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Double-strand RNA (dsRNA) delivery methods in Insects: *Diaphorina citri*

Yulica Santos-Ortega, **Alex Flynt**

Cellular and Molecular Biology, University of Southern Mississippi, 118 College Drive Hattiesburg, MS 39406. Mississippi, United States

Abstract

RNAi is a gene-silencing mechanism conserved in the vast majority of eukaryotes. It is widely used to study gene function in animals due to the ease of eliciting gene knockdown. Beyond research applications, RNAi technology based on exogenous dsRNA is a promising candidate for next generation insect pest control. An advantage of using RNAi is that design of dsRNA essentially requires only the sequence of the target gene. The greatest challenge, however, is dsRNA delivery for large scale insect control. Delivery methods that have widely been used are oral, injection, or via soaking. Unfortunately, each insect presents its own challenges owing to the differences in the presence of dsRNA degrading enzymes, cellular uptake efficiency, expression of core RNAi machinery, the nature of the target gene, the concentration and persistence of the dsRNA, as well as the particular way of feeding of each insect, which together cause variations in the efficiency of RNAi. In this chapter, a protocol for the synthetic production of dsRNA is described along with three methods for delivery that have been successful in one of the more problematic insects, *Diaphorina citri*.

Keywords

RNAi; dsRNA design; dsRNA synthesis; feeding; topical-feeding; soaking; insect

1. Introduction

Interference RNA (RNAi) is a highly conserved cellular mechanism present in a vast majority of eukaryotes (1). This mechanism is triggered through the processing of long precursor double-strand RNA (dsRNA) to yield small RNA fragments which load into Argonaute (Ago) family proteins. Ago proteins loaded with small RNAs form the RNA induced silencing complex (RISC). In this complex, the small RNA serves as a specificity factor to direct the complex to target nucleic acid molecules through complementary base-pairing. This leads to either mRNA degradation or repression of translation (1, 2) (Fig. 1).

Since its discovery, RNAi has been used for studying gene function at the cellular and organismal levels (3–5). It has facilitated large scale functional genomics in human cells

Corresponding Author: Alex Flynt. Cellular and Molecular Biology, University of Southern Mississippi, 118 College Drive Hattiesburg, MS 39406. Mississippi, United States Alex.Flynt@usm.edu.

and in other model organism systems (6, 7). It is also being used in the clinic as a gene modulatory therapy through negative regulation of the expression of specific genes in cancerous cells (8). Furthermore, in order to try to expand its use, RNAi has been studied in infectious disease, signaling, and ageing (9–12). However, the only use of RNAi approved by FDA, so far, is Patisiran in amyloidosis (13). In non-model organisms where genetic techniques are not available, RNAi has been essential to enabling functional genomics (14).

In insects, RNAi has been increasingly emphasized as an approach for insect pest management due to its high specificity to target genes and therefore species, as well as the lack of environmental persistence. RNAi shows significant promise in combating coleopteran insects. Indeed, in 2017 the transgenic corn product SmartStax Pro, which in addition to 6 transgenes producing *Bacillus thuringiensis* (Bt) toxin incorporates *Diabrotica virgifera* Snf7 (DvSnf7) dsRNA. DvSnf7 protein is a class E vacuolar sorting protein, shown to be essential for transmembrane protein sorting. Transgenic maize produce DvSnf7 dsRNA which when consumed by *D. virgifera* and other corn rootworm species enters the RNAi pathway leading to down-regulation of the targeted DvSnf7 gene and death (15–19). This shows the promise of RNAi for pest management, which will only be realized through effective delivery methods.

Successful RNAi is dependent on design, synthesis and delivery of dsRNA (20). Typically, dsRNAs are between 300–600 bp in size with homology to coding regions of the target gene. Ideally, the sequence should contain no more than 19 nucleotides of contiguous homology with non-target genes within its own species and others, lest the dsRNA could be non-specific (15, 21–23). There are many options to produce dsRNA such as chemical synthesis for siRNAs which mimic products of DICER cleavage. Other strategies include expression in microbes or in crop cells via transgenes. In this chapter, *in vitro* synthesis based on T7 RNA polymerase kits is described (24, 25). Regardless of method of production, insects will likely encounter the molecules through diet or environmental exposure. In this chapter we discussed feeding strategies along with methods for topical delivery and dsRNA soaking.

Delivery of dsRNA requires maintenance of integrity during application to uptake by cells, so that it can ultimately be processed by the RNAi machinery (26). In cell lines assays, the dsRNA is typically introduced via endocytosis (27). In insects, the soaking delivery method has been widely used in S2 cell lines derived from *Drosophila melanogaster* embryos (28–31), or Sf21 cells derived from ovaries *Spodoptera frugiperda* (32). Better results of gene silencing were observed upon combining soaking dsRNA delivery with different transfection reagents in Sf21 cells as well as CiE1, a cell line derived from *Chrysodeixis includens* embryos (33, 34). Although the S12 cell derived from *Spodoptera littoralis* was also used for dsRNA uptake, no efficient silencing was observed in this lepidopteran cell nor in Bm5 cell line derived from *Bombyx mori* (35). Thus generally, insect cells readily uptake dsRNA and mount an RNAi response, however, this suggests in some cases additives may be required to fully elicit gene silencing.

In whole insects, dsRNA delivery methods such as injection, microinjection, oral delivery and soaking have been tested or applied (3, 36–39) (see Table 1). Injection of various insect

species, such as *Tribolium castaneum* (40, 41), *Locusta migratoria* (42–45), and *Blattella germanica* (35, 46) showed success in down regulations of target genes. The advantages of injection include the immediate, direct delivery of a known amount and concentration of dsRNA into the insect body at various developmental stages or even to specific body parts (43) as well as avoidance of the structural barriers such as the integument, that prevent penetration of dsRNA. However, injection is more time-consuming and is sometimes challenging due to the small body size of some insects and is not applicable to control insect pests in the field.

In contrast, synthetic dsRNA can be introduced with food or artificial diet for oral delivery to insects, which has been applied with success in some lepidopterans (36, 47)), coleopterans (48), hemipterans (49) and dipterans (50). In contrast, neither artificial diet that was coated with dsRNA nor food that was mixed with dsRNA could induce RNAi in four *Drosophila* species (*D. melanogaster*, *D. pseudoobscura*, *D. sechellia*, *D. yakuba*) (49).

Two more ways of oral dsRNA delivery are dsRNA droplet-feeding and topical feeding (36, 38). Although the techniques are similar, the difference is that during droplet-feeding, the insects are placed in a petri dish with a drop so they may acquire the dsRNA when ingesting the drop. In topical-feeding, the insects are placed face up and the drop containing dsRNA is applied over their mouthparts. In both methods, the gene silencing effect has been observed in lepidopterans and hemipterans (36, 38). However, one disadvantage to the methods of oral delivery is that the dsRNA can be degraded by nucleases in the insect saliva, gut lumen or hemolymph preventing take up by cells. Several solutions have emerged to counteract these effects. One is that following identification of nucleases genes (dsRNases) in the insect of interest co-administer the dsRNA target to the dsRNases alongside dsRNA against the main target as seen in work with *Bemisia tabaci* (51). Feeding insects with a diet containing microorganism such as *Escherichia coli* and *Saccharomyces cerevisiae* or with endosymbionts that expressed the dsRNA of interest are strategies that protect dsRNA from endonucleases and improve oral delivery (20, 26). In transgenic plant feeding delivery a concern is dsRNA processing in plants cells, which hampers the effectiveness. One possible solution could be expression from chloroplasts (REF).

Soaking dsRNA delivery method may be the most convenient, especially for functional genomics in the laboratory. It was first reported in *Caenorhabditis elegans* that RNAi can be induced by soaking nematodes in dsRNA solution. Owing to the ease of this technique, soaking has been used in large-scale analysis of gene function by high-throughput RNAi (52, 53). On the other hand, delivery of dsRNA in whole insect bodies is possible, despite the extra barriers such as the insect cuticle, provided the development state of the insect is considered. For example, considerable mortality ranging between 40–70% correlated with downregulation of target gene expression was found after spray of dsRNA on newly hatched *Ostrinia furnacalis* larvae (54) when the cuticle was soft. On the other hand, spray chitosan or other nanocarrier-based dsRNA nanoparticles increase the stability of the dsRNA as well as cellular uptake in insects (20). 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 (55, 56), facilitate dsRNA penetration through the insect body wall (57), and likely improve the cellular uptake and endosomal escape of dsRNA (58).

The efficiency of all dsRNA delivery methods in insects, is subject to the presence of dsRNA degrading enzymes, cellular uptake efficiency, the expression of the core components of the RNAi machinery, the nature of the target gene, the concentration and persistent of the dsRNA, as well as the feeding behavior of each insect. For these reasons, it is common to observe variations in the effectiveness of RNAi and even to find no effect whatsoever. In this sense in depth study of RNAi pathways biogenesis in insects may help to identify the most effective mechanism to generate superior methodologies for the control of insect pests.

In this chapter, two enzymatic synthesis methods for obtaining dsRNA and three delivery methods are described that have been successful in one of the most challenging insects, *Diaphorina citri*, a major pest devastating the citrus industry (59–61). Its manner of feeding and size make successful delivery of dsRNA to *D. citri* difficult. The three methods for delivering dsRNA are: feeding, topical-feeding and soaking. Each is able to elicit silencing of target genes, leading to lifespan reduction and high mortality (62–66). A part of success in *D. citri* may be due to insufficient dsRNase activity in the digestive tract and hemolymph as well as efficient cell uptake of dsRNA (67).

2. Materials

1. Acid-Phenol-Chloroform, 5:1 solution, pH 4.5+/−0.2 (99%) Ambion.
2. Ethanol 70 and 100 % (v/v).
3. 2-Propanol, Isopropanol, Fisher Scientific.
4. D-Sucrose, biological molecular grade, Fisher Scientific.
5. Q5 high-fidelity DNA Polymerase (New England, BioLabs).
6. Phire Hot Start II DNA Polymerase (Thermo Fisher).
7. gBlock Gene Fragment. Integrated DNA Technologies (IDT) company.
8. Primers. Integrated DNA Technologies (IDT).
9. QIAquick PCR Purification Kit (QIAGEN). Supplied with the Kit: pH indicator, buffer PE, buffer PB, Filter Cartridge and collection tubes.
10. MEGAScript RNAi Kit (Ambion). Supplied with the Kit: T7 Enzyme, 10X T7 Reaction Buffer, ATP, GTP, CTP, UTP Solution, 10X Binding Buffer, Elution Solution, RNase, DNase I, 10X Digestion Buffer, 2X wash solution, Nuclease free water, Filter Cartridge and collection tubes.
11. MEGAScript Kit (Thermo Fisher). Supplied with the Kit: T7 Enzyme, 10X T7 Reaction Buffer, ATP, GTP, CTP, UTP Solution, 10X Binding Buffer, Elution Solution, RNase, DNase I, 10X Digestion Buffer and 2X wash solution, Nuclease free water, ammonium acetate stop solution, TURBO DNase.

12. Eppendorf Mastercylcer Pro 6321 PCR Thermal Cycler w/vapo protect lid.
13. Sorvall Legend Micro 21R Centrifuge, Thermo Scientific.
14. Growth room.
15. Insect aspirator # 654135, CAROLINA.
16. Carbon dioxide (CO2).
17. Parafilm.
18. Distillate water.
19. Plants of *Citrus machrophyla* (small size).
20. Rearing cages (12 × 12 × 12 inches).
22. Petri dishes (100 × 15 mm).
23. Stereoscope.
24. Concave slides.
25. 2115 Economical Camel Hair Touch Up Brush size 2.
26. Filter paper 320 mm (Whatman).
27. Pipet of 200 μl.
28. Small Berlese funnel trap.
29. 10 μl Hamilton syringe.
30. PB600 Dispenser.
31. DNase-RNase free-water.

3. Methods

D. citri belongs to the order Hemiptera, which feed exclusively on sucrose rich plant sap (phloem) through the introduction of its stylet. To avoid damaging the stylet before removing insects from *Citrus macrophyla,* the plants must first be shaken. This provokes the insects to pull their stylet back so as not to break it during the aspiration. In this way, the feeding structure remains intact. In the case of nymphs, they can be gently touched with a paint brush before being carefully removed from the plants. The identification of the 4–5 instar is done under stereoscope. For each methodology, the insects were kept without food for 3 hours before dsRNA delivery and were kept in temperature-controlled growth rooms set at 25 ± 3°C, 60 % ± 5 % relative humidity (RH), and with a 16:8 (light/dark) photoperiod.

3.1 dsRNA: enzymatic synthesis from gBlock Gene Fragment.

1. Use NCBI database to identify the Open Reading Frame (ORF) of the gene of interest.

2. Copy the ORF sequences in Primer Plus 3 free on-line software to pick up the primers from the ORF sequences (*see* Note 1).

3. Run a Blast of the DNA sequence of the target against the genome of the insect that you want to avoid as target.

4. Do a second pair of primers using the sequences of the primers already designed and add the T7 promotor sequence (*see* Note 2)

5. Order the gBlock Gene Fragment (*see* Note 3).

6. Re-suspend the gBlock following the instructions from the IDT and do two PCR reaction using the primers with the T7 attached:

   6.1 Prepare the reaction:

Component	uL	Concentration
Q5 high-fidelity 2X	25	1 X
Forward Primer T7 X	2.5	0.5 μM
Reverse Primer T7 X	2.5	0.5 μM
Template DNA (gBlock)	2	<1.000 ng
DNase-RNase free-water	18	
TOTAL VOL	50	

   Gently mix the reaction and run the PCR.

   6.2 Run an agarose gel in order to check the integrity of the DNA. Then mix the two PCR reactions

7. Purify the PCR using QIAquick Kit

   7.1 Mix (1:250 volume) pH indicator I and Buffer PB. Add 5 vol of this mix at 1 vol of PCR.

   7.2 Transfer the mix to the column QIAquick (load in the center) to bind the DNA and centrifuge at 8,944 × g for 1 min. Discard flow-through and re-used the tube

   7.3 Add 750 μL of Buffer PE at the column to wash and centrifuge and repeat twice.

   7.4 Transfer the column to a clean 1.5 mL microcentrifuge tube, add 30 μL of DNase-RNase free-water to elute DNA and centrifuge. (*see* Note 4)

   7.5 Read on nanodrop and do an agarose gel in order to get the quantity and quality of the DNA.

8. Synthesize the dsRNA using MEGAScript RNAi Kit.

   8.1 Prepare the *in vitro* transcription reaction. (X: uL necessary to reach 2 μg or 20 μL)

Component	µL
PCR + gBlock (2 µg)	X
10x T7 Reaction Buffer	2
ATP Solution	2
CTP Solution	2
GTP Solution	2
UTP Solution	2
T7 Enzyme Mix	2
Nuclease-Free Water	X
TOTAL VOL	20 µL

8.2 Incubate at 37°C overnight (~16 hrs).

8.3 Transfer the reaction into thermocycler and heat it at 75°C for 5 min. Then cool to room temperature for around 15 min (*see* Note 5).

8.4 Prepare the following reaction on ice and incubate at 37°C for 1 hr (do not continue this incubation for longer than 2 hrs):

Component	µL
dsRNA reaction	20
Nuclease-free water	21
10X Digestion buffer	5
DNase I	2
RNase	2
TOTAL VOL	50 µL

8.5 Purify of dsRNA

Component	µL
dsRNA reaction from Step 4	50
10X Binding buffer	50
Nuclease-free water	150
100% Ethanol	250
Total Volume	500 µL
Gently mix the reaction by pipetting up and down.	

8.6 Transfer the dsRNA reaction into the Filter Cartridge and collection tubes. Centrifuge at 12,878 × g for 2 min

8.7 Wash the Filter twice with 500 µL of 2X wash solution and centrifuge (12,878 × g for 2 min)

8.8 Centrifuge one more time. Then transfer the filter into the new collection tube.

8.9 Add 50 – 100 μL of hot Elution Solution into the Filter and incubate at Room Temperature for 10 min. Then centrifuge 12,878 × g for 3 min and read in nanodrop. Do an agarose gel in order to check the dsRNA integrity (*see* Note 6).

3.2 dsRNA: enzymatic synthesis from genomic DNA.

1. Use NCBI database to select a region exon or transcript in the Genome Browser Viewer.
2. Copy the sequence in the Plasmid Editor APE (https://jorgensen.biology.utah.edu/wayned/ape/), for design Forward and Reverse Primers to have fragment size between 300–600 bp and add the T7 promotor sequence to each primer.
3. Amplify through PCR the target fragment using the primers previously designed and the genomic DNA extracted from *D. virgifera*.

   3.1 Prepare the following reaction. (X: uL necessary to reach 25 μL)

Component	μL	Final Concentration
5X Phire Reaction Buffer	4	1x
10mM dNTPs	0.4	200 μM each
T7 Forward Primer	1	0.5 μM
T7 Reverse Primer	1	0.5 μM
gDNA	50ng	
DNase-RNase free water	X	
Phire Hot Start II enzyme DNA Polymerase	0.4	
TOTAL VOL	25	
Check the PCR through agarose gel		

4. Synthesize the dsRNA using MEGAScript Kit. Use directly the PCR.

   4.1 Prepare the *in vitro* transcription reaction. (X: uL necessary to reach 2 μg or 20 μL)

Compound	μL
T7 10x Reaction buffer	2
T7 ATP solution	2
T7 CTP solution	2
T7 GTP solution	2
T7 UTP solution	2

Compound	µL
DNA template (PCR: 2µg)	X
Nuclease free water.	X
T7 Enzyme Mix	2
TOTAL VOL	20

4.2 Incubate the reaction for 4 hrs. at 37°C.

5. Clean up the reaction:

5.1 Add 1µL of TURBO DNase to the *in vitro* transcription reaction (20 µL) and incubate the reaction at 37°C for 10 min

5.2 Add 20 µL of ammonium acetate stop solution and 100 µL of DNase-RNase free water

5.3 Add 200 µL 99% of Acid-Phenol-Chloroform, take aqueous part and transfer in new tube

5.4 Add 600 µL of isopropanol and chill for 20 min at 80°C

5.5 Speed at 17,530 × g for 15 min and remove supernatant

5.6 Wash three times with 70% of Ethanol

5.7 Spin down at 17,530 × g for 10 min and discard the supernatant

5.8 Let the sample dry and resuspend in 20 µL of DNase-RNase free-water

5.9 Read concentration in nanodrop and do an agarose gel in order to check the integrity of the dsRNA.

3.3 Feeding dsRNA delivery in adults (Fig. 2A)

1. Collect 30 insects from their colonies with the aspirator. The aspirator come with small cylindrical containers, each with a hole bored into each side so that oxygen may enter throughout the ingestion period for dsRNA.

2. Prepare the artificial diet which consists of 20 % (w:v) D-sucrose mixed with dsRNA. Use DNase-RNase free water to prepare the solution of sucrose.

3. Plug the holes in the container with hand and add a little CO_2 in order to immobilize the insects for a couple of minutes and thus prevent them from flying out when the lid is opened.

4. Stretch a layer of parafilm over the mouth of the container and create a slight depression in this cover by pressing down with the thumb in the center.

5. Add 150 µl of dsRNA into the depression and place a second layer of well-stretched parafilm on top.

6. Return the insects with the container to the growth room, and they will feed from the dsRNA solution.

7. Change the dsRNA every 24 hrs. for three days. Remove only the second layer to add a new 150 μl of dsRNA (*see* Note 7).

8. Transfer the adults of *D. citri* to rearing cages in the growth room that contain plants with small sized *C. macrophylia* (*see* Note 8)

3.4 Soaking dsRNA delivery in Nymphs in 4–5th instar (Fig. 2B)

1. Remove nymphs in 4–5 instar from leaves of the plants and maintain them in petri dishes.

2. Prepare the dsRNA with DNase-RNase free-water at the desired concentration.

3. Place 4 nymphs in the concave slide under stereoscope and immediately add 50 μl of dsRNA with pipet. Wait 20 min (*see* Note 9).

4. Remove all the excess solution and put it in a new Eppendorf tube. Put the filter paper in the floor of the petri dish and transfer the nymphs with camel hair brush and cover it (*see* Note 10).

5. Place 4 more nymphs on the same slide and add the excess solution of dsRNA reserved in the Eppendorf tube. Add the quantity of solution necessary to again reach 50 μl (*see* Note 11).

6. Transfer the nymphs with the camel hair brush to a petri dish furnished with tender *C. macrophylla* leaves to feed nymphs. Make sure the leaves are placed face down (*see* Note 12).

7. Cover the petri dish with well-stretched parafilm. Use a pin to make small holes in the parafilm.

8. Repeat the application of dsRNA after 24 hrs.

9. Transfer the nymphs with camel hair brush to the leaves and stems of the *C. macrophylla* plant and place on a small Berlese funnel trap. Transfer the trap to the growth room (*see* Note 13).

3.5 Topical-feeding dsRNA delivery in Nymphs in 4–5 instar and adults (Fig 2C)

1. Prepare the solution of dsRNA in DNase-RNase free water.

2. Place the filter paper under the stereoscope and moisten it with distilled water using a camel hair brush (*see* Note 14).

3. Place the nymphs onto the filter paper in ventral position with the help of the camel hair brush. The adults must first be immobilized with CO_2 and then placed face up with the brush.

4. Moisten the nymphs passing the camel hair brush several times over the bodies gently. Adults must be principally moistened on the wings (*see* Note 15).

5. Place lines of ten nymphs for more control. Adults should be placed in lines of five (*see* Note 16).

6. Have the syringe ready with the dispenser calibrated to apply 0.2 μl of solution of dsRNA.
7. Press the button of the dispenser to form a drop on the tip of the needle.
8. Gently bring the drop to the mouth of the insect and just touch it (*see* Note 17).
9. Keep the insect in that position feeding on the drop for 20 minutes (*see* Note 18).
10. Transfer the nymphs and the adults on the leaves with the help of the camel hair brush and close the small Berlese funnel trap. Transfer the trap to the growth room (*see* Note 19).

4. Notes

1. The final fragment should be around between 300–500 bp which is the gBlock. (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) this is the page of Primer Plus 3 free on-line software.
2. This pair of primers will use to amplify the gBlock with the T7 promotor through PCR.
3. https://www.idtdna.com/pages/products/genes-and-gene-fragments/double-stranded-dna-fragments/gblocks-gene-fragments this is the page to get all the information of the company IDT. Following the indications of the company (IDT) send the information of DNA sequence target for synthetized the gBlock Fragments and primers. The cost depends on the size of the fragments.
4. One recommendation is elute the DNA with DNase-RNase free-water.
5. Never let it cool down on ice.
6. In this step, it is necessary to heat at 95°C in the thermocycler the Elution Solution and apply it hot in the center of the column.
7. The insects will stick their stylet through the parafilm to ingest the artificial diet of dsRNA. The layer of parafilm mimics the leaf and, given that stylet is in the ventral part, they must be placed face up to feed. When changing the dsRNA, it is possible to see small bubbles between the layers of the parafilm, which indicates that the insects have stuck their stylets through there.
8. From this moment, it is possible to consider doing survival studies a/o evaluate the silencing of the gene of interest.
9. The nymphs will try to escape rapidly. To keep them in the solution, it's important to push them below the surface with the camel hair brush.
10. Place a weight on the lid of the petri dish, because the nymphs are very active and will try to get out.
11. Repeat this procedure until the number of desired insects is reached in each repetition.

12. It is very important that the leaves be soft and in very good state so that they may hold up for 13 hours. If they are dry, the insects will die during this period.

13. Funnel trap is removed to place the small plants contained in standard round pots. Reinforce with fine screen the airway in the upper part. This will prevent the nymphs or some emerged adult from escaping during the treatment.

14. It's important to maintain the paper filter moist during the application time.

15. It's important to moisten the bodies of the nymphs and the wings of the adults during the application because this, together with the moistened paper filter, keeps them adhered to the paper filter.

16. With a little practice, it is possible to place up to 50 nymphs in rows of 25 and quickly apply the drop to each one. With adults, as many as 10 is manageable, while always keeping the wings moist. Otherwise, they dry, the insects flip over, and they fly away.

17. The mouth in both nymphs and adults is easily identifiable under stereoscope due to its appearance as a black beak. Moistening the insect just before applying the drop helps to avoid lifting the insect with the attraction effect

18. In some cases, the insects turn over or the drop gets dissipated by the moisture already in the paper filter. If this happens, place the insect in the ventral position and place the drop again.

19. It is advisable to not apply the dsRNA to nymphs and adults at the same time.

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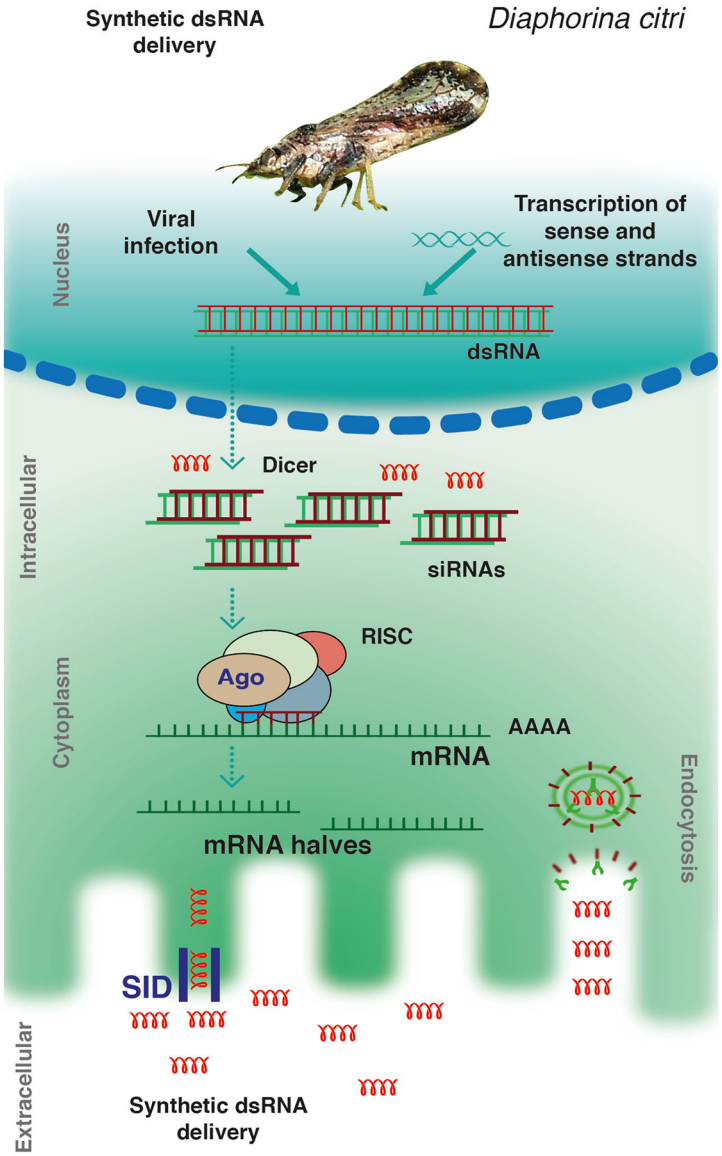

Figure 1. siRNA pathway.

After the synthetic dsRNA taken up by the cell, long dsRNA is cleaved by a RNaseIII-enzyme called DICER into small interfering RNA (siRNA) molecules of 20–25 nucleotides long. Subsequently, these siRNA associate with an Argonaute protein to form the RNA-induced silencing complex (RISC), which then targets and destroys mRNA complementary to the siRNA in the RISC.

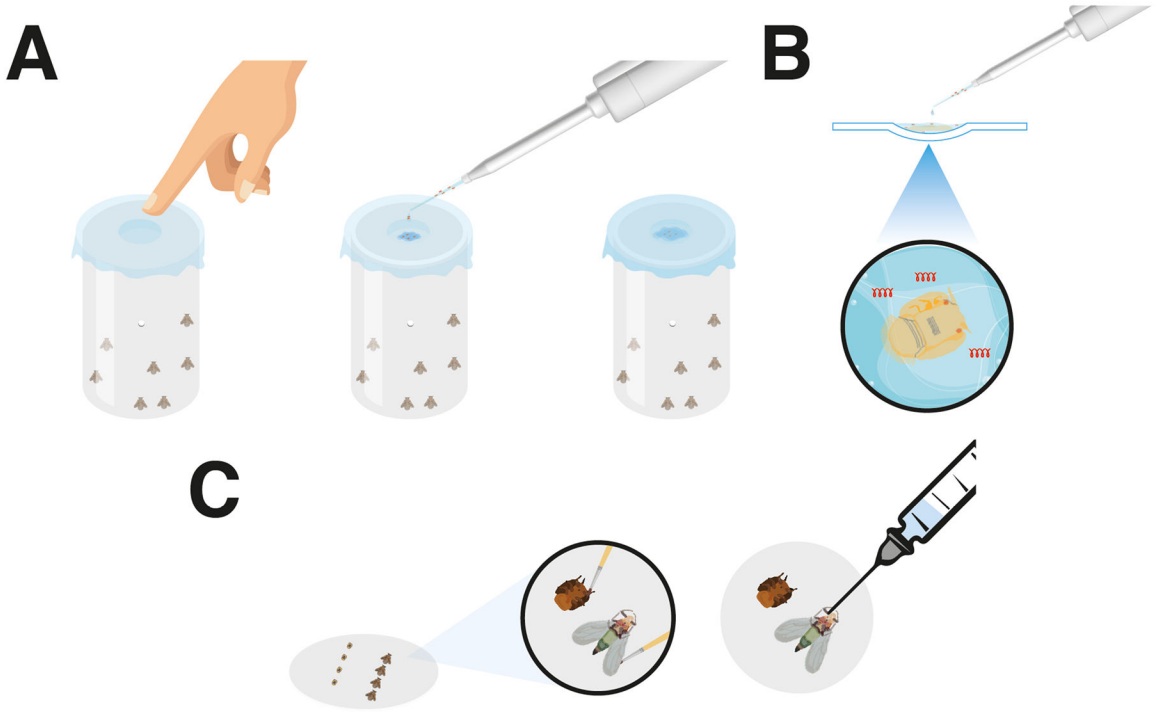

Figure 2. dsRNA delivery methods in *Diaphorina citri*.
A) Artificial diet-feeding, B) Soaking and C) Topical-feeding.

Table 1.

List of genes targeted by RNAi and dsRNA delivery methods in different insect orders

			dsRNA Delivery Methods			
Orden	**Specie**	**Target**	**Oral**	**Injection/ Soaking**	**Stage**	**Ref**
Coleoptera	*Tribolium castaneum*	V-ATPase E	Artificial diet		Neonates	(49)
Chewing-feed	*Diabrotica virgifera*	Multiple targets	Artificial diet		Neonates	(68)
	Virgifera	Snf 7	Artificial diet		Neonates	(15)
		Bol and vgr	Transgenic Plant		Larva/ Adults	(69)
	Leptinotarsa	Multiple targets	Leaf tissue		Neonates	(68)
	decemlineata	V-ATPase A and E	*TransgenicEcoli*		2nd Instar	(70)
	Diabrotica	Snf 7	Artificial diet		Neonates	(15)
	undecimpuctata	V- ATPase A and E	Artificial diet		Neonates	(68)
	howardi	α-Tubulin	Artificial diet		Neonates	(68)
	Phyllotreta striolata	Arginine kinase	Leaf tissue		Adults	(71)
Diptera	*Aedes aegypti*	V-ATPase A Multiple targets	Artificial diet		Adults	(72)
			Water		1st Instar	(50)
Blood-nectar plant-insect juice Sucking-Feed		ATP-dependent efflux Pump	Water		2nd Instar	(73)
	Anhopeles gambiae	Chitin synthase 1, 2	Artificial diet		3rd Instar	(74)
	Anopheles stephensi	3-HKT	Transgenic algae		1st Instar	(75)
	Bactrocera dorsalis	Multiple targets	Transgenic *E.coli*		Adults	(76)
	Glossina morsitans	Tsetse EP	Blood meal		Male Adults	(77)
	Morsitans	Transferrin	Blood meal		Male Adults	(77)
Hemiptera	*Acyrthosiphon pisum*	Aquaporin	Artificial diet		6-day-old nymphs	(78)
		V-ATPase E	Artificial diet		1st Instar	(49)
Phloem/Xylem Sucking-feed		V-ATPase E	Artificial diet		Neonates	(79)
		Hunchback (hb)	Artificial diet		Neonates	(80)
		Salivary protein COO2		Microinjection	Adults	(81)
		Gut digestive enzyme cathepsin-L	Artificial diet	Microinjection	1st, 3rd Instar	(82)
		Structural sheath protein SHP		Microinjection	Adults	(83)
		Angiotensin-converting enzymes ACE1 and ACE2		Microinjection	Adults	(84)
		Peroxiredoxin 1 gene ApPrxl		Microinjection	Adults	(85)
		Macrophage migration inhibitory factor ApMIF1		Microinjection	Adults	(86)
		Calreticulin, Cathepsin-L		Microinjection	Adults	(87)
	Myzus persicae	Rack1 and COO2	Transgenic Plants		Adults	(88)
	Aphis gossypii	Carboxylesterase geneCar	Artificial diet		Adults	(89)

			dsRNA Delivery Methods			
Orden	**Specie**	**Target**	**Oral**	**Injection/ Soaking**	**Stage**	**Ref**
		Cytochrome P450 monooxygenase gene CYP6A2	Artificial diet		3rd instar, Adults	(90)
		Odorant-binding protein 2 AgOBP2	Artificial diet		Adults	(91)
	Bactericera cockerelli	Multiple targets	Artificial diet		Adults	(92)
	Bemisia tabaci	V-ATPase subunit A, rpL19 V-ATPase subunit A AChE and EcR	Artificial diet		Adults	(93)
			Transgenic Plants		Adults	(94)
			Transgenic Plants		Adults	(95)
	Nilaparvata lugens	Trehalose PO4 synthase	Artificial diet		3rd instar	(74)
		V-ATPase E	Artificial diet		2nd Instar	(96)
	Grain aphid	Catalase gene CAT	Artificial diet		3rd instar	(97)
		Acetylcholinesterase gene SaAce1		Microinjection	Adults	(98)
		Multiple targets	Artificial diet		3rd instar	(99)
		Multiple targets	Artificial diet		Adults	(100)
		Olfactory coreceptor gene SaveOrco	Artificial diet		Adults	(90, 101)
	Rhopalosiphum padi	Acetylcholinesterase gene RpAcel		Microinjection	Adult s	(98)
	Schizaphis graminum	Salivary protein C002	Artificial diet		Adults	(102)
	Peregrinus maidis	V-ATPase B and D	Artificial diet		3rd instar	(103)
	Rhodnius prolixus	Nitrophorin 2	Artificial diet		2nd Instar	(104)
	Lygus lineolaris	Inhibitor of apoptosis	Artificial diet		Neonates	(105)
	Diaphorina citri	*awd* gene muscle protein 20 carboxyesterases CYP4 genes sucrose hydrolase *boule* homologue gene glutathione S-transferase transformer 2 homologue	Topical		5th instar	(106)
				Water	4rd, 5th instar	(63)
			Topical		4rd, 5th instar	(62)
			Topical		Adults	(107)
			Topical		4–5th instar, Adults	(44)
			Artificial diet		Adults	(64)
			Artificial diet		Adults	(108)
			Artificial diet		Female Adults	(66)
Hymenoptera	*Apis mellifera*	Vitellogenin	Natural diet		2nd Instar	(109)
Chewing and Sucking-Feed	*Solenopsis invicta*	PBAN/pyrokinin	Artificial diet		4rd instar	(110)
		Guanine nucleotide binding GNBP	Artificial diet		Workers	(111)
Isoptera	*Reticulitermes flavipes*	Cellulase	Paper disc		Workers	(112)
Chewing-Feed		Hexamerin	Paper disc		Workers	(112)
Lepidoptera	*Chilo infuscatellus*	CiHR3 molting factor	Corn kernels		3rd instar	(113)
Chewing and Sucking-Feed	*Epiphyas postvittana*	Carboxylesterase	Droplet		3rd instar	(36)

		dsRNA Delivery Methods				
Orden	**Specie**	**Target**	**Oral**	**Injection/ Soaking**	**Stage**	**Ref**
		Pheromone bp	Droplet		3rd instar	(36)
	Helicoverpa armigera	AchE receptor	Artificial diet		Neonates	(114)
		AchE receptor	Leaf tissue		Neonates	(114)
		Ecdysone receptor EcR	Artificial diet		3rd instar	(115)
		HaHR3 molting factor	Artificial diet		3rd instar	(116)
		CYP6B6	Artificial diet		3rd instar	(26)
		CYP6AE14	Transgenic Plant		Adults	(117)
		Ultraspiracle protein, EcR	Artificial diet		3rd instar	(118)
	Manduca sexta	V-ATPase E	Artificial diet		Neonates	(49)
	Ostrinia nubilalis	Chitinase	Artificial diet		Neonates	(119)
	Plutella xylostella	CYP6BG1	Artificial diet		4rd instar	(120)
		Rieske protein	Artificial diet		2nd Instar	(121)
		AchE receptor	Artificial diet		2nd Instar	(122)
	Sesamia nonagrioides	JH esterase JHER	Droplet		1st – 6th instars	(123)
	Spodoptera exigua	Chitin synthase A β1 integrin subunit	Leaf tissue		Neonates	(124)
			Leaf tissue		4rd instar	(125)
	Spodoptera litura	Aminopeptidase N	Artificial diet (Ec)		Neonates	(126)
	Bombix mori	*atg* gene bursicon gene		Injection	Larva	(127)
				Injection	Pupae	(128)
	Spodoptera frugiperda	Allatostatin C	Droplet	Injection	1st instar, larva	(129)
		Allototropin 2	Droplet		1st instar	(129)
		SfT6 serine protease	Droplet		4rd instar	(130)
Orthoptera	*Gryllus bimaculatus*	Sulfakinins	Droplet		Adults	(131)
Chewing-Feed	*Locusta migratoria*	Multiple targets	Artificial diet		4rd instar	(132)
	Schistocerca gregaria	Tubulin, GAPDH	Artificial diet		Adults	(133)

Modified 744 of Kumar *et al.*, 2018