

*Annual Review of Entomology*  
**Mechanisms, Applications,  
 and Challenges of Insect  
 RNA Interference**

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Annu. Rev. Entomol. 2020. 65:293–311

First published as a Review in Advance on  
 October 14, 2019

The *Annual Review of Entomology* is online at  
[ento.annualreviews.org](http://ento.annualreviews.org)

<https://doi.org/10.1146/annurev-ento-011019-025224>

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**Keywords**

dsRNA delivery, RNAi efficiency, RNAi pathway, systemic RNAi, pest management

**Abstract**

The RNA interference (RNAi) triggered by short/small interfering RNA (siRNA) was discovered in nematodes and found to function in most living organisms. RNAi has been widely used as a research tool to study gene functions and has shown great potential for the development of novel pest management strategies. RNAi is highly efficient and systemic in coleopterans but highly variable or inefficient in many other insects. Differences in double-stranded RNA (dsRNA) degradation, cellular uptake, inter- and intracellular transports, processing of dsRNA to siRNA, and RNA-induced silencing complex formation influence RNAi efficiency. The basic dsRNA delivery methods include microinjection, feeding, and soaking. To improve dsRNA delivery, various new technologies, including cationic liposome-assisted, nanoparticle-enabled, symbiont-mediated, and plant-mediated deliveries, have been developed. Major challenges to widespread use of RNAi in insect pest management include variable RNAi efficiency among insects, lack of reliable dsRNA delivery methods, off-target and nontarget effects, and potential development of resistance in insect populations.

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**RNA interference (RNAi):**

small RNA-mediated downregulation of gene expression

**Argonaute (Ago):** the main siRNA-induced silencing complex component aiding in target recognition and cleavage during RNAi

**Short/small interfering RNA (siRNA):**

double-stranded, approximately 19–21-base-pair RNA processed from long dsRNA by Dicer

**MicroRNA (miRNA):**

single-stranded, approximately 21–24-nucleotide-long RNA processed from hairpin RNA precursors by Dicer

**P-element induced wimpy testis (Piwi):** highly conserved RNA-binding proteins

**Piwi-interacting RNA (piRNA):**

single-stranded, approximately 23–36-nucleotide-long RNA processed mainly from transposons by Dicer-independent processes

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## INTRODUCTION

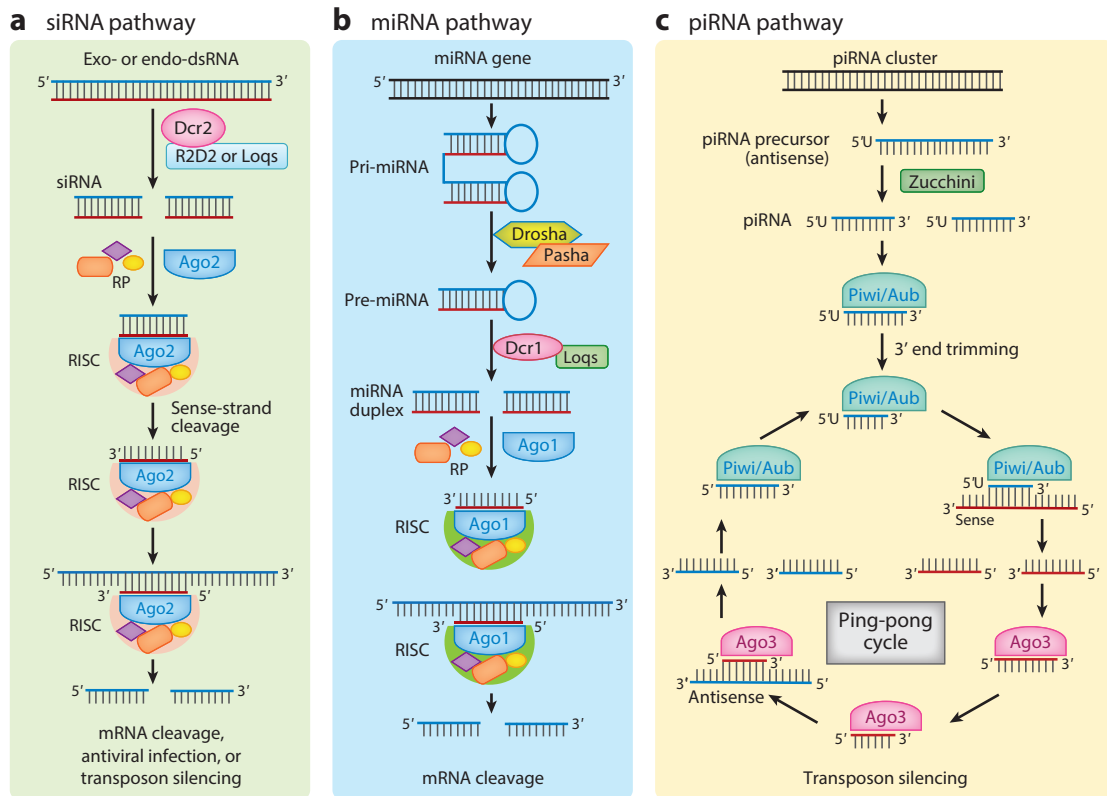
RNA interference (RNAi) refers to highly conserved cellular mechanisms in which Argonaute (Ago) family proteins bind small RNAs to trigger the degradation of longer RNAs (i.e., targets) through the sequence complementarity between the small and target RNAs (71). RNAi mechanisms were first described in the nematode (*Caenorhabditis elegans*) by Andrew Z. Fire, Craig C. Mello, and their colleagues (24), who received the 2006 Nobel Prize in Physiology or Medicine. RNAi has been found in all eukaryotic organisms, including protozoans, invertebrates, vertebrates, fungi, algae, and plants (1), and is believed to have initially evolved as an antiviral innate immune response (19). There is compelling evidence to support a key role for RNAi in antiviral immunity in insects (51, 112). It has been hypothesized that RNAi efficiency among insects is correlated with the prevalence of viral infections in them (112).

Since its discovery, RNAi has rapidly emerged as a powerful reverse genetics tool for studying gene function, regulation, and interaction at the cellular and organismal levels (8, 78, 110). RNAi has also shown great potential in pest management (6, 91, 98). One breakthrough was the development of transgenic maize engineered to express double-stranded RNA (dsRNA) of an essential insect gene to protect maize roots from damage caused by feeding of the western corn rootworm (*Diabrotica virgifera virgifera*) larvae (6). The current rapid pace of target gene identification and the development of new dsRNA delivery systems will soon lead to an increase in the use of RNAi-based technologies for the management of insect pests (44, 154).

## DIFFERENT RNA INTERFERENCE PATHWAYS IN INSECTS

### RNA Interference Pathways and Mechanisms

In insects and other animals, three different classes of small RNAs have been known to trigger three corresponding RNAi pathways (150). These classes of the small RNAs include short/small interfering RNAs (siRNAs) that mediate the siRNA pathway, microRNAs (miRNAs) that mediate the miRNA pathway, and P-element induced wimpy testis (Piwi)-interacting RNAs (piRNAs) that mediate the piRNA pathway (29, 78) (**Figure 1**). The siRNAs are dsRNAs typically 19–21 bp in length, which are processed from long dsRNA molecules that originate either exogenously or endogenously (**Figure 1a**). Exogenous dsRNAs include viral RNAs and experimentally introduced dsRNAs, whereas the vast majority of endogenous dsRNAs are derived from repetitive or transposable elements (e.g., transposon RNAs), structured loci, and overlapping transcripts (9, 18, 20, 25). Accordingly, siRNAs can be divided into exogenous siRNAs (exo-siRNAs) and endogenous siRNAs (endo-siRNAs) (14). In contrast, miRNAs are single-stranded RNAs 21–24 nucleotides (nt) in length (14). They are derived from endogenous primary miRNAs (pri-miRNAs) that are transcribed by RNA polymerase II from miRNA genes and constitute a large family of noncoding RNAs (49) (**Figure 1b**). Both siRNAs and miRNAs are generated by the type III ribonuclease Dicer (Dcr). However, piRNAs are single-stranded RNAs that are generally approximately 23–36 nt in length (124). At least two mechanisms have been identified for biogenesis of piRNAs in the fruit fly (*Drosophila melanogaster*) germline nurse cells, which include the de novo pathway and the ping-pong cycle (**Figure 1c**). In insects and other arthropods, different RNAi pathways have been found to play crucial roles, including (a) defenses against viral infections and transposable elements (transposons) via the siRNA-mediated pathway (9, 75, 113), (b) regulations of gene expression via the miRNA-mediated pathway (59, 107, 136, 137), and (c) suppression of germline transposon expression via the piRNA-mediated pathway (29, 75, 78, 81, 106, 121).



**Figure 1**

Mechanisms of different RNA interference (RNAi) pathways identified in insects. (a) In the short/small interfering RNA (siRNA)-mediated pathway, long double-stranded RNA (dsRNA) is cleaved by Dcr2 in association with R2D2 in the exo-siRNA pathway or Loqs in the endo-siRNA pathway (67). After a siRNA duplex is loaded into Ago2, an RNA-induced silencing complex (RISC) is assembled from Ago2 and other RISC-associated proteins (RP). The degradation of the sense strand of the siRNA in the RISC allows the antisense strand to guide the cleavage of complementary target RNA (e.g., mRNA) by Ago2 of the RISC (69). (b) In the microRNA (miRNA) pathway, the dsRNA-specific endoribonuclease (Drosha) and its partner (Pasha) trim the primary miRNA (pri-miRNA) into hairpins to form precursor miRNA (pre-miRNA). The pre-miRNA is then sliced by Dcr1 to yield a miRNA duplex. The duplex is unwound by RNA helicase to yield mature single-stranded miRNA, which is subsequently loaded into Ago1. After the RISC components are assembled, the active Ago1 enzyme within the RISC cleaves complementary target mRNA. (c) In the piwi-interacting RNA (piRNA)-mediated pathway, de novo piRNA production begins with the transcription of long single-stranded antisense RNA called the piRNA precursor by RNA polymerase II from particular genomic sites (piRNA clusters) that contain transposable elements (106). The piRNA precursor is then cut into primary piRNA by the endonuclease Zucchini (Zuc) (65). The resulting antisense piRNA is then loaded into the Piwi family proteins, Piwi and Aub (82). The Piwi/Aub complex cleaves a sense transposon transcript, which not only degrades the target, but also creates new sense piRNA. A different Piwi family protein, Ago3, then uses the new sense piRNA to guide the cleavage of the complementary antisense transposon transcript to generate more antisense piRNA, which can be loaded into the Piwi/Aub complex to cleave additional complementary sense transposon transcripts (82).

## Core Machinery Genes and Their Variations in Insect RNA Interference Pathways

The core machinery genes of the three RNAi pathways were found to undergo various duplications and deletions in insects (23, 83). In the siRNA pathway, gene duplications have been reported for *Ago2*, *Dcr2*, and/or *R2D2*, which encodes the dsRNA-binding protein (dsRBP) R2D2 for at least some insect species (27, 52, 76, 120). The only exception is Blattodea; however, only the

**Dicer 2 (Dcr2):**  
RNase III nuclease family enzyme that cuts long dsRNA into siRNA

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**R2D2:**

a dsRBP that forms the Dicer 2/R2D2 complex

**dsRNA-binding protein (dsRBP):**

a class of proteins responsible for recognizing dsRNA

**Dicer 1 (Dcr1):**

RNase III nuclease family enzyme that cuts precursor microRNA (pre-miRNA) to yield the miRNA duplex

**Aubergine (Aub):**

a Piwi family protein that binds RNA

**Systemic RNA interference defective 2 (Sid2):**

transmembrane protein involved in cellular uptake or cell-to-cell transport of dsRNA in *C. elegans*

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German cockroach (*Blattella germanica*) of this order has been examined (57). Specifically, both the common house mosquito (*Culex pipiens*) and the house fly (*Musca domestica*) were reported to have two *Ago2* genes (12, 52), whereas the Tsetse fly (*Glossina morsitans*) has three *Ago2* genes (76). The red flour beetle (*Tribolium castaneum*) has two *Ago2* and two *R2D2* or *R2D2*-like genes (120).

In the miRNA pathway, two *Ago1* genes in the yellow fever mosquito (*Aedes aegypti*) and the Russian wheat aphid (*Diuraphis noxia*) and two *Dcr1* genes in *M. domestica* have been reported (52, 76). However, the pea aphid (*Acyrthosiphon pisum*) appears to have two genes for each of *Ago1*, *Loqs*, which encodes the dsRBP Loquacious (Loqs), and *Dcr1*, and four genes of *Pasha*, which encode Pasha, a partner of the dsRNA-specific endoribonuclease Drosha (15, 23, 37, 76).

In the piRNA pathway, two or three *Ago3* genes have been reported in *A. pisum* and *G. morsitans* (15, 23, 76). Two *Aub/Piwi* genes, which encode the Piwi family proteins, Aubergine (Aub) and Piwi, in the African malaria mosquito (*Anopheles gambiae*) (52, 76), and 2–3 *Aub* genes in the jewel wasp (*Nasonia vitripennis*), the Florida carpenter ant (*Camponotus floridanus*), and the Indian jumping ant (*Harpegnathos saltator*) have been reported (76). However, 5–8 *Piwi* genes have been reported in various insect species, including *A. pisum*, *A. aegypti*, *A. gambiae*, and *D. noxia* (23, 52, 76). Duplications of core RNAi-related genes may lead to subfunctionalization or neofunctionalization in RNAi pathways, which may increase RNAi efficiency in insects, whereas loss of these genes may decrease RNAi efficiency in certain insect lineages (18, 23).

## CELLULAR UPTAKE AND SYSTEMIC RNA INTERFERENCE

### Mechanisms of Double-Stranded RNA Cellular Uptake

The first identified cellular uptake mechanism represents a passive transport of dsRNA through an intestinal luminal transmembrane protein called systemic RNA interference defective 2 (Sid2), where it functions as an endocytic receptor for ingested dsRNA in *C. elegans* (70, 128). Although no *Sid2* homologs have been identified in insects (18, 38, 120), it is possible that the proteins that function in transport of macromolecules may help in cellular uptake of ingested dsRNAs. In the *D. melanogaster* S2 cell line and the desert locust (*Schistocerca gregaria*), the endocytosis-mediated pathway was identified as a mechanism for dsRNA uptake (100, 131). In *T. castaneum*, studies using selective endocytosis inhibitors and the RNAi of RNAi strategies showed that clathrin-dependent endocytosis is a major mechanism of dsRNA cellular uptake (134). Clathrin-dependent endocytosis has now been identified or implicated as a mechanism for cellular uptake of dsRNA in other insects or insect cells (13, 54, 86, 130, 140). Also, macropinocytosis has been implicated in cellular uptake of dsRNA in the boll weevil (*Anthonomus grandis*) (26). Thus, the cellular uptake of dsRNA seems to be highly complex in insects not only because different species may have different mechanisms, but also because the same species may rely on more than one mechanism (13).

### Intercellular Transport of Double-Stranded RNA and Systemic RNA Interference

The highly conserved transmembrane protein Sid1 has been known to function as a dsRNA-selective channel that enables passive bidirectional transport of dsRNA between cells in *C. elegans* (103, 127). However, most insect species do not possess *Sid1* orthologous genes, but rather only *Sid1-like* genes (3, 13, 45, 62, 73, 135). Most studies on insect *Sid1-like* genes have mainly focused on cellular uptake of dsRNA, whereas the uptake of dsRNA into alimentary canal cells is mediated by the *Sid2* gene in *C. elegans* (128). In *T. castaneum*, silencing three *Sid1-like* genes, which share high sequence identities with another *C. elegans* gene (*Tag130*), did not affect RNAi efficiency

against other genes, suggesting that these genes do not play any significant role in either cellular uptake or intercellular transport of dsRNA (120). Nevertheless, research has shown that *C. elegans* Sid1 expressed in *Drosophila* S2 cells can enable the S2 cells to gain passive uptake of dsRNA from the culture medium (103). However, the migratory locust (*Locusta migratoria*) Sid1-like protein expressed in *Drosophila* S2 cells did not enhance dsRNA uptake (62), suggesting a functional difference between the *C. elegans* Sid1 and *L. migratoria* Sid1-like proteins.

Systemic RNAi refers to the intercellular spreading of RNAi silencing throughout the body or even into its progeny after dsRNA is delivered into a specific tissue or region. In *C. elegans*, systemic RNAi requires Sid1-mediated transport of dsRNA between cells (103, 128). Although systemic RNAi has been widely documented in different insect species (11, 36, 62, 73, 133), the mechanism(s) that mediate systemic RNAi in insects are largely unknown (18). However, nanotube-like structures have been found to mediate systemic RNAi in *D. melanogaster* by transporting dsRNA and components of the RNAi machinery between adjacent cells (39). In addition, exosome-like vesicles, which originate in hemocytes and participate in immune priming, have also been found to transport siRNAs and thereby to mediate the systemic RNAi-based adaptive antiviral response (116). However, these mechanisms and their roles need to be explored in other insects.

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**Systemic RNAi:**

a process in which local administration of dsRNA leads to RNAi responses in the whole body or even its progeny

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## DELIVERY OF INTERFERING RNA TO INSECTS

Various methods have been tested or applied to deliver dsRNA in insect species (38, 78, 84, 141). Basic dsRNA delivery methods include microinjection, feeding, and soaking. In recent years, however, various technologies have been developed and incorporated into these basic methods, including cationic liposome-assisted, nanoparticle-enabled, symbiont-mediated, and plant-mediated deliveries.

### Basic Delivery Methods

The most commonly used method for delivery of dsRNAs is microinjection, in which a small amount of dsRNA in an appropriate solution is directly injected into an insect embryo or insect body (38, 84, 141). The first successful dsRNA microinjection experiment was reported for silencing the *frizzled* and *frizzled 2* genes in the wingless pathway by injecting their corresponding dsRNAs into *D. melanogaster* embryos in 1998 (40). Since then, microinjection has become a popular technique to deliver dsRNA into the embryos or bodies of various insect species, such as *T. castaneum* (58, 120), *L. migratoria* (53, 142, 145), and *B. germanica* (33, 68). The advantages of microinjection include the immediate and direct delivery of a known amount of dsRNA into the insect body at various developmental stages or even to specific body parts (102, 145). This method can also avoid the structural barriers, such as the integument, that prevent penetration of dsRNA and that are encountered by other delivery methods, such as topical applications and soaking. However, microinjection is more time-consuming and is sometimes challenging due to the small body size of the insects.

Another basic delivery method is to feed insects a diet containing synthetic or microorganism-expressed dsRNA (i.e., oral delivery) (141). The microorganisms that have been used to express dsRNAs for insect RNAi include *Escherichia coli* (119, 153) and *Saccharomyces cerevisiae* (77). Oral delivery of dsRNA is highly effective for RNAi in many coleopterans (6, 42, 84, 94). It is not laborious, is easy to perform, and is more applicable to insect pest management. However, oral delivery results in low RNAi efficiency in many other insect species. In *L. migratoria*, for example, RNAi efficiency is high if dsRNA is delivered by injection but extremely low if it is delivered

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**dsRNase:**

double-stranded  
ribonuclease capable  
of degrading dsRNA

**Nanocarrier:**

nanomaterial used as a  
transport module for  
other substances, such  
as RNA

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by feeding (107). Such differential RNAi efficiencies between the two dsRNA delivery methods appear to be mainly due to dsRNA degradation by double-stranded ribonucleases (dsRNases) in the insect gut. In addition, supplementing dsRNA into the cell culture medium is a method widely applied to silence gene expressions in insect cell cultures. Applications of this delivery method have been extensively reviewed (129).

### Cationic Liposome-Assisted Delivery

Cationic liposomes are lipid-based transfection reagents that can provide a simple and efficient means to transfer dsRNA into animal cells in a process known as lipofection (48). The interactions of positively charged cationic lipids and negatively charged dsRNA can form lipoplexes through electrostatic interactions (155). Cationic liposomes have been widely used to assist in the delivery of dsRNA in insect cell cultures (129). However, several studies have also reported an increase in RNAi efficiency after feeding or injecting the RNAi-recalcitrant arthropod species (e.g., *D. melanogaster*, *Drosophila suzukii*, *Rhipicephalus haemaphysaloides*) with the lipoplexes (114, 126, 148). However, different cationic liposomes have not been thoroughly evaluated in conjunction with basic delivery methods.

### Nanoparticle-Enabled Delivery

The first proof-of-concept study on nanoparticle-enabled delivery used chitosan/dsRNA nanoparticles to silence two chitin synthase genes in *A. gambiae* larvae, for which the naked dsRNAs were ineffective with feeding as the delivery method (147). Chitosan is made up of highly deacetylated  $\beta$ -1,4-linked *N*-acetylglucosamine polymers and can form nanoparticles with dsRNA based on the electrostatic interactions between positively charged amino groups of chitosan and negatively charged phosphate groups of dsRNA. The increase in RNAi efficiency when chitosan/dsRNA nanoparticles were used is likely due to an increase in dsRNA stability and cellular uptake in insects (147). Li-Byarlay et al. (56) also developed a unique method to treat a large number of honey bees (*Apis mellifera*) quickly and noninvasively by coupling siRNA to perfluorocarbon-nanoparticles (PFC-NPs) for aerosol application. To date, chitosan and various other nanocarrier-based dsRNA nanoparticles have been used to improve RNAi efficiency in other insect species (21, 31, 93, 146). More recently, guanidine-containing polymers, nanocarrier/dsRNA/detergent formulation, and branched amphiphilic peptide bilayer conjugated gold nanoparticles have been reported to protect dsRNA against nucleolytic degradation (115), facilitate dsRNA penetration through the insect body wall (149), and likely improve the cellular uptake and endosomal escape of dsRNA (79), respectively.

### Symbiont-Mediated Delivery

Many insect species frequently harbor culturable symbiotic bacteria in their guts. Whitten & Dyson (125) used an intriguing strategy to create RNase III-deficient, dsRNA-producing bacterial strains from the symbionts of the kissing bug (*Rhodnius prolixus*) and the western flower thrips (*Frankliniella occidentalis*). Once ingested by the host insect, the genetically manipulated symbiotic bacteria can colonize the insect, which allows constitutive synthesis of dsRNA to trigger the RNAi pathway. Furthermore, the dsRNA-producing symbionts can be horizontally transmitted to other individuals via ingestion of feces in *R. prolixus*. The conspecific spread of such dsRNA-producing symbiont bacteria through an insect colony represents sustained RNAi and can be used to study gene function and develop new RNAi-based pest management strategies.



## Plant-Mediated Delivery

The first proof-of-concept study on plant-mediated delivery of dsRNA for insect pest management was reported by Baum and colleagues in 2007 (6); they developed transgenic maize expressing an insecticidal V-ATPase A dsRNA to protect maize roots from feeding damage by *D. v. virgifera* larvae. Similar studies have also been carried out to generate transgenic plants expressing dsRNAs targeting insect detoxification genes (e.g., cytochrome P450 genes). The target insects feeding on these plants demonstrated an increase in susceptibility to phytotoxins (66) or to spider predation (46). In plants, however, most of the expressed long dsRNA can be processed into siRNAs. This process can reduce RNAi efficiency because insect cells take up siRNAs much less efficiently than do long dsRNAs (11). To overcome this problem, researchers generated transgenic potato plants expressing long dsRNA in the chloroplasts (plastids), where long dsRNA is accumulated but is not processed into siRNA, as chloroplasts are devoid of the RNAi machinery. When the Colorado potato beetle (*Leptinotarsa decemlineata*) ingest transgenic potato plants, the long dsRNA expressed in the chloroplasts can be taken up by the insect gut cells to initiate the RNAi process (144).

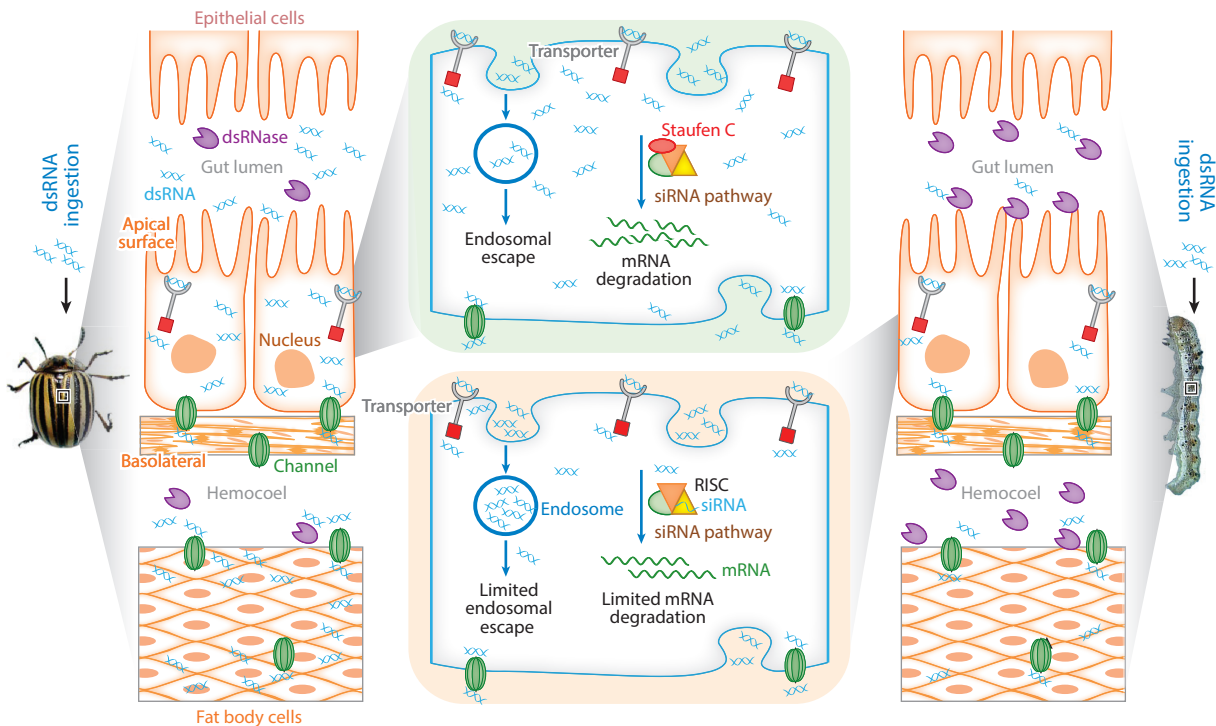
## MECHANISMS AFFECTING RNA INTERFERENCE EFFICIENCY

### Variations in RNA Interference Efficiency Among Insect Groups

RNAi efficiency varies considerably among insects. RNAi is highly efficient and systemic in coleopterans, especially those belonging to the families Tenebrionidae (e.g., *T. castaneum*) and Chrysomelidae (e.g., *L. decemlineata* and *D. v. virgifera*). In general, RNAi functions well in beetles; injection or oral delivery of dsRNA has been shown to induce systemic and robust RNAi response in many economically important coleopterans (10, 47, 63, 89, 90, 96, 97). RNAi has been shown to function efficiently for knockdown of target gene expression after injection of dsRNA into hemimetabolous insects such as the milkweed bug (*Oncopeltus fasciatus*) (34), the brown marmorated stink bug (*Halyomorpha halys*) (74), the bed bug (*Cimex lectularius*) (151), *B. germanica* (17), *S. gregaria*, and *L. migratoria* (61, 133). RNAi efficiency is variable in insects belonging to the orders Lepidoptera, Diptera, Hymenoptera, and Hemiptera, which include major pests, disease vectors, and beneficial insects (15, 28, 72, 104, 117). Furthermore, RNAi efficiency varies among insects even within the same order. For example, in the order Hemiptera, RNAi has been reported to be effective in *Cimex*, *Halyomorpha*, and *Oncopeltus* but not highly effective in *Acyrtosiphon*.

### Differences in Stability of Interfering RNA

Nucleases, such as dsRNases produced by host cells, have been identified and shown to degrade dsRNA (2, 108, 132). In *L. migratoria*, injected, but not ingested, dsRNA triggers efficient RNAi (61). dsRNases produced in the alimentary canal and capable of degrading dsRNA have been identified in this and other insects (108). Degradation of dsRNA by nucleases is a major problem in hemipterans such as aphids (15). In *L. migratoria* and *L. decemlineata*, silencing of genes encoding dsRNases by injection of dsRNA targeting these genes improved RNAi triggered by orally delivered dsRNA (108, 109). Also, knockdown in expression of genes coding for dsRNases improved RNAi in other insects including aphid and whitefly (16, 60). Comparative studies on the degradation of dsRNA by nucleases in insects that showed variable RNAi efficiency revealed significant differences in their nuclease activity. In general, lepidopterans and hemipterans showed higher nuclease activity compared to that in other insects (105, 123). Comparison of dsRNA degradation by nucleases from *L. decemlineata* (highly efficient systemic RNAi) and the tobacco budworm



**Figure 2**

Comparison of uptake, intracellular transport, and processing of dsRNA in coleopteran (RNAi-efficient) and lepidopteran (RNAi-inefficient) insects. In coleopterans (*left*), the ingested dsRNA is taken up by gut epithelial cells through clathrin-dependent endocytosis and other dsRNA transporters. The dsRNA is then released into the cytoplasm, and there is no major accumulation of dsRNA in endosomes (104). The dsRNA in the cytoplasm is processed to siRNA to trigger RNAi. Some of the dsRNA is exported to the hemocoel and taken by tissues such as fat body, where RNAi is induced. In contrast, in lepidopterans, some of the ingested dsRNA is degraded by dsRNases, and the rest is taken up by gut epithelial cells. Most of the dsRNA is trapped by the endosomes and is therefore not efficiently processed to siRNA, resulting in inefficient RNAi (138). Some of the dsRNA may also be exported to the hemocoel, but the dsRNases in the hemocoel degrade most of the dsRNA, resulting in inefficient transport into tissues such as fat body. In addition, an RNA binding protein, Staufen C, that is involved in processing dsRNA to siRNA for initiating RNAi, has so far been detected only in coleopterans and not in other insects (139). Abbreviations: dsRNA, double-stranded RNA; RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, short/small interfering RNA.

(*Heliothis virescens*) (inefficient RNAi) showed that the dsRNA-degrading nucleases are much more active in *H. virescens* than in *L. decemlineata* (104) (**Figure 2**).

### Differences in Cellular Uptake and Intracellular Transport of Double-Stranded RNA

As discussed in the section titled Cellular Uptake and Systemic RNA Interference, Sid2 and Sid1 mediate gut cellular uptake and intercellular transport of dsRNA, respectively, in *C. elegans*. However, the proteins that function as dsRNA channels and transporters, similar to SID proteins of *C. elegans*, have not been identified in insects. Studies on insects showed that clathrin-dependent endocytosis and macropinocytosis might be involved in dsRNA uptake (100, 131, 134). Differences in the uptake of dsRNA among tissues in an insect have been reported. For example, in *L. migratoria*, the injected dsRNA is not taken up by the follicle cells and oocytes, and thus RNAi



is not triggered in these tissues (95). The dsRNA taken up by cells through clathrin-dependent and other endocytic pathways is transported through endosomes to these cytoplasmic sites. In coleopterans, such as *T. castaneum* and *L. decemlineata*, where RNAi is efficient, the dsRNA is delivered to the cytoplasm, where it is converted to siRNA (104). In contrast, in lepidopterans, such as *H. virescens* and the fall armyworm (*Spodoptera frugiperda*), where RNAi does not work efficiently, the intracellular transport is inefficient, and most of the dsRNA is not able to escape endosomes and thus is not converted to siRNA (104, 138) (**Figure 2**).

### Differences in Processing of Double-Stranded RNA to Short/Small Interfering RNA

Upon entering cells, dsRNA is transported into the cytoplasm and processed to siRNA by Dicers (43, 50, 64, 85, 92). The processing of dsRNA to siRNA varies among insects. For example, in cells and tissues of *L. decemlineata* where RNAi is efficient, the <sup>32</sup>P-labeled dsRNA is processed to siRNA (104). In contrast, in cells and tissues of *S. frugiperda*, the <sup>32</sup>P-labeled dsRNA is not efficiently processed to siRNA (104). Comparative analysis of processing of dsRNA to siRNA in insects belonging to suborder Heteroptera and orders Lepidoptera, Orthoptera, and Diptera showed that the dsRNA was processed to siRNA but not as efficiently as in coleopterans (105). Differences in the structure and activity of Dicers and their substrate specificity could contribute to differences in processing of dsRNA to siRNA among insects. However, further studies are needed to verify this hypothesis. In addition, recent studies showed that an RNA binding protein, Stauf C, that is involved in processing dsRNA to siRNA is detected only in coleopterans and not in other insects (139). The function of RNA binding proteins such as Stauf C also needs further investigation.

### Differences in Argonaute Proteins

Recent studies on the evolution of *Ago* genes in insects suggest that these genes undergo frequent evolutionary expansions resulting in their functional divergence (23, 52). The number of *Ago* genes varies among insects. For example, four members of *Ago* genes coding for Ago1, Ago2, Ago3, and Piwi were identified in the silkworm (*Bombyx mori*) genome (122). In *D. melanogaster*, five genes coding for Ago1, Ago2, Ago3, Aub, and Piwi were identified (52, 111). Studies in *D. melanogaster* showed that Ago1 and Ago2 participate in siRNA and miRNA pathways, whereas Ago3, Aub, and Piwi interact with piRNAs (14, 30, 106). *B. germanica* seems to use both Ago1 and Ago2 in the miRNA pathway, but uses only Ago2 in the siRNA pathway (99). Interestingly, silencing of not only *Ago2*, but also *Ago1* and *Aub* genes blocks dsRNA-triggered RNAi in the *L. decemlineata* cell line (140). In addition, *Ago2* is duplicated in this insect, and both *Ago2* genes are required for dsRNA-triggered RNAi (140). Enhancement of RNAi efficiency after expression of *Ago2* in *B. mori* suggests that Ago2 may be one of the factors contributing to lower RNAi efficiency in lepidopterans (55).

### Physiological Conditions Affecting RNA Interference Efficiency: Expression of Genes Coding for RNA Interference Pathway Components

Studies in *D. melanogaster* showed that feeding naked dsRNA did not trigger RNAi efficiently, but adding a transfection reagent such as Lipofectamine improved response to fed dsRNA, suggesting that the fruit fly lacks mechanisms for efficient uptake of dsRNA from the lumen (126). The physiological conditions of the target insect could influence RNAi efficiency. For example, the pH of

the lumen, especially the highly alkaline pH observed in the lepidopterans, could be detrimental to successful RNA (22). In some lepidopterans, such as *B. mori* and the tobacco hornworm (*Manduca sexta*), the dsRNA triggers RNAi more efficiently during the nonfeeding wandering stage of the final instar larvae than during the feeding stages of the larvae. This may reflect changes in expression of genes involved in dsRNA degradation or RNAi response, as well as the physiological state of target tissues. For example, declines in food intake and digestion and thus in the titers of the dsRNA-degrading enzymes may enhance RNAi efficiency during the wandering stage.

## OPPORTUNITIES AND CHALLENGES OF RNA INTERFERENCE

### RNA Interference as a Powerful Research Tool

Before the discovery of RNAi, the analysis of gene function was limited to a few model insects such as *Drosophila*. RNAi has helped researchers perform large-scale functional genomics studies in many model insects, as well as in economically important non-model insects. This led to significant advances in our understanding of insect biology. Recent studies on genome and transcriptome sequencing and proteomic analyses identified thousands of genes, mRNAs, and proteins in hundreds of insect species. RNAi played a critical role in uncovering the function of the genes, RNA, and proteins identified by these studies (7). These advances facilitated an increase in our understanding of the developmental, physiological, and molecular processes that govern every aspect of insect life (32). Indeed, about 1,300 papers published during the past two decades studied RNAi or employed RNAi to investigate gene function in *Drosophila*. In addition, research in other model insects, such as *A. aegypti*, *A. gambiae*, *B. mori*, *Locusta* sp., *Spodoptera* sp., *T. castaneum*, the brown planthopper (*Nilaparvata lugens*), the cotton bollworm (*Helicoverpa armigera*), and the western honey bee (*Apis mellifera*), used RNAi methods to learn about the molecular mechanisms of physiological processes that are specific to each group of insects. RNAi methods also helped to identify targets for insecticide discovery and make significant advances in our understanding of the mode of action of insecticides and molecular mechanisms of insecticide resistance (4, 151, 152).

### RNA Interference for Development of Novel Pest Management Methods

Soon after the discovery of RNAi and development of its methods for gene silencing, entomologists initiated research to apply this technology to pest management. The US Environmental Protection Agency and other regulatory agencies have approved commercial products based on RNAi technology to control *D. v. virgifera*. Products for management of *L. decemlineata* and canola flea beetles are under development. Successful knockdown of target gene expression in the insects feeding on plants expressing dsRNA has been demonstrated in *H. armigera* (80), *L. decemlineata* (144), *M. sexta* (88), *N. lugens* (143), the green peach aphid (*Myzus persicae*) (87), and the silverleaf whitefly (*Bemisia tabaci*) (118). Although several publications document successful knockdown in expression of genes by dsRNA in lepidopterans and hemipterans, whether the knockdown reaches the levels required for the successful development of commercial products needs further research. dsRNA targeting house-keeping genes in *L. decemlineata* produced in *E. coli* and delivered as a foliar application of heat-killed bacteria causes significant insect mortality (101, 153). This approach may be further developed for controlling this insect pest in the field.

### Challenges in Widespread Use of RNA Interference Products

The major challenges to widespread use of RNAi to manage insect pests include the cost of dsRNA triggers, efficient delivery of dsRNA to the site of action, off-target and nontarget

effects of dsRNA, and potential resistance development. Whyard et al. (126) fed in vitro synthesized dsRNA to insects and successfully silenced target genes; this suggests that it is possible to achieve silencing of target genes by feedings dsRNA. However, achieving insect control by feeding of dsRNA is challenging due to the lack of cost-effective methods for producing large quantities of dsRNA. One could produce large quantities of dsRNA using cell-free synthesis methods. Hunter et al. (35) successfully field-tested dsRNA produced using such methods to control Israeli acute paralysis virus in *A. mellifera*. Effective delivery of dsRNA to the site of action is another major hurdle faced by RNAi applications in agriculture and medicine. The half-life of naked siRNA in serum ranges from several minutes to about an hour (5). It is difficult to achieve efficient RNAi if the trigger is degraded rapidly in an organism unless a high dose of dsRNA or siRNA is applied.

Another significant roadblock to RNAi that is preventing aggressive investment in the development of dsRNA-based products for pest management is the concern that insects may evolve resistance to dsRNA-based products, as they do for conventional pesticides. Insects could use several different mechanisms to develop resistance to these products. These include mutations of target genes, mutations of RNAi core machinery genes, enhanced dsRNA degradation, increased dsRNA excretion, decreased dsRNA uptake and transport, and decreased dsRNA endosomal escape. Some of the resistance mechanisms could be mitigated more easily than the others. For example, target site mutations could be managed by employing a dsRNA that targets a different gene. However, if insects develop resistance to dsRNA products through mutations or change in expression of a critical gene required for RNAi mechanisms, management of resistance could become a difficult task. Recent studies on resistance development in *D. v. virgifer* to *Snf7* dsRNA (41) and the *L. decemlineata* cell line to *LAP* dsRNA (139) showed that the resistance is not due to mutations in the dsRNA target site. Changes in the expression of proteins involved in uptake and intracellular transport of dsRNA are thought to be the major mechanisms for the resistance.

## SUMMARY POINTS

1. RNAi is triggered by the siRNA, miRNA, and piRNA pathways in insects.
2. Different cellular uptake mechanisms for dsRNA have been found or implicated, whereas the mechanisms of intercellular transport of dsRNA and systemic RNAi are largely unknown in insects.
3. Basic dsRNA delivery methods include microinjection, feeding, and soaking, but various new technologies have been developed and incorporated into the basic methods; these new technologies include cationic liposome-assisted, nanoparticle-enabled, symbiont-mediated, and plant-mediated deliveries.
4. Mechanisms affecting RNAi efficiency among insects include dsRNA degradation, cellular uptake and intracellular transport of dsRNA, efficiency in the processing of dsRNA to siRNA, the difference in RNA-induced silencing complex formation, and various physiological factors.
5. RNAi has provided not only powerful tools to study the developmental, physiological, and molecular processes that govern every aspect of insect life, but also great opportunities to develop new methods for insect pest management.
6. Major challenges to widespread use of RNAi for insect pest management include variable RNAi efficiency among insects, lack of reliable dsRNA delivery methods, off-target and nontarget effects, and potential resistance development.

## FUTURE ISSUES

1. What are the mechanisms governing cellular uptake, inter- and intracellular transport of dsRNA, and systemic RNAi?
2. What are the exact mechanisms causing significant differential RNAi responses among different taxonomic groups of insects?
3. How do duplication or deletion and upregulation or downregulation of the RNAi core machinery genes affect RNAi efficiency?
4. What are practical strategies to enhance RNAi efficiency by increasing the stability, cellular uptake, inter- and intracellular transport, and endosomal escape of dsRNA?
5. How can off-target and nontarget effects of dsRNA and potential development of resistance to RNAi be identified and managed?

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

The authors acknowledge that only a fraction of more than 3,000 papers published on RNAi in insects could be included in this review due to space restrictions. We particularly thank Zhitao Yu for help in organizing the references. Relevant research in the Zhu laboratory is supported by the US Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA), including the Foundational Program (2014–67013–21714), Hatch (371324), and Hatch Multistate (372103) projects. Other support comes from a collaborative grant funded by the National Natural Science Foundation of China (NSFC31730074). Research in the Palli laboratory is supported by grants from the National Institutes of Health (GM070559–13 and 1R21AI131427–01), the National Science Foundation (Industry/University Cooperative Research Centers, the Center for Arthropod Management Technologies under grant IIP-1821936), and the USDA/NIFA (under Hatch Project 2351177000 and Agriculture and Food Research Initiative Competitive Grant 2019-67013-29351). This manuscript is contribution No. 19–208-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kansas.

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