Video Article

Double-stranded RNA Oral Delivery Methods to Induce RNA Interference in Phloem and Plant-sap-feeding Hemipteran Insects

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Abstract

Phloem and plant sap feeding insects invade the integrity of crops and fruits to retrieve nutrients, in the process damaging food crops. Hemipteran insects account for a number of economically substantial pests of plants that cause damage to crops by feeding on phloem sap. The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Heteroptera: Pentatomidae) and the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) are hemipteran insect pests introduced in North America, where they are an invasive agricultural pest of high-value specialty, row, and staple crops and citrus fruits, as well as a nuisance pest when they aggregate indoors. Insecticide resistance in many species has led to the development of alternate methods of pest management strategies. Double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) is a gene silencing mechanism for functional genomic studies that has potential applications as a tool for the management of insect pests. Exogenously synthesized dsRNA or small interfering RNA (siRNA) can trigger highly efficient gene silencing through the degradation of endogenous RNA, which is homologous to that presented. Effective and environmental use of RNAi as molecular biopesticides for biocontrol of hemipteran insects requires the *in vivo* delivery of dsRNAs through feeding. Here we demonstrate methods for delivery of dsRNA to insects: loading of dsRNA into green beans by immersion, and absorbing of gene-specific dsRNA with oral delivery through ingestion. We have also outlined non-transgenic plant delivery approaches using foliar sprays, root drench, trunk injections as well as clay granules, all of which may be essential for sustained release of dsRNA. Efficient delivery by orally ingested dsRNA was confirmed as an effective dosage to induce a significant decrease in expression of targeted genes, such as juvenile hormone acid O-methyltransferase (JHAMT) and vitellogenin (Vg). These innovative methods represent strategies for delivery of dsRNA to use in crop protection and overcome environmental challenges for pest management.

Video Link

The video component of this article can be found at https://www.jove.com/video/57390/

Introduction

Hemipteran insects comprise some of the most economically significant pests of agriculturebecause of their ability to attain elevated population growth and spread diseases in plants. The BMSB, *H. halys* Stål, is an invasive pest that was accidentally introduced in the Western hemisphere in Allentown, Pennsylvania from Asia (China, Taiwan, Korea, and Japan) with the first sighting reported in 1996[1]. Since its introduction, BMSB has been detected in 43 states, with the highest populations in the Mid-Atlantic (DE, MD, PA, NJ, VA, and WV), as well as in Canada and Europe, and represents a potential threat to agriculture[2]. As a polyphagous pest, BMSB can instigate damage to approximately 300 identified plant hosts including high-value crops such as apples, grapes, ornamental plants, seed crops, soybeans, and corn. Damage is caused primarily due the mode of feeding known as lacerate and flush where the animal pierces the host crop with its needle-like stylet to gain access to the nutrients from the vascular tissues[2,3]. BMSB is also an indoor pest as they may find residence in living areas such as schools and houses during autumn through winter[2]. Chemicals and aeroallergens released by BMSB were reported to illicit allergic reaction in fruit crop workers. BMSB may also contribute to allergic disease leading to contact dermatitis, conjunctivitis, and rhinitis in sensitive individuals[4,5]. Another hemipteran insect, the ACP, *D. citri* Kuwayama (Hemiptera: Liviidae), is a serious pest of citrus fruits, and transmits the phloem-limited bacteria (*Candidatus* Liberibacter asiaticus) causing Huanglongbing (HLB), better known as citrus greening disease[6,7]. HLB was first reported from Southern China and has spread to 40 different Asian, African, Oceanian, South and North American countries[7]. Citrus greening is a worldwide problem with threatening economic and financial losses due to citrus fruit loss; hence, management of ACP is considered of utmost importance to prevent and control HLB.

Measures for effective control of these insect pests usually requires the application of chemical pesticides that are relatively short lived. Chemical insecticide control strategies often lack safe environmental management strategies or have decreased susceptibility due to pesticide resistance in pest populations[8,9]. Hence, the biological control of pests with molecular biopesticides is a potential alternative, but its use globally remains modest, and various species of parasitoids (*e.g.*, *Trisolcus japonicus*) may also be effective as natural biological controls. RNAi is a potential emerging technology for managing invasive insect pests with molecular biopesticides[10]. RNAi is a well described gene regulatory mechanism that facilitates the effective posttranscriptional gene silencing of endogenous as well as invading dsRNAs in a sequence-specific manner, that

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eventually leads to the regulation of gene expression at the mRNA level[11,12]. Briefly, when exogenous dsRNA is internalized into a cell it is processed into siRNAs by a member of the bidentate nuclease RNase III superfamily, called Dicer, which is evolutionarily conserved in worms, flies, plants, fungi, and mammals[13,14,15]. These 21-25 nucleotide siRNA duplexes are then unwound and integrated in the RNA-induced silencing complex (RISC) as guide RNAs. This RISC-RNA complex allows Watson-Crick base pairing to the complementary target mRNA; this eventually leads to cleavage by the Argonaute protein, a multi domain protein containing an RNase H-like domain, which degrades the corresponding mRNA and reduces protein translation, thereby leading to posttranscriptional gene silencing[16,17,18].

RNAi for pest management requires the introduction of dsRNA *in vivo* to silence the gene of interest, thereby activating the siRNA pathway. Various methods that have been used for dsRNA delivery to insects and insect cells to induce systemic RNAi include feeding[10,19], soaking[20,21], microinjection[22], carriers such as liposomes[23], and other techniques[24]. RNAi was first demonstrated in *Caenorhabditis elegans* to silence *unc-22* gene expression by Fire and Mello[25], followed by knockdown in expression of the frizzled genes in *Drosophila melanogaster*[26]. Initial functional studies utilized microinjection to deliver dsRNA in insects, such as *Apis mellifera*[22,27], *Acyrthosiphon pisum*[28], *Blattella germanica*[29], *H. halys*[30], and lepidopteran insects (reviewed by Terenius *et al.*[31]). Microinjection is advantageous to deliver an accurate and precise dose to the site of interest in the insect. Albeit such septic punctures may elicit expression of immune related genes due to trauma[32], hence, ruling out its practicality in agricultural biopesticides development.

Another method of delivering dsRNA *in vivo* is by soaking, which involves ingestion or absorption of dsRNA by suspension of animals or cells generally in extracellular medium containing dsRNA. Soaking has been used to efficiently induce RNAi in *Drosophila* S2 tissue culture cells to inhibit Downstream-of-Raf1 (*DSOR1*) mitogen-activated protein kinase kinase (MAPKK)[20], as well as in *C. elegans* to silence the *pos-1* gene[33]. However, dsRNA delivered using soaking is less efficient to induce RNAi compared to microinjection[20]. RNAi mediated silencing in a chewing insect was first shown in the Western corn rootworm (WCR) (*Diabrotica virgifera virgifera*) by infusing the dsRNA into an artificial agar diet[10]. Earlier reports have summarized methods to deliver dsRNA infused in natural diets specific to arthropods[34]. These delivery methods were further determined to be comparably effective to artificial means of delivery; such as the case of the tsetse fly (*Glossina morsitans morsitans*), where equal knockdown of an immune-related gene was observed when dsRNA was delivered either through blood meal or microinjected[35]. Similarly, delivery of dsRNA through droplets in light brown apple moth (*Epiphyas postvittana*)[36], diamondback moth (*Plutella xylostella*) larvae[37], as well as honey bees[38,39] induced efficient RNAi. Most effective RNAi experiments in hemipteran have utilized injection of dsRNA[40] because oral delivery of dsRNA in hemipteran insects is arduous since it must be delivered through the host plant's vascular tissues. Effective RNAi was also observed in ACP and glassy-winged sharpshooter leafhopper (GWSS), *Homalodisca vitripennis*: dsRNA was delivered through citrus and grapevines that had absorbed dsRNA into the vascular tissues through root drench, foliar sprays, trunk injections, or absorption by cuttings[41,42,43,44,45,46]. This also resulted in the first patent for dsRNA against the ACP (2016, US 20170211082 A1). Delivery of siRNA and dsRNA using carriers such as nanoparticles and liposomes imparts stability, and increases in delivered dsRNA efficacy are rapidly emerging[23,47,48,49,50]. A new class of nanoparticle-based delivery vehicles for nucleic acids for *in vitro* and *in vivo* that was summarized specifically for therapeutic applications may impart immense potential as suitable delivery vectors[51]. Nanoparticles as a delivery vehicle for dsRNA may have disadvantages including solubility, hydrophobicity, or limited bioaccumulation[52], but a suitable polymer aiding delivery may compensate these disadvantages. Development and use of self-delivering nucleotides are also emerging called 'antisense oligonucleotides', which are single stranded RNA/DNA duplexes[46].

Vitellogenesis in arthropods is a key process controlling reproduction and regulated by juvenile hormone (JH) or ecdysone, which are the key inducers of Vg synthesis by the body fat; the Vg is eventually taken up by the developing oocyte via Vg receptor mediated endocytosis[53]. Vg is a group of polypeptides synthesized extraovarially, which is essential for development of the major egg yolk protein, vitellin[54,55], and therefore, it is important in reproduction and aging[56]. Vg has been successfully silenced in nematodes[57] as well as in honey bee (*Apis mellifera*) where RNAi mediated depletion of Vg was observed in adults and eggs[22]. RNAi mediated posttranscriptional gene silencing of Vg was tested because it was thought its depletion would lead to an observable phenotypic effect such as reduced fertility and fecundity, to potentially aid in BMSB control. The JHAMT gene that encodes the S-adenosyl-L-methionine (SAM)-dependent JH acid O-methyltransferase, catalyzes the final step of the JH biosynthesis pathway[58]. In this pathway farnesyl pyrophosphate (FPP) is sequentially transformed from farnesol, to farnesoic acid followed by conversion of methyl farnesoate to JH by JHAMT. This pathway is conserved in insects and arthropods specifically for metamorphosis, a process that is developmentally regulated by hormones[59,60,61]. In *B. mori*, JHAMT gene expression and the JH biosynthetic activity in the Corpora allata suggest that the transcriptional suppression of the *JHAMT* gene is crucial for the termination of JH biosynthesis[58]. Therefore, the *JHAMT* and *Vg* genes were selected for targeted depletion using RNAi. RNAi was also tested in citrus trees for control of ACP and GWSS. Citrus trees were treated with dsRNA through root drench, stem tap (trunk injections), as well as foliar sprays with dsRNAs against insect specific arginine kinase (AK) transcripts[42,44]. The topical application of dsRNA was detected all over the canopy of citrus trees, indicating efficient delivery through the plants vascular tissues, and resulted in increased mortality in ACP and GWSS[41,42,45].

In the current study, we have identified a natural diet delivery method for treatments such as dsRNA. This newly developed technique was subsequently used for silencing the JHAMT and Vg mRNA using gene specific dsRNAs in BMSB nymphs as demonstrated earlier[62]. These new delivery protocols demonstrated here supersede conventional RNA delivery systems that use topical sprays or microinjections. Vegetables and fruits, stem tap, soil drenching, and clay absorbents in may be used for delivery of dsRNA, which is critical to the continued development of biopesticide pest and pathogen management.

Protocol

1. BMSB Rearing

1. Rear BMSB insects as per standard lab practice and previously described[63].
2. Raise ACP (*D. citri*) insects on *Citrus macrophylla* in a glasshouse (22 °C) and natural light. Use adult ACP, at approximately 5-7 days post eclosion.

2. Selection of Gene Regions and *In Vitro* Synthesis of dsRNA

1. Select genes specific to BMSB from previously published transcriptome profiles[32].
2. Ensure the regions of interest selected vary between 200 to 500 base pairs.
3. Perform polymerase chain reaction (PCR) using the conditions described below to generate fragments associated with the selected gene of interest from genomic DNA. See **Table 1** for the gene-specific oligonucleotides.
    1. PCR reaction: In a 0.25 mL PCR tube, combine 5 µL of 10X PCR Buffer, 4 µL of dNTP Mixture (2.5 mM each), 2 µL of DNA template (50 ng/µL), 2.5 µL each of Primers 1 and 2 (10 µM), 0.25 µL of DNA polymerase (5 U/µL), and DNase/RNase free water up to 50 µL.
    2. PCR condition: Cycle the PCR reaction to amplify the region of interest at 95 °C for 3 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, 72°C for 1 min. Incubate the reaction at 72 °C for an additional 10 min. Purify the PCR reaction using a purification kit.
4. Amplify the obtained PCR fragments further with gene specific primers flanked with the T7 RNA polymerase promoter sequence (5'-GAA TTA ATA CGA CTC ACT ATA GGG AGA-3') as mentioned earlier[62].
5. Use LacZ gene as a negative control (mock) for RNAi.
   NOTE: LacZ is a gene that encodes β-galactosidase amplified from *Escherichiacoli* genomic DNA (the primers used are listed in **Table 1**).
6. Perform *in vitro* transcription to yield dsRNA as described earlier[62].
7. Dissolve and resuspend the resulting dsRNA in 150 µL DNase/RNase free water, measure the concentration, and store at -80 °C for future use.

3. Delivery of dsRNA Using Green Beans

1. Select early 4th instar BMSB nymphs hatched from the same egg mass and starve them for 24 h prior to dsRNA feeding.
2. Select slender certified organic green beans (*Phaseolus vulgaris* L.) and wash with 0.2% sodium hypochlorite solution for 5 min.
   NOTE: Slender green beans were selected so that the beans can easily be accommodated in the 2 mL microcentrifuge tubes.
3. Wash 3 times with ddH_2O and allow to air dry.
4. Trim the green beans from the calyx end to a total length of 7.5 cm using a clean razor blade.
5. Immerse the washed and trimmed green beans in a cap-less 2 mL microcentrifuge tube containing 300 µL of control solution (a 1:10 dilution of green food coloring (ingredients: Water, Propylene Glycol, Fd&C Yellow 5, Fd&C Blue 1, and Propylparaben as preservative)).
6. Make dilutions of the *in vitro* synthesized LacZ, JHAMT, or Vg dsRNAs by diluting 5 µg or 20 µg in 300 µL of RNase/DNase free water to yield final concentrations of 0.017 µg/µL or 0.067 µg/µL, respectively.
7. Immerse the washed and trimmed green beans in a cap-less 2 mL microcentrifuge tube containing 300 µL of dsRNA solution (from step 3.6).
8. Wrap and seal the edges of the microcentrifuge tubes enclosing the immersed beans to avoid evaporation of the dsRNA solution and to prevent the animals from entering the microcentrifuge tube.
9. Position these tubes in an upright manner at room temperature for 3 h to allow the dsRNA solution to be loaded throughout the green bean by capillary action.
10. Place these tubes in clean culture vessels (polypropylene). Place three starved 4th instar BMSB nymphs in the culture vessels.
11. Treat three animals per culture vessel each containing three green beans with green food coloring or dsRNA solution. Maintain the insects at 25 °C and 72% relative humidity, under a 16L:8D photoperiod in an incubator.
12. Allow the insects to feed on the green beans (immersed and absorbed with dsRNA) for 5 days but replenish with fresh diets of dsRNA treatment green beads after 3 days.

4. Real-time Quantitative (qPCR) Analysis of Gene Expression Following RNAi Mediated Silencing in BMSB

1. Measure the effect of RNAi on the levels of transcript expression by qPCR.
2. Isolate the total RNA from the dsRNA treated animals and synthesize the cDNA[62].
3. Setup the qPCR reactions using a real-time PCR system and the primers listed in **Table 1**. Use the following qPCR cycling condition: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, along with dissociation step including 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s.
4. Determine the qPCR standards: use the serial dilution of cDNA prepared from total RNA isolated from a normal animal as a reference standard for the quantification.
5. Use BMSB 18s RNA as an internal standard to correct for differences in RNA recovery from tissues[32].

5. Foliar Spray Application in Large Potted Citrus Trees and Seedlings

Note: Plants of the citrus cultivar '*Carrizo*' citrange (*Citrus sinensis XPoncirus trifoliata*, Rutaceae), were maintained in a glasshouse under natural light and temperature, grown in 1.2 L containers. The plants were constantly pruned to promote growth of new foliar shoots, called 'flush'. ACP prefers to feed and oviposition on the new growth of citrus[64].

1. Select plants or seedlings and do not water them for 2-3 days prior to use to let the soil dry out to damp but not completely dry.
2. Using a hand pump spray bottle apply a 200 mL of dsRNA solution (0.5 mg/mL) to the lower canopy (**Figure 4A**).
   NOTE: Prepare the above mentioned dsRNA solutions in DNase/RNase free water.
3. Post-spray application, allow the applied dsRNA solution to be completely absorbed by the leaves.
   NOTE: Citrus trees absorbed the applied dsRNA, and then leaves from either new growth or from branches that were covered prior to application, were extracted; the dsRNA was detectable using qPCR and showed systemic movement into the tree top canopy leaves in 3-4 h[44,45,46].

4. Sample the new growth from previously topped trees after 25-40 days by collecting approximately 10 leaves from the tips of four branches. Extract the total RNA and analyze it by reverse transcription PCR (RT-PCR) and qPCR for the presence of the applied dsRNA trigger using the primers listed in Table 1 and previously described methods[46].
   NOTE: The sample collection, total RNA isolation, RT-PCR, and qPCR were performed as previously described[46].
5. Similarly, topically spray 10 mL dsRNA to the lower region of a seedling or small potted tree foliage.
   NOTE: Hemipteran insects (ACP and GWSS) were given feeding access normally at 24 h, post treatments on new growth (leaves) which had not been directly sprayed, or which grew weeks later, or whole plants. This produced insects which tested positive for the dsRNA at 3, 6, and 10 days, post feeding.

6. Soil/Root Drench Application in Large or Small Potted Citrus Trees and Seedlings

1. Select plants or seedlings and do not water them for 2-3 days prior to use to let the soil dry out to damp but not completely dry (this creates air space to hold the liquid solution to be applied).
2. Add 1 L of dsRNA solution (0.2 mg/mL) to the soil of large potted plants (approximately 2.5 m) and add 1 L of water (chaser) after 1 h.
3. Apply 100 mL of dsRNA solution (1.33 mg/mL) to the soil of 1 m tall potted trees in partially dry soils.
4. For small seedlings, apply 10 mL of dsRNA solution (1 mg/mL) to the soil in the cones or to the bare roots (**Figure 4B, C**).
5. Allow the plants that receive the dsRNA solution applied as a soil drench to soak for 30 min. Then apply plain water only treatment to aid absorption by roots (20 mL for plants in yellow containers, or 100 mL if larger plant pots are > 1 gallon).
   NOTE: Topically applied dsRNA to foliage resulted in detection at most distal tips of branches within 3-6 h post treatments, showing systemic movement through trees. New growth branches tested positive for dsRNA at 60-90 days post treatments. Cuttings are provided to insects (ACP and GWSS) in a dsRNA feeding bioassay[44].

7. Stem Tap (Tree Trunk Injection) Application in Large Potted Citrus Trees and Seedlings

1. Select citrus seedlings, new, or approximately 3.5 years old plants for injecting dsRNA using the stem tap (trunk injections) method.
2. Drill holes in the citrus plants using a drill and a 10 mm drill bit, taking care not to exceed 2 cm, or about half the diameter of the stem.
3. Wrap the copper tip of each injector 4-6 times with a 0.6 cm (¼ inch) wide strip of sealing film to prevent leakage near the tip.
4. Fill the tree trunk injectors with 6 mL (1.7 mg/mL) of dsRNA solution diluted in DNase/RNase free water (denoted here as colored solution).
5. Inject the solution into the trunk of the tree and leave the injector in the trunk for 6-10 h to allow absorption of the dsRNA solution. Allow the insects to feed on the cuttings from treated trees at 3, 10, and 30 days post treatment.
   NOTE: The dsRNA injected using the trunk injection method persisted in the trees for a period of 30-60 days[41,42,44]. Validation for RNAi was performed by qPCR using the primers listed in **Table 1**.

8. dsRNA Treated Clay Granules for Delivery to Insects Through Soil

1. Pour out the clay absorbent into a 50-mL conical tube to the 35 mL mark (approximately 30 g of clay absorbent) on the tube.
2. Pour 20 mL of dsRNA solution diluted in DNase/RNase free water (100 µg/mL) into the tube to wet all the absorbent. Cap the conical tube, tip the tube to help remove air, and cap the tube.
3. Place the tube upright and let it stand for 1-2 min for the clay particles to absorb the solution.
4. Add enough dsRNA-soaked clay into the soil mix to fill a 1 gallon pot.
5. Mix and turn the soil by hand to thoroughly mix the dsRNA-soaked clay into the soil.
6. Use this soil to repot seedlings selected for the dsRNA treatment.
7. Water the soil with 200 mL of plain water without dsRNA. After 30 min to 1 h, follow with 100 mL of plain water. After 24 h, put the plant on a normal watering schedule.
8. Test 4-6 leaves of the treated potted plants with clay absorbents and dsRNA each month post treatment for dsRNA by collecting the most apical leaves of new plant growth.
   NOTE: Plants or cuttings from these treated plants are fed to insects any time after 24 h post treatment and have been able to delivery RNAi for up to a year to insects (unpublished data).

Representative Results

Vegetable mediated dsRNA delivery through feeding in BMSB 4th instar nymphs was tested for the development of molecular biopesticides using RNAi for invasive insect pests. BMSBs feed using their needle-like stylets by a mechanism known as lacerate and flush, which causes considerable damage to crops. Slender organic green beans, *P. vulgaris* L., were used to test if nutrients or dsRNA could be delivered *in vivo* to BMSB through feeding[3]. Segments of green beans were immersed in DNase/RNase free water or a solution of water and green food coloring to test delivery in BMSB (**Figure 1A**). The green food coloring was used as a visual indication to imitate dsRNA. The vascular tissue of the green beans were saturated with green food coloring due to the flow of green colored solution through the phloem by capillary action[62]. BMSB nymphs were seen feeding on the segments of green beans by inserting their stylets into the vascular tissues of the green beans (**Figure 1B**). Green colored excreta droplets were observed after days 2 and 3 in insects that were fed on beans saturated with green food coloring indicating the delivered material had been ingested orally and passed through the gut before excretion (**Figure 1C**, **D**).

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Subsequently we tested if significant depletion of targeted gene expression was accomplished using the green bean mediated delivery of BMSB specific dsRNA through ingestion in BMSB. Green bean segments were immersed and absorbed a solution of either 0.067 µg/µL (20 µg in 300 µL of DNase/RNase free water) or 0.017 µg/µL (5 µg in 300 µL of DNase/RNase free water) of *in vitro* synthesized dsRNA specific to BMSB JHAMT and Vg, respectively. Green beans were also immersed in water alone, 0.067 µg/µL or 0.017 µg/µL LacZ dsRNA (Mock) as respective controls. Transcript levels were evaluated using qPCR indicating that expression of JHAMT and Vg mRNA was significantly reduced *in vivo* by almost 4.5- and 2.2-fold, respectively (**Figure 2A**, **B**). Consequently, the results indicate that dsRNA may be delivered through the vascular tissues of green beans to induce successful RNAi.

Another hemipteran pest, the Harlequin bug (HB) (*Murgantia histrionica*), that causes damage to cole crops were also tested if successful delivery of nutrients or dsRNA treatments could be delivered using the vegetable mediated delivery. The 4th instar HB nymphs were allowed to feed on baby collard greens (*Brassica oleracea var. viridis*) immersed in either water or a solution of water with green food coloring. Results indicated that HB fed and ingested the green food coloring, which was apparent from their green colored excreta, as compared to HB that ingested the vegetables immersed in water, which had clear excreta (**Figure 3**).

Additional techniques for the transient delivery of dsRNA of spraying or root/soil soaking results in dsRNA uptake in all of the plant tissues[65,66]. Sprayable RNAi-based products are in development and may be available soon pending approval. Delivery of dsRNA in the fields using sprays or root drench may aid in managing invasive insects[42,67]. Full-sized citrus trees and seedlings were exposed to the dsRNA either by foliar spray, or by soil or bare root drenching, respectively (**Figure 4**). Results indicated that dsRNAs delivered by either sprays or soil/bare root drench could be detected in Citrus plants (2.5 m tall) for 7 weeks post a single exposure of 2 g of dsRNA[42]. Delivery and ingestion of psyllid dsRNA-AK (20 ng/µL) increased psyllid mortality by 30-45% (**Figure 4D**)[42].

In vitro transcribed dsRNA can be efficiently delivered to polyphagous insects using stem tap (trunk injections) of gene specific dsRNA directly into the vascular tissues of the plant, which can be acquired by the insects when preying on such plants[44] (**Figure 5**). For citrus trees that were exposed to the dsRNA by trunk injections, the dsRNA could be detected in aged citrus plants (approximately 1 m tall) for 7 weeks post a single exposure using 6 mL of 1.7 mg/mL of dsRNA in a solution of DNase/RNase free water (**Figure 5A**, B). Phloem-feeding hemipteran insects were then allowed to feed on these host plants treated with dsRNA. It is assumed that the dsRNA was successfully infused and moved through the vascular system of the citrus plants for ingestion by ACP, which demonstrated mortality when fed on dsRNA-AK[42].

Bioassays developed from 2008 to 2012, have been screened across a wide variety of potted plants and shown to provide dsRNA delivery in a manner by which plants can absorb and translocate the dsRNA for systemic dsRNA dissemination[41,42]. One method uses a clay absorbent component with dsRNA adsorption into the clay matrix; this includes a wide variety of clays, Fuller's Earth, Zeolite, and others, as well as other absorbent materials, cellulose, agars, bioplastics, *etc.* Clay particles are dust free nucleic acid carriers that may be used for delivering active ingredients such as dsRNA to soils for plant uptake and ultimately delivery to insects (**Figure 6**). The clay complex can also be chemically configured to release the dsRNA under specific pH or ionic conditions. Clay delivery methods of dsRNA into plants have been shown to provide dsRNA into potted citrus trees, and other plants for over 14 months (data not shown). This delivery system can be used to alter plant traits, such as flower colors, plant height (dwarfing), or others. Currently, the primary use of this method is for the development of an effective insect pest and pathogen (virus) control or management. The approach can be cost effective for nurseries and homeowners, and is a non-transgenic method.

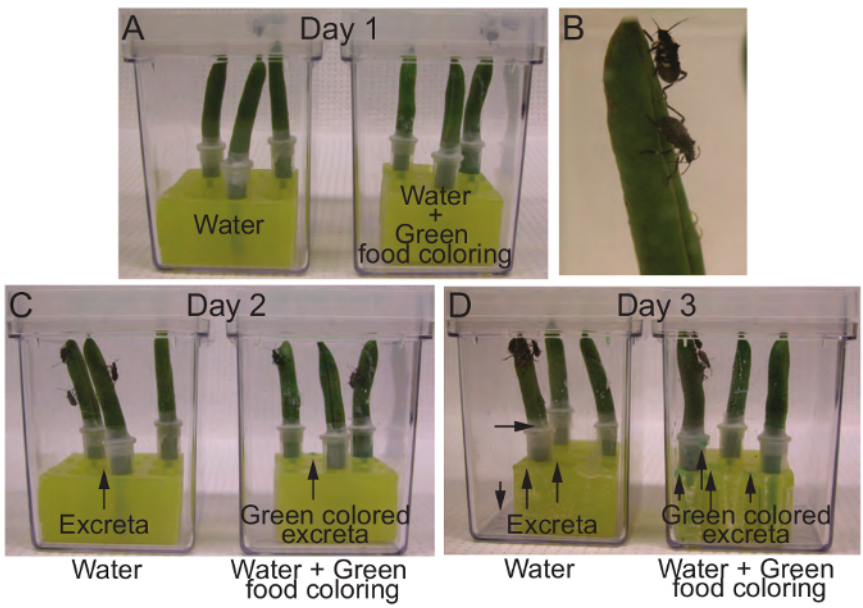

Figure 1. Delivery of nutrients or dsRNA through green beans. (**A**) Segments of slender organic green beans were immersed and absorbed the solution in a 2 mL microcentrifuge tube containing 300 µL of either ddH_2O alone or ddH_2O with green food coloring, for 3 h. Three 4th instar BMSB nymphs were starved for 24 h and placed in culture vessels along with 3 green beans per vessel. (**B**) BMSB feed on segments of green beans immersed in water by piercing through the green beans to reach the nutrients with their stylets. (**C**) Day 2 of the BMSB feeding bioassay, arrows denote excreta. (**D**) Day 3; Increased BMSB excreta (denoted by arrow) observed post ingestion of a solution of ddH_2O and green food coloring through green beans. Please click here to view a larger version of this figure.

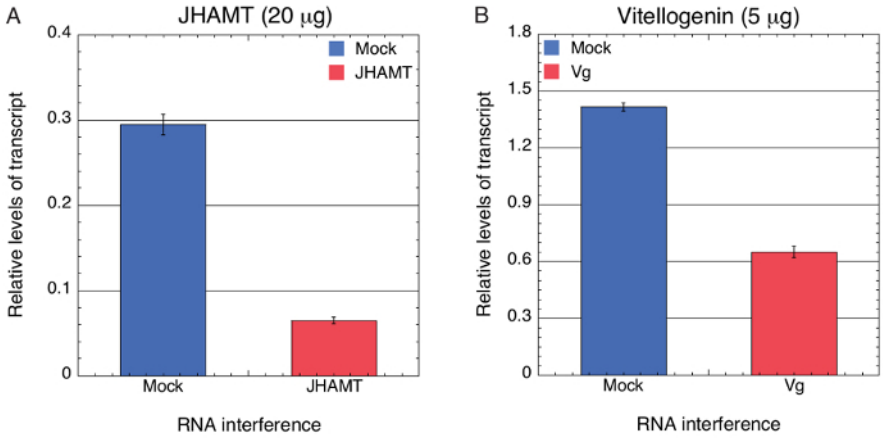

Figure 2. Quantitative RT-PCR analysis to measure the level of transcript after RNAi-mediated depletion of JHAMT and Vg in BMSB. Total RNA from 3 individual BMSB 4th instar nymphs fed on JHAMT (**A**) 20 µg (0.067 µg/µL), and *Vg* (**B**) 5 µg (0.017 µg/µL) dsRNAs in 300 µL of ddH_2O delivered through segments of green beans, was isolated and the transcript levels were measured by qPCR. LacZ dsRNA (Mock) served as a negative control. BMSB 18s RNA was used as an internal standard to correct for differences in RNA recovery from tissues. Results reported are from three biological replicates, and error bars indicate SEM. A one-way analysis of variance (ANOVA) was performed to test for statistical significance of data, $p < 0.0001$. Results reproduced from Ghosh *et al.*[62] Please click here to view a larger version of this figure.

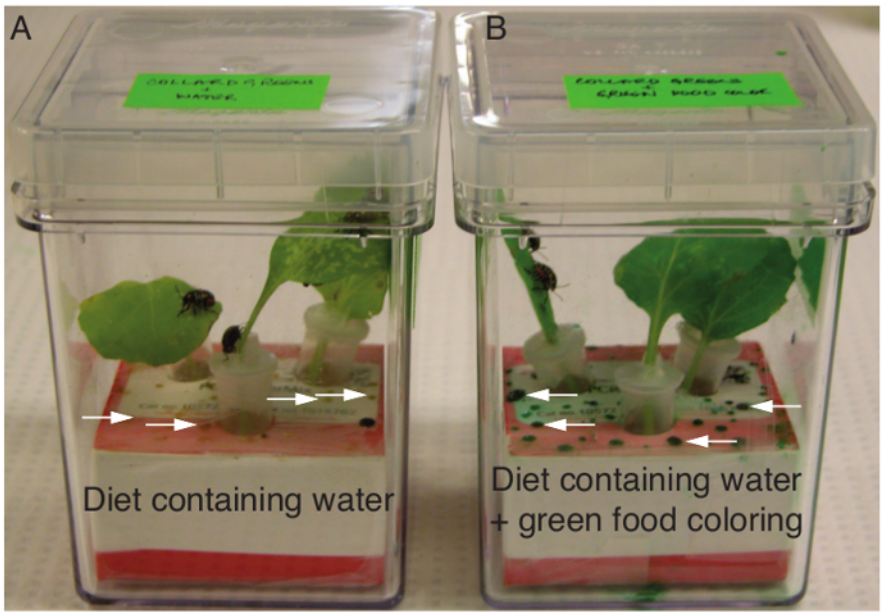

Figure 3. Oral delivery of treatment in Harlequin bug (*M. histrionica*) using baby collard greens. Organic baby collard greens were washed with 0.2% sodium hypochlorite, trimmed, and immersed in a 2 mL cap-less microcentrifuge tube containing 300 µL of solution of (**A**) DNase/RNase free ddH_2O, or (**B**) DNase/RNase free ddH_2O solution with green food coloring, for a period of 3 h. Three 4th instar HB nymphs were starved for 24 h and then placed in culture vessels and allowed to feed on these collard greens for 3 days. White arrows indicate excreta observed on day 3 post feeding. Please click here to view a larger version of this figure.

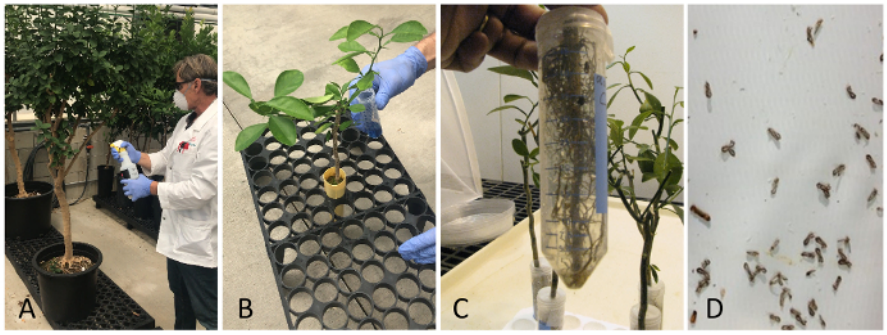

Figure 4. Foliar spray and root drench application in citrus trees and seedlings. The selected plants or seedlings prior to use are not watered for 2-3 days to let the soil dry out to damp but not completely dry. (**A**) The trees were first topped and 200 mL of dsRNA solution (0.5 mg/mL) in DNase/RNase free water was applied by hand pump spray bottle to the lower canopy. (**B**) 100 mL of dsRNA solution (1 mg/mL) in DNase/RNase free water was applied to the soil of seedlings in partially dry soils. (**C**) 100 mL of dsRNA solution (1 mg/mL) in DNase/RNase free water was applied to the bare roots of seedlings for approximately 3 h. (**D**) ACP feeding on seedlings that had absorbed dsRNA by the bare roots, showed increased ACP mortality. Please click here to view a larger version of this figure.

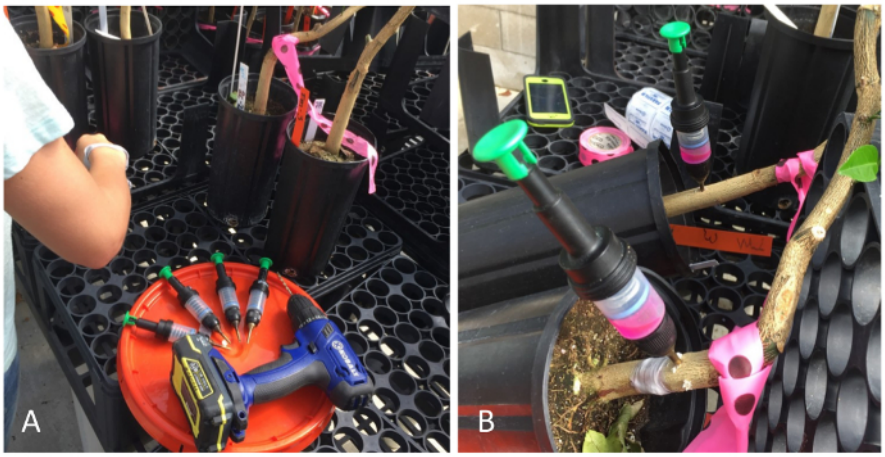

Figure 5. Citrus Seedling tree trunk injection. Citrus seedlings approximately 1 m tall were injected with 1.7 mg/mL of dsRNA. (**A**) Drill holes in citrus seedlings using a 10 mm diameter drill bit to insert the injector into the stem of the plant. The exposed copper tip of each injector was wrapped with a 0.6 cm (¼ inch) wide strip of sealing film to prevent leakage. (**B**) Injectors filled with 6 mL of colored solution were applied to the stem (trunk) of the seedlings. Injectors were left in place for approximately 6-10 h for complete uptake of the solution. Please click here to view a larger version of this figure.

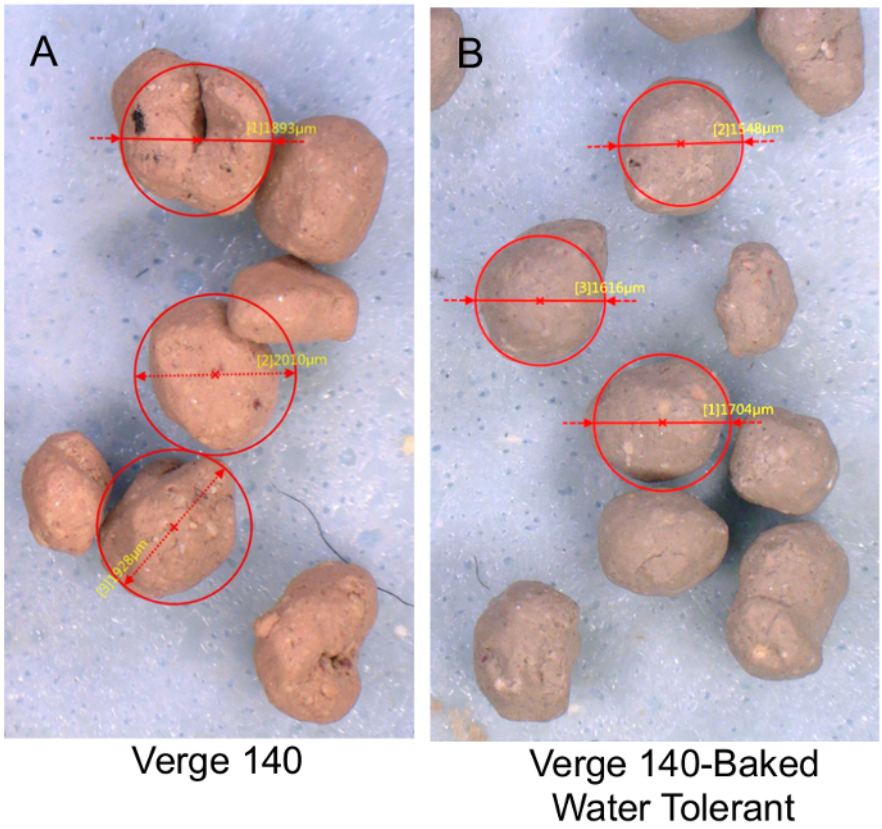

Figure 6. Clay soil amendment delivery of dsRNA into plants through soil. A new line of delivering material: clay that is a dust free nucleic acid carrier for use in delivering active ingredients such as dsRNA to soils for uptake in plants and ultimately into insects. (**A**) Unbaked clay and (**B**) baked clay depicting water tolerance/retention. Please click here to view a larger version of this figure.

Potential H. halys target genes		
Accession	Size	Gene Name/Homology
XP_014293026.1	491	*Vitellogenin-A1-like* (Vg) (Possible isoforms: *vitellogenin-2-like* isoform X1 XP_014291483.1; *vitellogenin-2-like* isoform X2 XP_014291484.1).
XP_014290953.1	545	*Juvenile hormone acid O-methyltransferase-like (JHAMT)* (Possible homolog: juvenile hormone acid O-methyltransferase XP_014283772.1).
Primers		
PCR		
Gene Name/Homology	Primer Name	Sequence
Vg	BMSB Vitellog P2 F	CAATTTGATCCACCGACTGTT
Vg	BMSB Vitellog P2 R	CCGCATGAATCTTACTCTGGA
JHAMT	BMSB JH P1 F	GGATGCTTATGAATAATCCAG
JHAMT	BMSB JH P1 R	GTATAGGATTGCCATTTTGG
T7 PCR		
Vg	T7 BMSB Vitellog P2 4263 F	GAATTAATACGACTCACTATAGGGAGACCAAAGTTGGAAGGGAATGA
Vg	T7 BMSB Vitellog P2 4753 R	GAATTAATACGACTCACTATAGGGAGACCGCATGAATCTTACTCTGGA
JHAMT	BMSB JH T7 P1 F	GAATTAATACGACTCACTATAGGGAGAGGATGCTTATGAATAATCCAG
JHAMT	BMSB JH T7 P1 R	GAATTAATACGACTCACTATAGGGAGAGTATAGGATTGCCATTTTGG
LacZ	T7 LacZ RNAi F	GAATTAATACGACTCACTATAGGGAGATGAAAGCTGGCTACAGGA
LacZ	T7 LacZ RNAi R	GAATTAATACGACTCACTATAGGGAGAGCAGGCTTCTGCTTCAAT
AK	dsAK-F	TAATACGACTCACTATAGGGAGTGGCATTCTTGTATGGCGTA
AK	dsAK-R	TAATACGACTCACTATAGGGAGGCCTGCAAGAATCTGTCTCC
AK	dsAK 50-F	TAATACGACTCACTATAGGGAGTGGCATTCTTGTATGGCGTA
AK	dsAK 50-R	TAATACGACTCACTATAGGGAGTGAAGCCCTTGTGGTAGTC
AK	dsAK 30-F	TAATACGACTCACTATAGGGAGACCCGGACTCTGGAGTAGG
AK	dsAK 30-R	TAATACGACTCACTATAGGGAGGCCTGCAAGAATCTGTCTCC
GFP	dsGFP-F	TAATACGACTCACTATAGGGAGCCAACACTTGTCACTACTTTCTCTT
GFP	dsGFP-R	TAATACGACTCACTATAGGGAGGTAATGGTTGTCTGGTAAAAGGA
qPCR		
Vg	RT Vitellog P2 F	TTGATAGTTGTTTGGATTTTGAAGGT
Vg	RT Vitellog P2 R	TCTTACTTGATCAGCGCTCAGAA
JHAMT	BMSB JH RT P1 F	AGGAAAACCCAAAATGGCAAT
JHAMT	BMSB JH RT P1 R	ATGTATTCTTCTTTTGGATCTTTTCTTGAG
18S	BMSB 18S F3	ATGCCCCCGCCTGTCCTTATT
18S	BMSB 18S R3	TGAAAGCAGCCTGAATAGTGG
GFP	GFP-F	GGTAAAAGGACAGGGCCATC
GFP	GFP-R	TCAAGGAGGACGGAAACATC
AK	AK quant-F	CGGACTTGAGGGAGAACTGA
AK	AK quant-R	GTGGTAGATACCGCGACCAG
a-Tub	a-Tub-F	GCGTCTCTTCGGTTTGACGG
a-Tub	a-Tub-R	CACTTCACCATCTGGTTGGC

Table 1. Oligonucleotide sequences for RNAi. Listed are the genes and oligonucleotides used for generating PCR fragments, dsRNA, and qPCR primers to analyze the transcript levels.

Supplementary Video 1: Agars and gels as absorbents for delivery and sustained release of dsRNA. Hydrated synthetic or natural agars and gels with dsRNA solution that may be used as bait or diets for various arthropods. Please click here to download this video

Discussion

RNAi has proven to be an important tool for exploring gene biological function and regulation, with great potential to be utilized for management of insect pests[19,68,69,70,71]. The design and selection of an appropriate gene(s) for silencing in a given insect species and the method of delivery of the corresponding dsRNA(s) to the insect are both of utmost importance. The optimal method for delivering dsRNA into an insect must be determined empirically, along with the relative dose selection for delivery, as certain methods may offer advantages and other limitations. For the development of a molecular biopesticide that may ultimately be suitable for environmental release, a feasible, efficient and advantageous delivery method is required. For example, topically applied dsRNA has shown to be effective for dsRNA delivery for insect control to citrus and grapevines[42,44]. Innovative strategies such as use of baits, sucrose solutions, yeast or bacterial dsRNA production for direct ingestion, topical application of dsRNA onto plants, or production of specific dsRNAs by modified transgenic plants, have advanced the development of effective RNAi-based controls[19,72].

A good example is demonstrated here with the use of green beans to deliver dsRNA (**Figure 1** and **Figure 2**) designed to specifically impact and reduce an insect pest of global importance. The newly developed vegetable mediated dsRNA delivery protocol using segments of green beans immersed and saturated with dsRNA has been used to effectively target genes specific for larval development in BMSB. Green beans are one of the many vegetables that are devoured by BMSB and so these were used as a medium to deliver dsRNA. Other mediums of dsRNA delivery were tested (data not shown) but were unsuccessful in supplying nutrition to the BMSB possibly due to differences in vascular texture. Therefore, we used segments of green beans to deliver *in vitro* synthesized dsRNA to the BMSB nymphs. The nucleic acids delivered through a plant or vegetable mediated technique may potentially be able to induce RNAi in the insect of choice as observed here with depletion of *JHAMT* and *Vg* genes in BMSB (**Figure 2**)[62].

This vegetable mediated dsRNA delivery protocol has been used successfully to induce RNAi not only in BMSB but also in HB using collard greens (**Figure 3**), though delivery strategies and methods must be optimized for each gene and insect. Hence, it is recommended that various methods be tested for delivery as well as to target multiple genes of interest individually or by stacking at least two dsRNAs in order to obtain better phenotypic penetrance. Several methods have been summarized for dsRNA delivery to insects and insect cells including feeding[13,22], soaking[73,74], microinjection[75], and other techniques[76] used for dsRNA uptake to induce systemic RNAi. Newer methods for oral delivery of dsRNA to induce RNAi in other insects by ingestion on treated non-transgenic plants also have been explored (**Figure 4**, **Figure 5**, and **Figure 6**). Citrus trees have been demonstrated to absorb dsRNAs either through roots, stem tap (trunk injections), or foliar sprays[42,44]. Previously, citrus trees and mature grapevines have been treated with insect specific dsRNA corresponding to the AK gene in both ACP and GWSS. These treated plants caused an increase in mortality for both the ACP and GWSS for up to various lengths of time[41,45]. Together these results demonstrate that dsRNA can impede expression of specific genes in the studied insects.

Challenges that affect successful silencing of gene expression include unique groups of dsRNA nucleases (dsRNases) expressed primarily in the gut tissue of insects and salivary secretions, or the gut pH which, may be responsible for degradation of dsRNA[47,77,78]. However, to overcome such degradation, systemic RNAi may be induced efficiently in insects by utilizing higher concentrations of dsRNA and/or using PEG in the diet for oral delivery of gene-specific dsRNA to overcome such degradation[62,79]. Delivery and uptake of dsRNA may also be stabilized with the help of carrier molecules, such as nanoparticles[80] like Chitosan[48], liposomes like Lipofectamine 2000 and Metafectene[76], polyethylene glycol[79], clay nanosheets[81], and carbon quantum dot[50]. Research is in progress to improve the effective delivery of dsRNA using agar and gel pieces infused with gene specific dsRNA (data not shown, see **Supplemental Video 4**). This technique may be used for delivery of an effective dose of dsRNA to ants that can carry the dsRNA infused resin or agar to the nest as food.

In addition to stability of dsRNA, long-term persistence of dsRNA in plant tissues is of importance, especially for agricultural crops. dsRNA distributed through vascular tissues of plants, fruits, or vegetables may accumulate in the xylem and phloem with reduced enzyme activity prior to being ingested by insects to induce the RNAi mechanism. It may be necessary for certain applications to have dsRNA persist for a short period, such as for 6 days in green beans or longer, for 36 h in the soil[62,82] so that the accumulation of nucleic acids in the environment becomes unlikely. However, Neena *et al.* demonstrated that nanosheets could protect large dsRNAs from premature degradation as well as mediate their sustained release on leaf surfaces over a period of at least 30 days[81].

Overall the dsRNA delivery strategies discussed here could be used to silence insect specific genes for pest management. Supply of dsRNA to plants through irrigation water, root drench, or trunk injection could be an effective strategy for pest insects, such as root feeders, for which no efficient control method is currently available. Delivery of dsRNA using a carrier or stabilizing medium such as clay or agar may be directly added to the soil for uptake by plants. One of the main causes for slow progress in the development of RNAi mediated molecular biopesticides has been the lack of efficient oral delivery techniques to initiate efficient RNAi and stability under environmental conditions[83]. The oral delivery methods outlined here may in the future be used for delivering nucleic acid mediated gene silencing treatments to plant sap feeding as well as chewing insects to reduce insect damage in global food production and develop sustainable ecological pest management.

Disclosures

The authors have nothing to disclose.

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