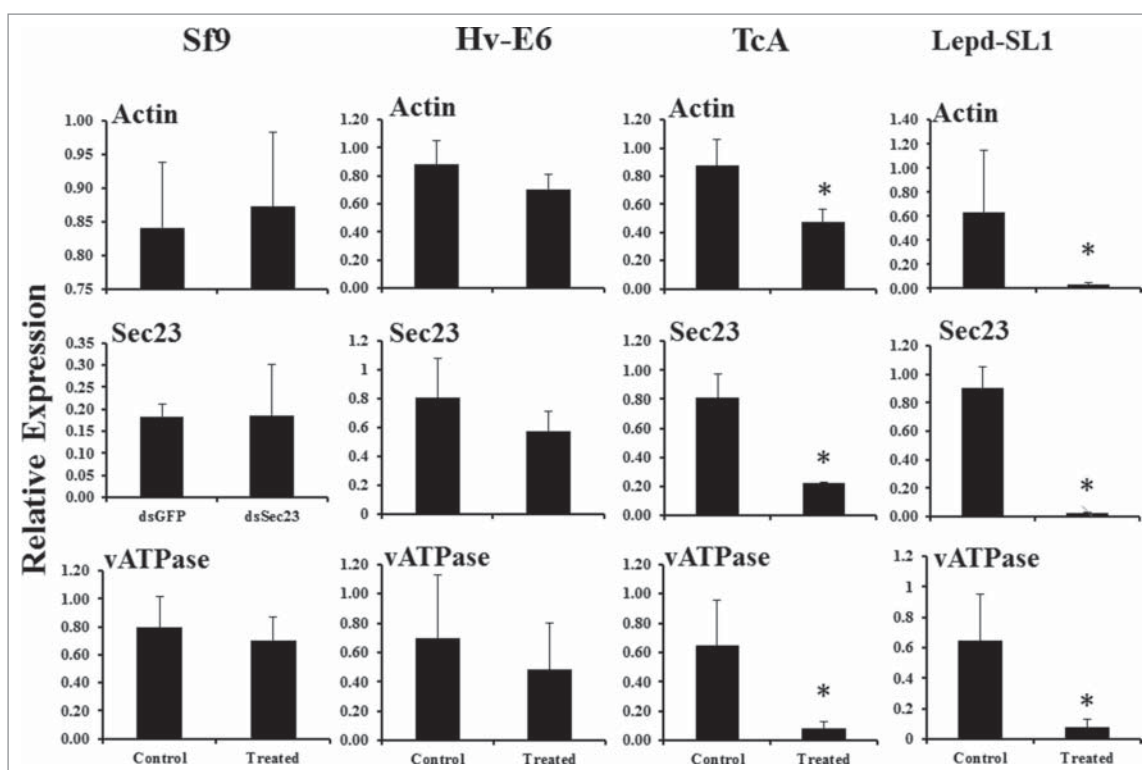


Figure 1. Knockdown efficiency of *actin* (top), *Sec23* (middle) and *V-ATPase* (bottom) genes in *Spodoptera frugiperda* (Sf9), *Heliothis virescens* (HvE6), *Tribolium castaneum* (TcA) and *Leptinotarsa decemlineata* (Lepd-SL1) cells. The mRNA levels of ribosomal protein genes were used for normalization. Mean + standard deviation ($n = 3$) are shown. * represents significant ($P < 0.05$) reduction in the target gene expression in TcA and Lepd-SL1 cells.

treatment with RNase III (Fig. 4). These data confirm that the fluorescence signal detected in Sf9 cells is indeed inside the cells.

dsRNA processing in cells

To test whether or not dsRNAs transported into lepidopteran and coleopteran cells are efficiently processed to small interfering RNA (siRNA), we incubated these cells with ^{32}P labeled dsRNA and the total RNA from these cells was

isolated and resolved on denaturing gels and the radioactivity in the gels was detected. A band of ~ 23 nt in size was detected in RNA isolated from coleopteran cells (Lepd-SL1 and TcA) at 72 hr after the exposure of these cells to ^{32}P labeled dsRNA (Fig. 5A). In contrast, RNA isolated from labeled dsRNA exposed lepidopteran cells (Sf9 and Hv-E6) did not show any band in the size range of 23 nt even after enhancing the contrast (Fig. 5B). To test whether or not Sf9 cells take up dsRNA, we exposed these cells to ^{32}P labeled

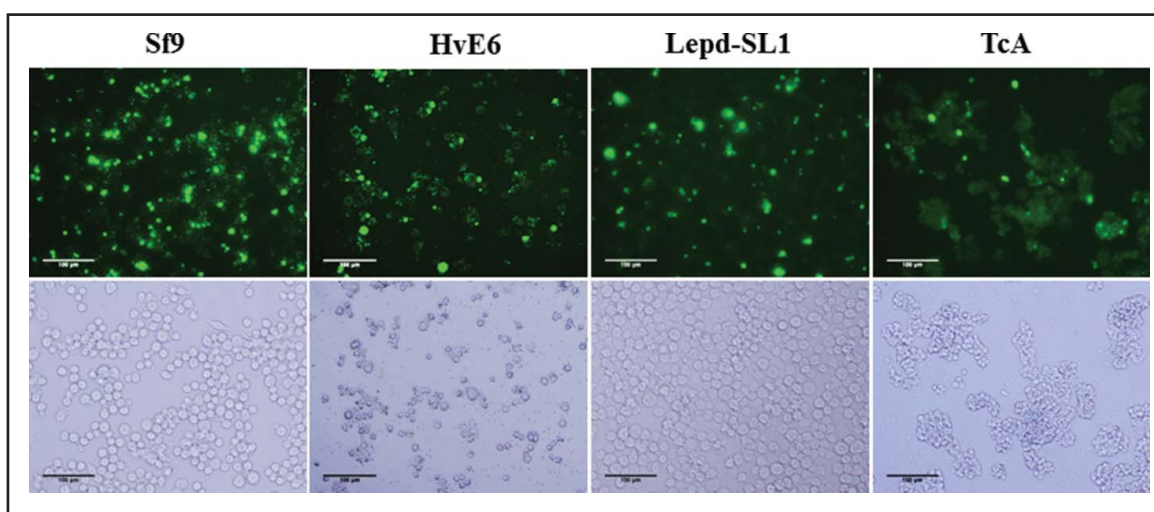


Figure 2. Internalization of fluorescein labeled dsRNA by lepidopteran and coleopteran cells. *Spodoptera frugiperda* (Sf9) *Heliothis virescens* (HvE6), *Tribolium castaneum* (TcA) and *Leptinotarsa decemlineata* (Lepd-SL1) were exposed to labeled dsRNA. The cells were then visualized under a fluorescence microscope. Photographs taken under fluorescent (top panel) and bright light settings (bottom panel) at 20X magnification are shown.

dsRNA for 30-90 min and the total RNAs isolated from these cells were resolved on denaturing gels and the radioactivity in the gels was detected. Intact dsRNA band was detected in cells exposed to 30-90 min and the intensity of this band increased with an increase in time of exposure to dsRNA suggesting that Sf9 cells take up dsRNA from the medium (Fig. 5C). These data suggest that coleopteran cells efficiently process dsRNA into siRNAs whereas lepidopteran cells are unable to process dsRNA into siRNA efficiently.

Therefore the siRNA band is not detected in total RNA isolated from these cells.

Processing of injected dsRNA

After 3 and 5 d of injection of labeled dsRNA, intact dsRNA bands were detected in *L. decemlineata* hemolymph (Fig. 6A) whereas degraded dsRNA bands were detected in *H. virescens* hemolymph (Fig. 6A). These data suggested that dsRNA was

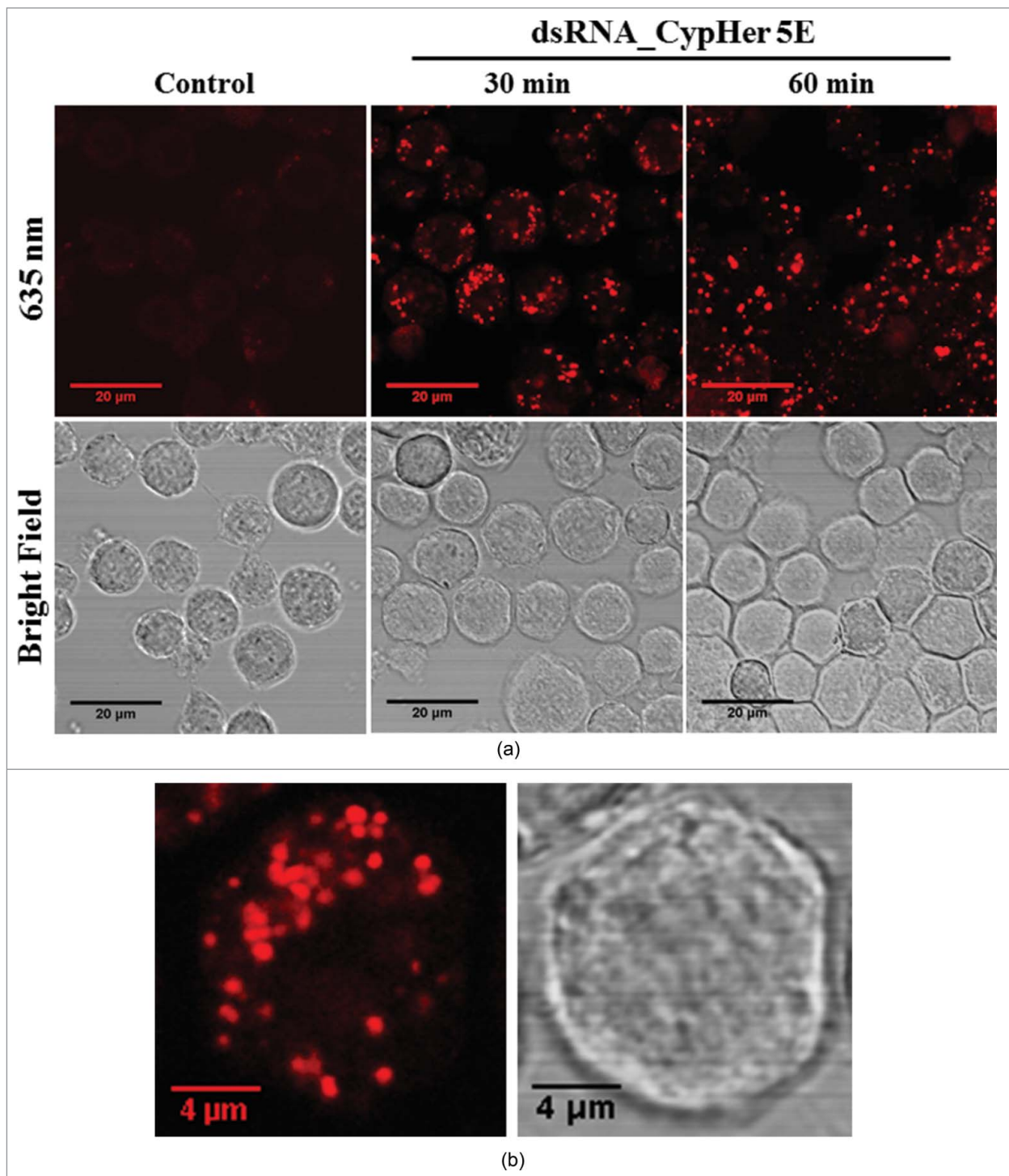


Figure 3. For figure legend see next page.

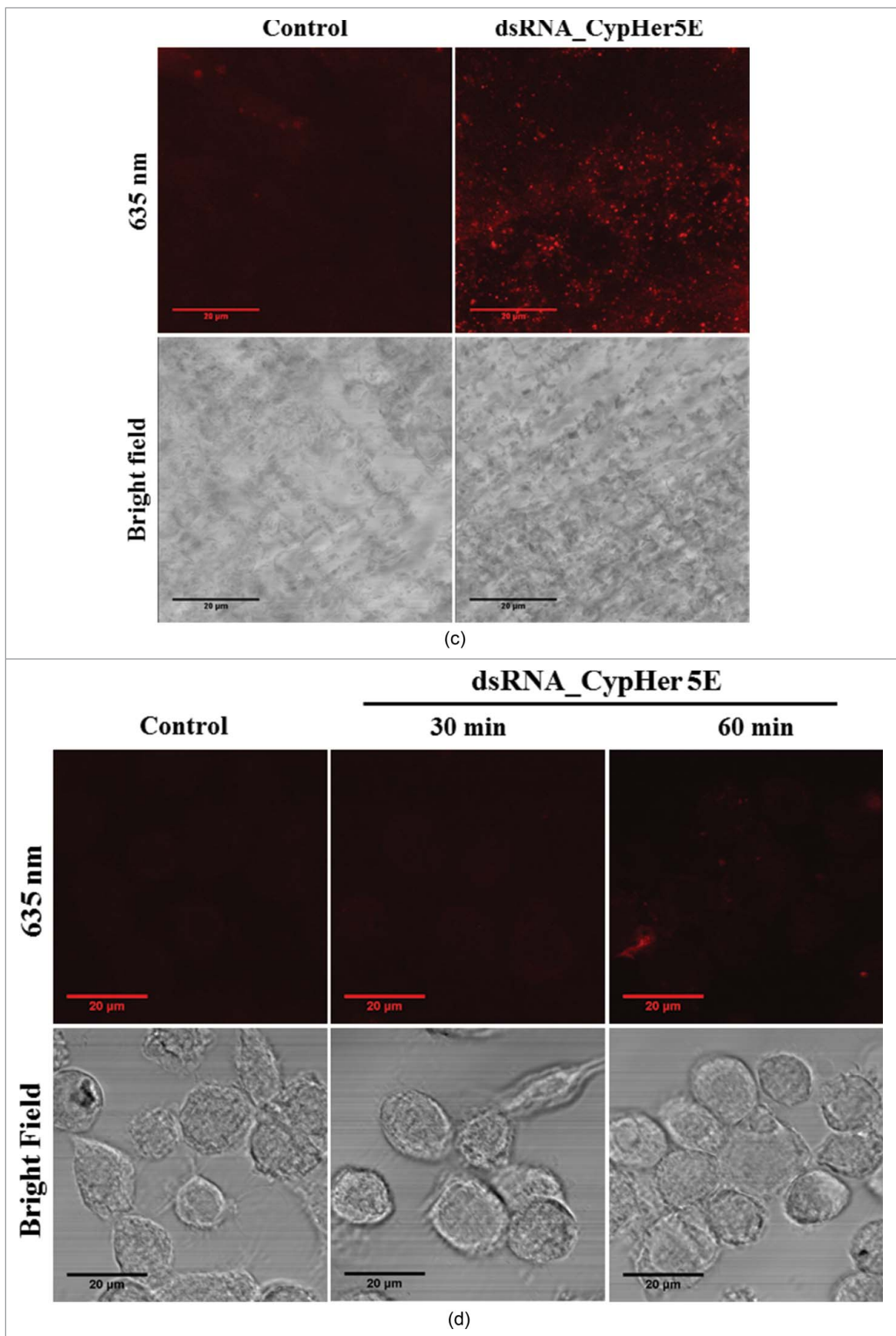


Figure 3. (See previous page) Internalization of CypHer-5E-labeled dsRNA into lepidopteran Sf9 cells and midgut tissue. (A) 100,000 cells/well (in chamber slide), were incubated with CypHer-5E-labeled dsRNA or unconjugated (control) dsRNA (10 ng/µl of media) in Sf-900 medium. Cells were washed and fixed after 30 min or 60 min of incubation time. Cells were then visualized under a confocal microscope. Photographs taken at 60X magnification under fluorescent (top panel) and bright light (bottom panel) are shown. (B) An enlarged view of a single cell under fluorescent (left panel) and bright light (right panel) are shown. (C) *S. frugiperda* midgut tissue incubated with CypHer5E-labeled dsRNA. (D) Detection of little or no fluorescence in Lepd-SL1 cells incubated with CypHer-5E-conjugated dsRNA (10 ng/µl of media).

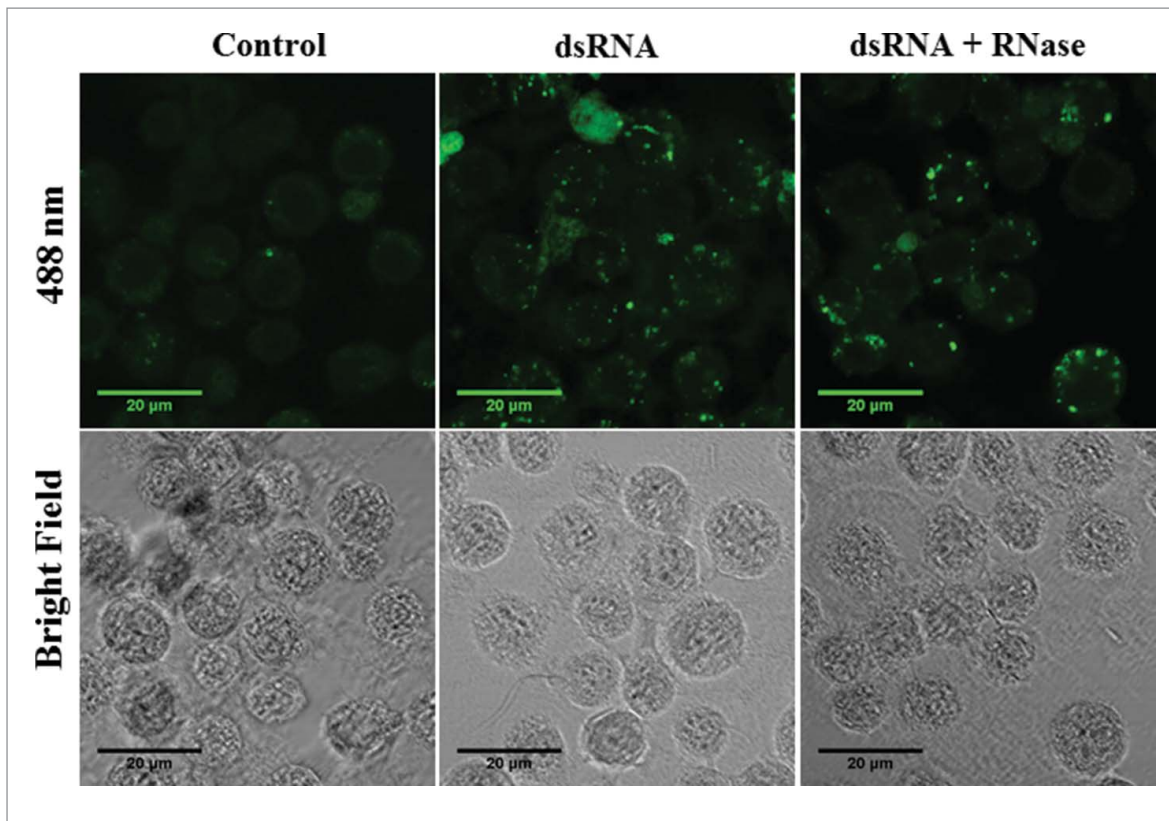


Figure 4. RNase III treatment does not eliminate dsRNA signal from Sf9 cells. 100,000 cells/well (in chamber slide), were incubated with fluorescein-labeled dsRNA (20 ng/ μ l of media) in SF-900 medium. After 1 hr of incubation, the medium was replaced with fresh media with or without RNase III and incubated for an additional hour. Cells were washed, fixed and visualized under a confocal microscope. Photographs were taken at 60x magnification under fluorescent (top panel) and bright light (bottom panel) are shown.

degraded faster in *H. virescens* than in *L. decemlineata*. To determine the stability of dsRNA in the hemocoel of *H. virescens*, labeled GFP dsRNA was injected and the hemolymph collected at 15, 40, 45 and 60 min after injection were analyzed on a gel. The results showed that the labeled dsRNA was completely degraded in the hemolymph by 60 min after injection (Fig. S4). Interestingly, the dsRNA taken up by the tissues was not degraded by 60 min after injection (Fig. S5).

An approximately 23 nt band likely comprising siRNAs was detected in the total RNA isolated from the gut and fat body tissues of *L. decemlineata* at 3 and 5 d after injection of labeled dsRNA (Fig. 6B). In contrast, siRNA band was not detected in the total RNA isolated (at different times after dsRNA injections) from *H. virescens* tissues (Fig. 6B and Fig. S5). These data indicated that gut and fat body tissues processed dsRNA into siRNA after taking up dsRNA from hemocoel in *L. decemlineata* larvae. However in *H. virescens* larvae, gut and fat body tissues did not process dsRNA to siRNA after its uptake from the hemocoel.

Fate of fed dsRNA

At 72 hr after feeding labeled dsRNA, most of the dsRNA in the hemolymph collected from *L. decemlineata* larvae was intact (Fig. 7A). In contrast, most of the dsRNA in the hemolymph of *H. virescens* was degraded (Fig. 7A). The total RNA isolated from the fat body, epidermis and gut dissected from labeled dsRNA fed larvae were resolved on urea-acrylamide

gels. Total RNA isolated from all the tissues dissected from *L. decemlineata* contained siRNA (Fig. 7B). In contrast, no siRNA was detected in RNA isolated from *H. virescens* tissues (Fig. 7B). These results suggested that dsRNA was processed to siRNA in *L. decemlineata* tissues, whereas dsRNA was not processed to siRNA in *H. virescens* tissues. To determine the contribution of food to the degradation of dsRNA, GFP dsRNA was incubated with *Heliothis* diet or potato leaf extract. The dsRNA incubated with *Heliothis* diet was not degraded at all concentrations tested. Whereas, the dsRNA incubated with the highest concentration of potato leaf extract caused degradation (Fig. S6). These data suggest that *Heliothis* diet does not contribute to dsRNA degradation.

dsRNA degradation in hemolymph and midgut lumen contents

dsRNA was degraded upon incubation in hemolymph samples collected from *H. virescens* and *L. decemlineata* (Fig. 8A). However, the dsRNA degradation efficiency of both samples varied drastically. The hemolymph of *H. virescens* serially diluted up to 12.5% caused complete digestion of dsRNA. In contrast, the hemolymph of *L. decemlineata* serially diluted up to 25% caused complete digestion of dsRNA (Fig. 8A). These data suggest that the hemolymph of *H. virescens* is more efficient at degrading the dsRNA as compared to that of *L. decemlineata*.

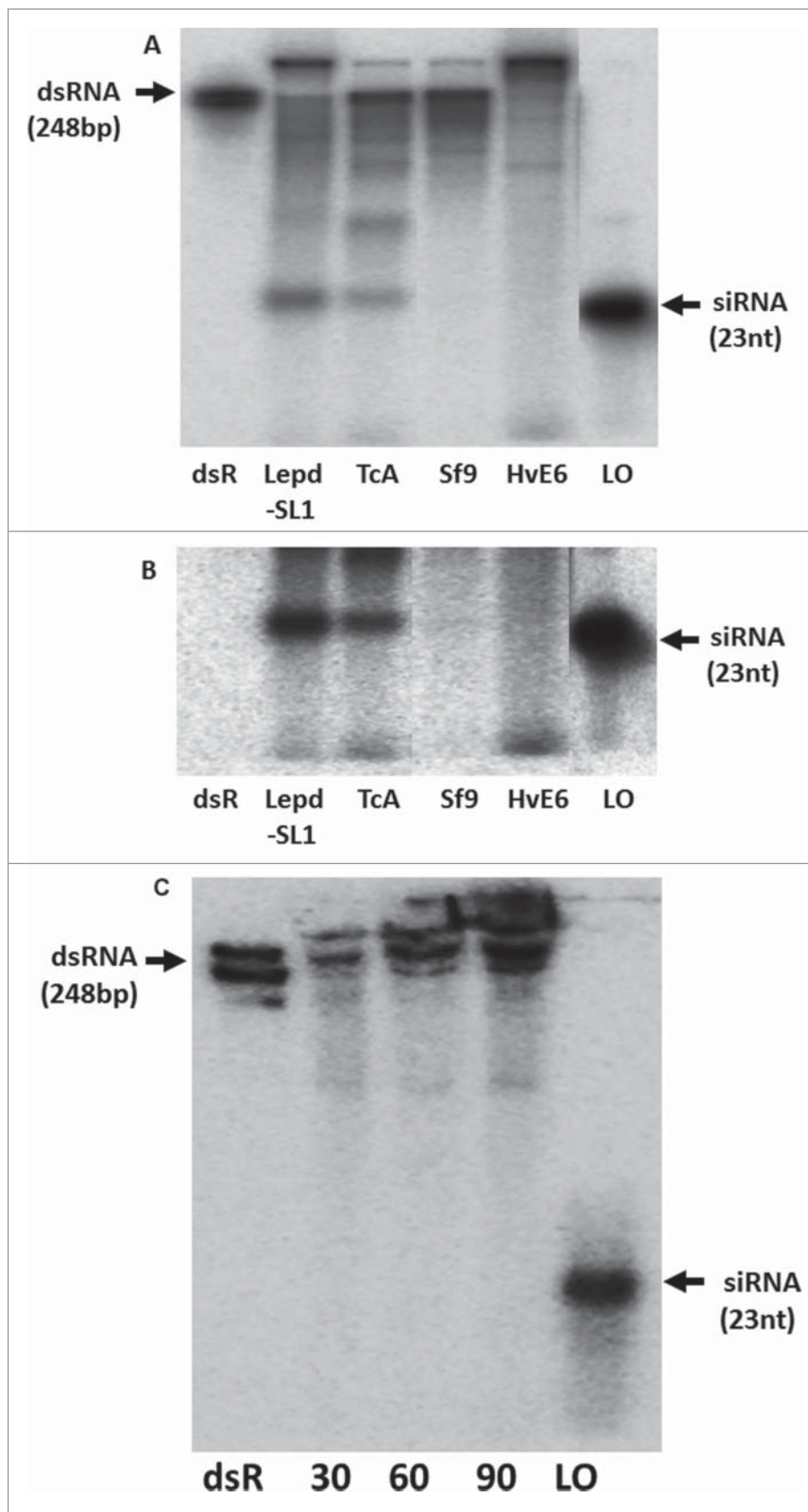


Figure 5. Transport and processing of dsRNA in coleopteran and lepidopteran cells. (A) Total RNA isolated from two coleopteran (TcA and Lepd-SL1) and two lepidopteran cell lines (Sf9 and Hve6) exposed to ^{32}P labeled dsRNA for 72 hr were resolved on 8M urea-20% polyacrylamide gel. The extreme left lane shows intact dsRNA and the extreme right lane shows $\gamma\text{-}^{32}\text{P}$ end labeled 23 nt oligo. (B) Enhanced contrast of the region of the gel presented in Fig. 5A where siRNA is detected. (C) dsRNA is detected in total RNA isolated from Sf9 cells after 30, 60 and 90 min exposure to dsRNA.

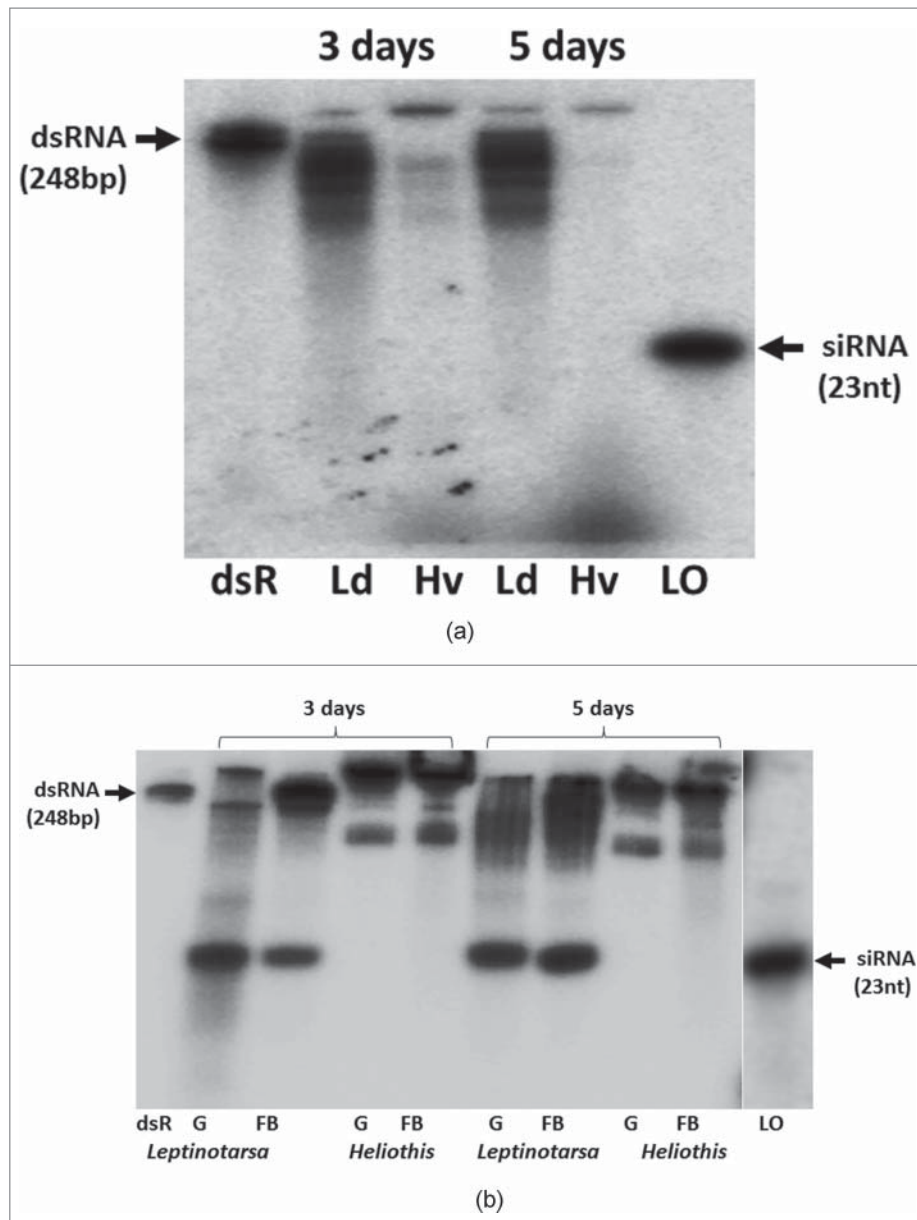


Figure 6. Transport of injected dsRNA into tissues in both *L. decemlineata* and *H. virescens*. dsRNA in the tissues was processed into siRNA in *L. decemlineata* but not in the tissues of *H. virescens*. (A) Hemolymph samples collected from *H. virescens* and *L. decemlineata* larvae at 3 or 5 d after injection of ^{32}P labeled dsRNA were resolved on 8M urea-20% polyacrylamide gel. The gel was dried and analyzed using a phosphorimager. The left lane shows intact-labeled dsRNA (dsR) and the right lane shows γ - ^{32}P end labeled 23 nt oligo (LO) run on the same gel. (B) Total RNA samples isolated from gut (G) or fat body (FB) dissected from *H. virescens* and *L. decemlineata* final instar larvae injected with ^{32}P labeled dsRNA at 3 d after injection were resolved on 8M urea-20% polyacrylamide gel. The gel was dried and analyzed using a phosphorimager. The left lane shows intact dsRNA (dsR) and the right lane shows ^{32}P labeled 23 nucleotide oligo (LO).

Serial dilutions of midgut lumen contents of *H. virescens* and *L. decemlineata* larva were also incubated with dsRNA for 90 min. Both the lumen contents digested dsRNA (Fig. 8B). The lumen contents of *H. virescens* diluted up to 25% caused complete digestion of dsRNA. However, the digestion was incomplete at 12.5% dilution followed by no digestion at 6.25% dilution. In contrast, the lumen contents of *L. decemlineata* caused a complete digestion of dsRNA only in the undiluted (100%) homogenate and even a dilution to 50% did not cause much degradation of dsRNA. These data suggest that the midgut lumen contents of the *H. virescens* are more efficient in degrading dsRNA as compared to those in *L. decemlineata*.

Discussion

RNAi is widely used as a powerful reverse genetic tool to study gene function.⁴²⁻⁴⁴ Development of RNAi-based technologies for crop improvement, pest control and therapeutic applications are also underway.^{16,17,45-48} One of the major challenges to the widespread use of RNAi in practical applications is the variable efficiency of RNAi among organisms. Insects are not an exception to this, as great variability in RNAi efficacy among insects has been reported.^{20,49,50} Robust and systemic RNAi response has been reported in coleopteran insects whereas RNAi response in lepidopteran insects has been reported to be poor.^{21,24,26,27,51} dsRNA degradation,^{29,30} reduced uptake of

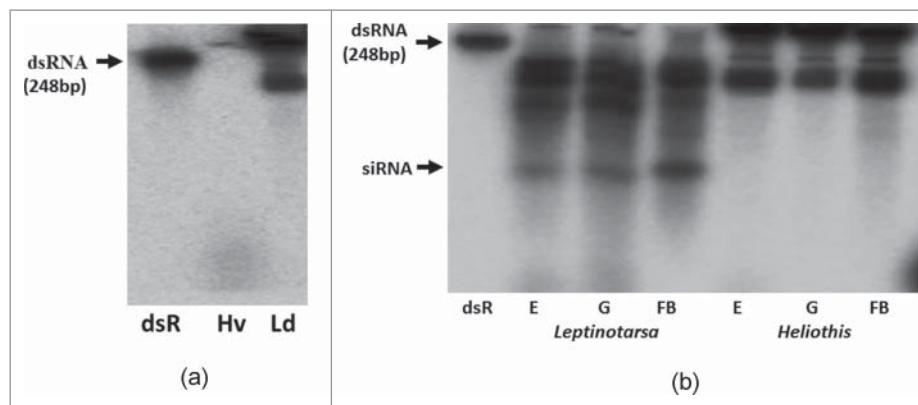


Figure 7. Transport of fed dsRNA into tissues by both *L. decemlineata* and *H. virescens*. dsRNA in *L. decemlineata* tissues but not in *H. virescens* tissues is processed into siRNA. A. Final instar *H. virescens* and *L. decemlineata* larvae were fed on diet containing nine million CPM ^{32}P labeled GFP dsRNA. At 72 hr after initiation of feeding on radioactive dsRNA, the hemolymph samples were collected and separated on 20% polyacrylamide-8M urea gel. The gel was exposed to PhosphorImager. 248 bp labeled GFP dsRNA (dsR) is also shown. B. Final instar *L. decemlineata* larvae were fed on diet containing nine million CPM ^{32}P -labeled GFP dsRNA. At 72 hr after initiation of feeding on radioactive dsRNA, epidermis (E), fat body (FB) and gut (G) were collected. Total RNA was isolated and the radioactivity was quantified. Equal CPM were separated on 20% acrylamide-8M urea gel. The gel was exposed to PhosphorImager. 248 bp labeled GFP dsRNA (dsR) is also shown.

dsRNA by the cells,³¹ reduced induction of genes coding for proteins involved in RNAi^{32,33} have been suggested as main reasons for differential efficacy of RNAi among insects studied.

Similar to the previous studies,^{29,30,52} the data presented here also showed the degradation of dsRNA in the lumen of the gut and in the hemocoel as among the factors responsible for the differential efficacy of RNAi between *L. decemlineata* and *H. virescens*. Differences in the stability of the injected or fed dsRNA between *L. decemlineata* and *H. virescens* were also observed. The injected labeled dsRNA was found to be

intact in the hemolymph of *L. decemlineata* larvae even at 5 d after injection, whereas it was completely degraded in the hemolymph of *H. virescens* at 3 d after injection (Fig. 6A). Similar results were obtained when hemolymph isolated from dsRNA fed larvae was analyzed (Fig. 7A). Further, *in vitro* incubation experiments showed that the hemolymph from both *H. virescens* and *L. decemlineata* were able to degrade dsRNA. However, the hemolymph from *H. virescens* was found to have more dsRNA degrading ability as compared to the hemolymph of *L. decemlineata* (Fig. 8A). We

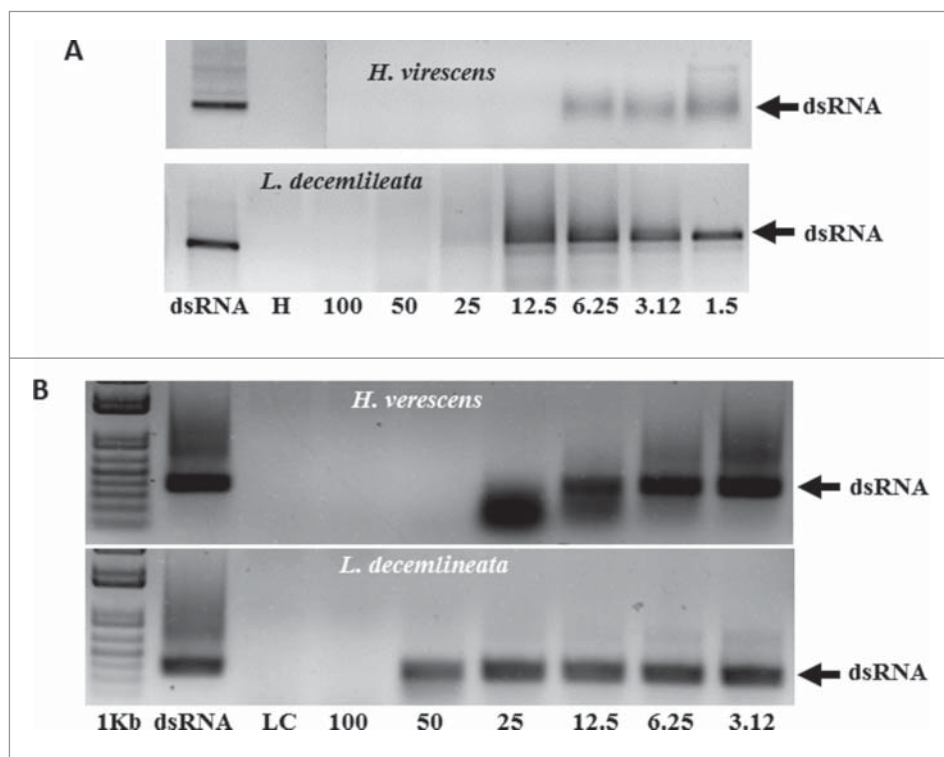


Figure 8. dsRNA degradation by hemolymph and midgut lumen contents. (A) dsRNA samples (1 μg) were incubated for 90 min at room temperature with various dilutions (100-1.5%) of hemolymph collected from *H. virescens* and *L. decemlineata* final instar larvae. The samples were separated on 1% agarose gel and visualized by staining with ethidium bromide. H, 100% hemolymph with no dsRNA added. (B) dsRNA samples (1 μg) were incubated for 90 min at room temperature with various dilutions (100-3.12%) of lumen contents from *H. virescens* and *L. decemlineata* larvae. The RNAs were separated on 1% agarose gels and visualized by staining with ethidium bromide. LC, undiluted lumen contents; dsRNA, the dsRNA that was not incubated with lumen contents.

also observed higher dsRNA degrading ability by the midgut lumen contents from *H. virescens* compared to *L. decemlineata* (Fig. 8B).

The most interesting finding we report here is that the transport of dsRNA within the cells seems to be the major factor responsible for the differential efficacy of RNAi between *L. decemlineata* and *H. virescens*. Several lines of evidence support this conclusion. 1. Labeled dsRNA was taken-up by both the lepidopteran and coleopteran cells. 2. dsRNA fed to *H. virescens* was detected in the peripheral tissues including epidermis and fat bodies. 3. A band of 23 nucleotides (nt) similar to the size of siRNA was detected in the coleopteran cells but not in the lepidopteran cells exposed to labeled dsRNA. 4. siRNA was detected in the gut, fat body and epidermis dissected from *L. decemlineata* injected or fed on labeled dsRNA but not in the tissues dissected from *H. virescens* injected or fed on labeled dsRNA. A recent study by Ivashuta et al.⁵³ showed the presence (in two coleopteran insects, *Diabrotica virgifera virgifera* and *L. decemlineata*) or absence (in lepidopteran insects, *Spodoptera frugiperda* and *Helicoverpa zea*) of siRNA (21 bp) derived from processing of plant originated dsRNA supports our result on dsRNA processing to siRNA in coleopteran but not in lepidopteran cell lines or tissues.

The absence of processed siRNA in the total RNA isolated from the lepidopteran cells and in the tissues of *H. virescens*

injected or fed with labeled dsRNA could be due to (1) reduced accessibility of dsRNA to RNAi machinery, (2) reduced activity of RNAi pathway component(s) required for dsRNA processing and (3) inability of the cells to take up and transport dsRNA within the cells. dsRNA mediated knockdown is effective in Sf9 and Sf21 cells by transfection and transduction; this argues against the lack of processing or other RNAi machinery components.^{54,55} Detection of radioactivity and detection of dsRNA bands in the total RNA isolated from Hv-E6 and Sf9 cells (Fig. 5A and 5C) as well as from the tissues dissected from dsRNA injected or fed *H. virescens* larvae (Fig. 6B, 7B and Fig. S5) suggest the uptake of dsRNA by Hv-E6 and Sf9 cells from the medium and by the gut, fat body and epidermis tissues from the hemolymph. It can also be argued that the detection of radioactivity in the RNA isolated from the tissues is due to the dsRNA adsorbing to the surface of the dissected tissues. However, detection of dsRNA bands in the total RNA isolated from the tissues washed multiple times (with 1X PBS) prior to RNA isolation (Fig. 6B and 7B) argue against such a possibility. Besides, potent dsRNA degradation ability of *H. virescens* hemolymph would have degraded any dsRNA sticking on the surface of tissues as dsRNA was found to be completely degraded in the *H. virescens* hemolymph within 60 min of injection (Fig. S4). Similar results were obtained when cells were incubated with fluorescently labeled dsRNA. Cells, from

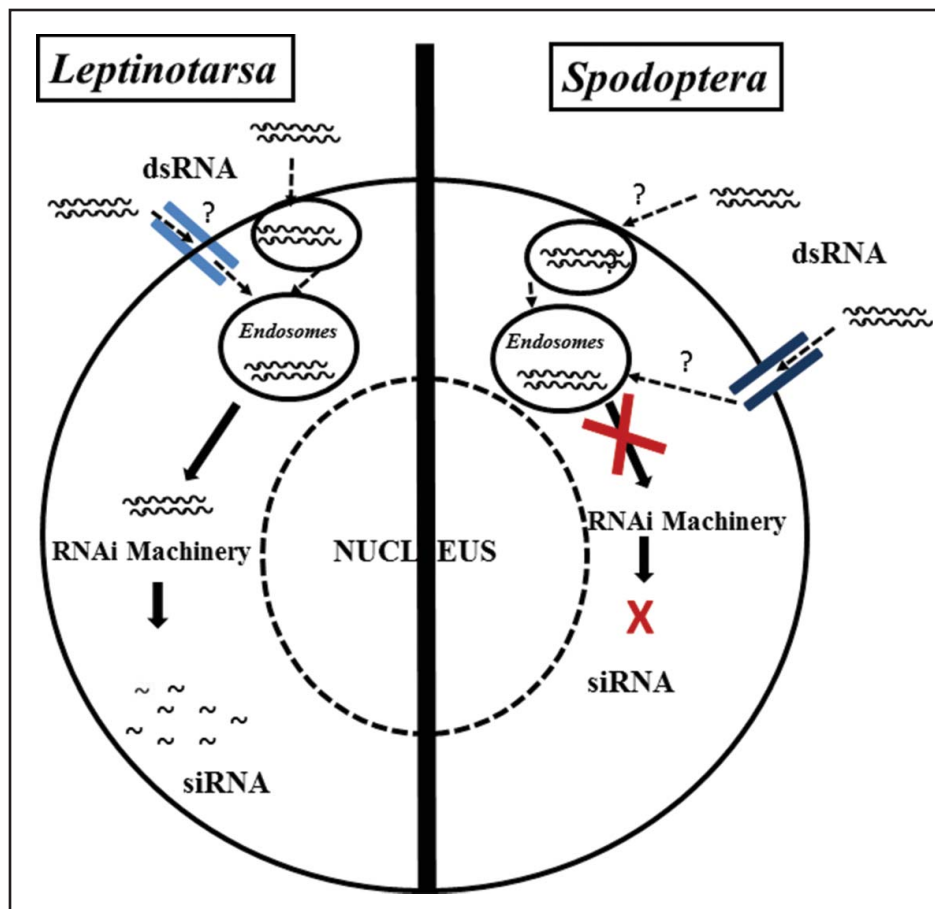


Figure 9. Model of the fate of dsRNA in *L. decemlineata* and *S. frugiperda* cells. In *L. decemlineata* cells, dsRNAs could enter through different pathways (e.g. through dsRNA transporters or endocytosis), escape from the endosomes and are processed by RNAi machinery, which leads to the production of siRNAs. In *S. frugiperda* cells, dsRNA is accumulated in acidic bodies after transport into the cells through endocytosis and/or transporters. Poor escape from the endosomes reduces their exposure to RNAi machinery hence no siRNA are produced.

both lepidopteran (Hv and Sf9) and coleopteran (TcA and Lepd-SL1) origin were able to take up the dsRNA from the media as evident from the fluorescence signal after incubation with labeled dsRNA (Fig. 2). Further, no difference was found in the fluorescence intensity between control and RNase III-treated Sf9 cells incubated with fluorescein-labeled dsRNA (Fig. 4). Treatment of Sf9 cells with RNase III followed by washing would have degraded and removed any dsRNA adhered to the cell surface (Fig. S3). Fluorescence emitted by cells incubated with CypHer5E-labeled dsRNA is an additional evidence suggesting the uptake of dsRNA by Sf9 cells (Fig. 3A and 3B) and *S. frugiperda* gut tissue (Fig. 3C). CypHer5E is a pH sensitive dye which excites only at acidic pH upon protonation. This feature of dye (combined with the extensive washing of cells and tissues after incubation with dsRNA) excludes any possibility of fluorescence emitted by dsRNA tethered to the plasma membrane. The detected fluorescence was also not due to free dye as the patterns of the fluorescence signal from Sf9 cells were different when cells were incubated with free CypHer-5E dye compared to the signals in cells that were incubated with dsRNA conjugated to CypHer-5E (Fig. 3A and Fig. S2). A possible explanation for the block in processing of dsRNA to siRNA in Hv-E6 and Sf9 cells and the tissues of *H. virescens* could be due to accumulation of dsRNA in the endosomal compartments due to less efficient endosomal escape (Fig. 9). Recent studies in insects showed that dsRNA enters cells predominantly through the endocytotic pathway.^{56,57} The absence of fluorescence in Lepd-SL1 cells incubated with CypHer5E conjugated dsRNA suggests that dsRNA may efficiently escape endocytotic compartments in these cells. Previous studies showed that overexpression of SID transporters improved RNAi efficiency in Sf9 cells³⁷ and *Bombyx mori* cells.³¹ It is possible that some of the dsRNA taken up through overexpressed transporters is able to escape endocytotic compartments and recruited to RISC complex resulting in an increase in RNAi efficiency. Further studies are required to test this hypothesis. Fig. 9 shows a model of the fate of dsRNA in *L. decemlineata* and *S. frugiperda* cells based on the present and the previous studies^{37,58} A detailed comparative analysis of dsRNA transport within the cells and its recruitment to the RISC complex in lepidopteran and coleopteran cells and tissues will help to understand the molecular mechanisms responsible for poor RNAi response in lepidopteran cells and tissues.

Disclosure of potential conflicts of interest

AS is current employee of, DuPont Pioneer; KN and EF are current employees of Dow AgroSciences.

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