Emerging strategies for RNA interference (RNAi) applications in insects

Raja Sekhar Nandety1, Yen-Wen Kuo1, Shahideh Nouri1, and Bryce W Falk*
Department of Plant Pathology; University of California; Davis, CA USA

1These authors equally contributed to this work.

Keywords: hemipterans, insects, next generation sequencing (NGS), RNA interference (RNAi)

RNA interference (RNAi) in insects is a gene regulatory process that also plays a vital role in the maintenance and in the regulation of host defenses against invading viruses. Small RNAs determine the specificity of the RNAi through precise recognition of their targets. These small RNAs in insects comprise small interfering RNAs (siRNAs), micro RNAs (miRNAs) and Piwi interacting RNAs (piRNAs) of various lengths. In this review, we have explored different forms of the RNAi inducers that are presently in use, and their applications for an effective and efficient fundamental and practical RNAi research with insects. Further, we reviewed trends in next generation sequencing (NGS) technologies and their importance for insect RNAi, including the identification of novel insect targets as well as insect viruses. Here we also describe a rapidly emerging trend of using plant viruses to deliver the RNAi inducer molecules into insects for an efficient RNAi response.

Biogenesis of Small RNAs

MicroRNAs

Mature miRNAs are single-stranded RNAs (ssRNAs) of ca. 22 nucleotides in length and are generated from endogenous primary transcripts by the action of 2 RNase III type nucleases, Drosha and Dicer (Fig. 1).6 The mechanisms of miRNA biogenesis differ slightly in insects vs. plants, here we discuss insects. The primary transcripts (pri-miRNAs) are generated by RNA polymerase II (Pol-II) and contain a characteristic stem-loop structure. In one of the maturation steps of miRNA processing, the stem-loop of the pri-miRNA gets excised by RNase III type activity of Drosha while still in the nucleus. The cleavage by Drosha along with a co-factor Pasha (DGCR8) results in the generation of a precursor microRNA (pre-miRNA).7,8 The pre-miRNAs are exported into the cytoplasm by the nuclear transport receptor family of proteins, exportin 5 by cooperative binding and association with GTP–bound form of the cofactor Ran.9,10 The pre-miRNAs that are released by GTP hydrolysis in the cytoplasm, are further processed by Dicer enzymes into ca. 22 nucleotide microRNA duplexes.11 Dicer (Dcr 1) cleavage is followed by the loading of miRNA duplexes into Argonaute proteins (Fig. 1). The 5’ terminal nucleotide was shown to influence the association of miRNAs or siRNAs (see below) with specific Argonaute proteins and thus the loading into RNA-induced silencing complex (RISC).12 This difference in the 5’ terminal nucleotide leads to loading of miRNAs into different Argonaute proteins; a 5’ uridine containing miRNA loads into AGO1.

Small interfering RNAs

Small interfering RNAs (siRNAs), like miRNAs, are dependent on Dicer processing and are ca. 19-24 nucleotides in length. The primary origins of siRNAs are from dsRNAs including virus-derived dsRNAs in the cytoplasm (Fig. 1).13 These RNAs are derived from endogenous transcripts, sense-antisense transcript
The siRNAs are specifically associated with the Argonaute 2 (AGO2) protein. In *Drosophila*, Heat-shock protein 90 (HSP90) is required for AGO 2 to receive the siRNA duplex from the RISC loading complex in RNAi suggesting an interaction between the 2 protein components. The endo-siRNA pathways in plants and some other eukaryotes also have signal amplification polymerases, RNA dependent RNA polymerase (RdRP), that are absent in insects and most metazoans. Small interfering endo-siRNAs are the result of processing by Dicer nuclease enzyme II (Dicer 2) compared to Dicer 1 for miRNAs. Interestingly, the insect antiviral RNAi defense pathway also uses the same inherent siRNA pathway to detect viruses and to induce an antiviral response. RNAi pathway mutants for Dicer 2 and, AGO 2 in *Drosophila* were shown to be highly sensitive to RNA virus infection, which correlated with an increase in virus RNA accumulation. Dicer 2 was shown to process viral dsRNAs in the cytoplasm into ca. 21 nucleotide virus-derived small RNAs (vsRNAs). Several studies have recently confirmed the presence of vsRNAs in virus-infected insects and cell lines, thus confirming the role of Dicer protein in processing the virus dsRNAs. The resulting siRNAs with 5’ adenosine

Figure 1. RNAi pathways in insects. The figure shows the biogenesis pathways for siRNAs, miRNAs and piRNAs in insects. In the siRNA pathway, the RNA transcript from a DNA construct encoding hairpin loop sequences, or from a virus-derived dsRNA will trigger siRNA generation in the cytoplasm, resulting from the recognition and dicing by Dicer 2. The siRNA duplexes will then load into the AGO 2 - RISC complex. The guide strand of siRNA is maintained in the RISC and will lead it to the target mRNA for degradation/cleavage or translation repression. For endogenous miRNAs, pri-miRNA is transcribed from genomic DNA, and processed by Drosha and cofactor Pasha (DGC8) into the pre-miRNA. Next, the pre-miRNA will be transported into cytoplasm by exportin 5 and Ran-GTP, and cleaved by Dicer 1 into miRNA duplexes. Similar to siRNAs, the miRNA duplexes will incorporate into the AGO 1 - RISC complex, followed by target mRNA cleavage/degradation or translation repression. In the piRNA pathway, piRNA precursors are transcribed from piRNA clusters in the genomic DNA sequences. The antisense transcript could transport into cytoplasm, processed by series of proteins of the Piwi-group, in Zuc dependent or independent manner and incorporated into the PIWI-RISC complex, or into the AUB-RISC complex in the ping-pong cycle. The sense strand, which is targeted by the antisense strand in AUB-RISC, will then be cleaved and incorporated into AGO 3 to target the antisense strand; hence the ping-pong cycle.
load into AGO2 and those with a 5’ cytidine load into AGO5.12 Similar to miRNAs, the duplex siRNAs are loaded into the RISC, followed by the retaining of the guide strand. RISC is then directed by the guide strand which determines the mRNA target.

Piwi-interacting RNAs
Piwi-interacting RNAs (piRNAs) are generally ca. 24 to 30 nucleotide long, endogenous, germ-cell-specific small RNAs that are distinct from miRNAs and siRNAs based on their size and means of biogenesis.4 The primary function of piRNAs is to silence transposable elements (TEs) and their role is highly conserved among many eukaryotes. They are derived from distinct transposable elements that are referred to as piRNA clusters, in a Dicer-independent manner.25 These piRNAs from each locus are characterized by a complex mixture of sequences spanning large portions of the transposons. They were previously referred to as repeat-associated siRNAs (rasiRNAs) due to their association with the repeat elements.6,26 The Piwi group of proteins mainly consists of 3 subclasses; PIWI, Aubergine (AUB) and AGO 3, and the small RNAs that associate with Piwi proteins are thus named piRNAs. These piRNAs from piRNA clusters are transcribed in the sense or antisense direction and the long single-stranded RNA serves as the basis for piRNA production.

There are divergent biogenesis pathways that are important for piRNA production. In one of the pathways, the long transposon transcript is sliced by zucchini (Zuc) nuclease that probably results in the generation of the 5’ ends of primary piRNAs (Fig. 1). In a ping-pong cycle, mature sense primary piRNAs guide Piwi clade proteins to complementary sequences on antisense transcripts from the same piRNA cluster. Piwi proteins, due to their slicer activity, cleave the target antisense transcript to generate a new 5’ end. These newly generated 5’ ends are bound by another Piwi protein. Subsequently, the 3’ ends are trimmed to the length of the mature piRNAs. In Drosophila, 2 Piwi proteins AUB and AGO 3 cooperate in the secondary piRNA production to generate sense and antisense piRNAs (Fig. 1).

AUB- and PiWI-associated piRNAs are mainly derived from antisense transcripts of retrotransposons, whereas AGO3-associated piRNAs are derived from sense transcripts. AUB- and PiWI-associated piRNAs show strong preferences for uracil at their 5’ terminal ends, while AGO3-associated piRNAs mostly have adenine at nucleotide 10, with no bias at their 5’ termini. In another divergent pathway, primary piRNAs that get loaded specifically on to PIWI, also generate secondary piRNAs that were found in nucleus and induce nuclear RNAi of targets (Fig.1). Both piRNAs and siRNAs are 2’-O-methylated by small RNA 2’-O-methyl transferase across eukaryotes. This methylation protects them from 3’ uridylation and degradation.27

RNAi Activity as a Gene Regulation and Defense Strategy in Insects
RNAsi activity in insects is achieved by all 3 classes of small RNAs, although they each show different specific activities. Some RNAi activities are important for natural gene regulation events and play important roles in insect development. For example, RISC, directed by the miRNA guide strand, locates target miRNAs containing specific nucleotide sequences complementary to the guide, binds to these sequences and blocks translation of the mRNA target. Recent functional studies have shown the regulation of ecdysone receptor (EcR) in an isoform specific manner by miRNAs in the Bombyx mori,28 and studies in the hemipteran insect, Nilaparvata lugens, have shown the critical role of the conserved miRNAs miR-8-5p and miR-2a-3p, for ecdysone-induced chitin biosynthesis.29

But RNAi activity in insects also includes several triggers and substrates that function to silence the expression of certain host endogenous genes along with parasite and pathogen invaders such as viruses and mobile genetic elements including transposons.6,30 Insects respond to a variety of external stimuli, both biological and non-biological stresses, much like plants and other animals, through the generation of siRNAs and miRNAs. The generation of vsRNAs results because insects use RNAi activity as an anti-viral defense against both RNA and DNA viruses.31-34 Abundant vsRNAs result during virus infections, and recent studies using next generation sequencing (NGS) approaches have demonstrated that it is possible to identify sufficient vsRNAs to assemble complete virus genomes.23,35 However, it is important to note that the resulting populations of vsRNAs is not uniform across the infecting virus genomes, suggesting that certain regions of the infecting virus RNAs (either genomic or mRNA) are targeted more than others. In a recent report, vsRNAs mapped to Homalodisca coagulata virus-1, (HoCV-1) and Homalodisca vitripennis reovirus (HoVRV) showed strikingly contrasting patterns for each of their genomic RNAs respectively. For example, in H. vitripennis, HoCV-1-specific vsRNAs were predominantly of positive strand polarity (Fig. 2), and hotspots of more abundant vsRNAs were observed in regions encoding the HoCV-1 genome.

![Figure 2. Mapping summary of vsRNA reads against HoCV-1 genomic RNA. The numbers of vsRNA reads mapped along the Y-axis and the HoCV-1 genomic RNA are represented across the X-axis. The gray colored peaks represent the positive-strand small RNAs while the red colored peaks represent the negative-strand small RNAs. This figure is adapted from Nandety et.al 201323 with permission from Virology, Elsevier Limited. This is just one example, several publications describe the distribution of small RNA reads on different viruses.22,24](image-url)
RdRP, and the intergenic region between the 2 ORFs (Fig 2). Such a bias for positive strands of genomic RNAs has also been reported for other viruses infecting plants and other invertebrate hosts. The vsRNAs can be so abundant in virus infections that they can even be used as strategies to identify new viruses. Studies on small RNA analysis of soybean aphids resulted in the identification of the single-stranded positive-sense RNA virus (Aphis glycines virus, AGV) and 2 other known aphid viruses (Aphid lethal paralysis virus, ALPV and Rhopalosiphum padi virus, RHPV). piRNAs also have been shown to play a pivotal role in the antiviral defense of mosquito cell lines against Rift valley fever virus (RVFV).

**RNAi Inducers**

Experimental studies have demonstrated that RNAi activity can be specifically induced using dsRNAs, siRNAs and miRNAs that are homologous to specific target mRNAs, and a variety of approaches have been used to deliver RNAi inducers to target insects. DsRNAs are the most commonly used RNAi inducers and can be produced in bacteria or in vitro. Also, siRNAs have been used to induce RNAi effects and down regulate target mRNAs in psyllids (Bactericerca cockerelli) and honeybees (Apis mellifera). The siRNAs used in the above examples were produced through the digestion of dsRNAs in vitro by RNase III to generate 18 – 25 bp siRNAs. Artificial microRNAs (amiRNAs) can also potentially be used for inducing RNAi effects in insects. For both plants and animals, both mRNAs and amiRNAs have been used for gene function analysis, therapeutic approaches against diseases, and regulating gene expression. Furthermore, a considerable number of studies have shown that miRNAs can affect insect morphology, behavior, immunity, and gene expression; some of those studies showed that stage-specific or tissue-specific expression of miRNAs during insect development in different insects. Therefore, using miRNA mimics or amiRNAs offers potential for practical RNAi applications toward insect pests.

**Target selection for efficient RNAi**

The potential for using RNAi specifically and efficiently in insects depends on several parameters including: dose dependency of the RNAi inducers, the efficiency differences of RNAi inducers against targets in different tissues of insects, the types of RNAi inducers to be used (siRNAs, piRNAs or miRNAs), their mode of delivery, and the availability of targets for RNAi. The successful use of RNA interference with insects also requires the identification of best targets for RNAi and selection of the best interfering RNAs (siRNAs, piRNAs, miRNAs or dsRNAs).

Previous studies have demonstrated an efficient RNAi process through selection of targets that are inherent part of the insect development, metamorphosis, or gene targets that are involved in key metabolic processes. Recent studies have demonstrated the role of important gene targets such as actin and V-ATPase in mealybugs and psyllids. Similarly, insect metamorphosis can be influenced by different classes and levels of juvenile hormones (JHs); the levels of JHs in turn can be influenced by JH metabolic enzymes (juvenile hormone esterase, JHE; juvenile hormone epoxide hydrolase, JHEH; juvenile hormone acid methyl transferase, JHAMT). Some of the recent studies in Tribolium showed enhanced RNAi effects when the targets were either juvenile hormone or juvenile hormone acid methyl transferase. In Bemisia tabaci, RNAi effects were prominent in nymphal stages when the genes in the edysone pathway were silenced.

Some of the bottlenecks in the efficient use of RNAi for insects are due to the limitations with target choice and hence efficiency, and due to the lack of genetic resources in non-model insects. With the availability of NGS technologies such as RNA-seq and Miseq and associated bioinformatic processing of larger datasets, generation of complete transcriptomic/genomic data and gene expression data for non-model insects can be accomplished quickly and efficiently. The transcriptome is a complete set of RNA transcripts produced inside an organism at a particular time, and represents a genomic blueprint of that organism. Unlocking the complexity of the transcriptome is essential for interpreting the functional real world elements of a genome which can be applied for more effective downstream applications including developing genome-based biorational control strategies such as those based on RNA interference. Transcriptome sequencing in insects, particularly in non-model organisms, can result in the preliminary understanding of host genes and can reveal the information associated with molting, metamorphosis, and development, necessary for the design of targets for RNAi. The current approaches are very suitable for transcriptome resequencing or for transcriptome assemblies for which the reference genomes are available. In organisms, where the reference genomes are absent, partial assembly of reference genome can be developed based on the assembly of small reads obtained through RNAseq. Additionally transcript information and annotation of novel transcriptome data can be obtained through BLAST homology and annotations from Gene Ontology databases.

**Delivery of RNAi inducers - in vitro Synthesized/Expressed RNAi Inducers**

Several approaches have been used to deliver RNAi inducers to insects, and even cultured insect cells. Some insect cells can be grown in multi-welled plates allowing for testing many candidate
RNAi inducers simultaneously, and this can be a rapid way to obtain information on RNAi effects at the cellular level. In some cases, RNAi-specific cell phenotypes can even be observed in cultured cells. For example, when cultured *H. viropex* Z-15 cells were transfected with dsRNAs targeting actin mRNA, actin filaments were greatly disturbed and cells showed an overall unhealthy appearance.

Microinjection into insect bodies has been widely used for RNAi studies in whole insects. Microinjection can ensure that the RNAi inducers are delivered to the tissue of choice, or more generally into the hemolymph. Furthermore, when specific volumes of known concentration are injected, the dosages used can be accurately compared, and different RNAi inducers can be mixed and simultaneously injected. For example, the efficiency of RNAi to reduce mRNA target expression was higher in double injections compared to single injections in *Rhodnius prolixus* and *N. lugens*.

Because of its many benefits, microinjection of specific dsRNAs or siRNAs has been used to study the regulation and expression of genes in both model and pest insects including *Drosophila melanogaster*, *T. castaneum*, *B. mori*, *B. tabaci*, *B. cockerelli*, *H. vitripennis*, and *A. mellifera*. Each of these studies yielded very good information, more than can be discussed here, so only limited examples are presented. Mutti et al. used microinjection to deplete a target salivary gland transcript *Coo2* in the pea aphid (*A. pisum*). Aphids injected with anti-Coo2 siRNAs showed earlier mortality than control insects. Microinjection of dsRNA into *A. pisum*, also induced mRNA degradation at about 40% for 2 targets, the *Actin* mRNA encoding a calcium binding protein (calreticulin) and the gut specific *Ap-cath-L* mRNA encoding a cysteine protease, *cathespin-L*. *Rong et al.* showed through dsRNA injection, that *β-N-acetylglucosaminidase (LmNAG1)* in *Locusta migratoria* plays an essential role in insect molting. Microinjection of dsRNAs against actin, chitin synthase 1 and V-ATPase in the citrus mealybug, *Planococcus citri* showed that the corresponding mRNAs are good targets for RNAi.

In addition to microinjection, RNAi effects have also been achieved by soaking (*C. elegans*), spraying (*Ostrinia furnacalis*), and electroporation (*Ixodes scapularis*) approaches to deliver RNAi inducers. In a recent study, El-Shesheny, et al. applied dsRNAs directly to the *Diaphorina citri* ventral epidermis by micro-application. The droplets containing dsRNAs were placed ventrally on the thorax region of *D. citri* nymphs. Treated nymphs showed target mRNA degradation and wing malformedion. Like microinjection, these are excellent approaches for fundamental RNAi studies, but are unlikely to have practical application potential, and RNAi applications for pest control will most likely require other means for delivering the interfering RNAs.

Oral delivery of RNAi inducers has been experimentally successful in many, but not all insects studied so far. A variety of approaches have been attempted including adding RNAi inducers to artificial diets, baits, and in some examples for plant-feeding insects, the plant has been used to deliver the RNAi inducers. While feeding RNAi inducers has great potential it is not as straightforward as is injection or other means of delivery. Inconsistent doses of RNAi inducers may be taken up by individual insects, frequency of feeding can be variable, and stability of the RNAi inducers in the oral delivery medium might be problematic. For example, for *A. pisum*, both salivary secretions and the hemolymph were able to degrade the dsRNAs; therefore, dsRNA-induced RNAi effects were not obtained through either ingestion or injection.

Oral delivery of RNAi inducers resulted in reduction of targeted mRNAs in several other agriculturally and medically important insects including *Epiphyas postvittana*, *Rhodnius prolixus*, *Glossina morsitans*, *Reticulitermes flavipes*, *Plutella xylostella larvae*, *Anopheles gambiae*, *B. cockerelli*, and *D. citri*. Depending on the insect stage, in some cases it was possible to see phenotypic effects on the target insects and in others it was only possible to see target mRNA reductions. For example, the results from feeding tests with *A. pisum* showed that reduced expression of the hunchback (*hb*) mRNA encoded by a gap gene, (*zinc finger transcription factor*), which is a key player for insect segmentation, led to pea aphid lethality. Similar artificial diet studies with *B. cockerelli* showed the efficiency of oral feeding dsRNAs and siRNAs by targeting endogenous mRNAs for the homologues of several essential genes including *Actin*, *ATPase*, *Hsp70*, and *CJC*.

Whitelines of the genus *Bemisia* are one of the most important global invasive agricultural insect pests in recent years. The potential of these phloem-feeding hemipterans to develop resistance to chemical insecticides combined with their high reproduction rate has allowed for rapid expansion among many worldwide agricultural areas, and RNAi has brought new hope for potential control strategies. In 2011, RNAi-induced mortality through oral delivery of dsRNA/siRNAs of 5 important target mRNAs, actin ortholog, ADP/ATP translocase, α-tubulin, ribosomal protein L9 (*RPL9*) and *V-ATPase A* for *B. tabaci* was reported. In this study, reducing the expression of the corresponding mRNAs caused mortality after 6 days of feeding. Oral feeding of siRNAs showed a high mortality rate for *RPL9* (84.61%) and *V-ATPase A* (85.62%), while the dsRNA treatment demonstrated the highest mortality rate for *V-ATPase A* with 97.5%. In that bioassay, the mortality rate was similar in both treatments after reducing the expression of ADP/ATP translocase mRNA. Interestingly, siRNAs were found stable in the insect diet for at least 7 days at room temperature. Later, this group successfully engineered tobacco plants expressing a dsRNA precursor against the whitelye *V-ATPase A* and the results illustrated that these modified plants formed siRNAs to downregulate the mRNA target and protect the plant.

Another successful example of oral delivery of dsRNA to induce RNAi was for *Diabrotica virgifera virgifera*. *Snf7* is a vacular sorting protein belonging to the ESCRT (Endosomal Sorting Complex Required for Transport)--III complex involved in sorting of transmembrane proteins resulting in lysosome degradation through the endosomal-automphagic pathway. RNAi of the WCR *Snf7* ortholog (*DvSnf7*) led to deficiencies of mRNA and protein levels in the insect midgut and fat body resulting in...
de-ubiquitination and autophagy, and death of WCR larva.93 This result suggests this mRNA as a potential target for generation of transgenic plants for management of this economically important pest in the field.93

Whether or not RNAi inducers acquired orally induce RNAi effects only in cells where they are taken up (e.g. midgut epithelial cells) is still an important question. Unlike in plants and C. elegans, insects are not known to exhibit a robust, systemic or distal RNAi response. Thus, if only the cells that are exposed to the RNAi inducer show RNAi effects, then care must be taken in interpreting results from oral feeding experiments.

**Delivery of RNAi Inducers-Microbial-Mediated RNAi**

In recent years several studies have identified ways to use microbial mediated-RNAi inducers to overcome some of the limitations mentioned above. A recombinant *Escherichia coli* was engineered to produce specific dsRNAs fed to *C. elegans* for inducing RNAi.94,95 Similarly, *E. coli* was engineered to produce dsRNAs for successful RNAi of 5 different mRNA targets in the Colorado potato beetle, *Leptinotarsa decemlineata*.96 The plasmids were transformed into a RNase III-deficient *E. coli* strain for dsRNA production and after ingesting the bacteria this resulted in significant mortality and loss of body weight in *L. decemlineata*. This system of microbial/bacterial delivery of dsRNAs worked efficiently in suppression of *Peneaus merguiensis*, densovirus (PmergDNV), which is a serious pathogen of the house cricket.97 In that study, mortalities and viral loads in house crickets were significantly lower in treatments challenged with PmergDNV following exposure to bacterially expressed PmergDNV-dsRNAs.97

For many plant-feeding insects, a more natural means of ingesting interfering RNAs would be by feeding on plants that generate the RNAi inducer. The use of transgenic plants engineered to produce dsRNAs91,98 might be desirable in many cases, but transgenic plant generation requires time. As an alternative, several groups have investigated using recombinant plant viruses as tools for expressing RNAi inducers50,51 in plants. The main advantage here is that RNAi activity is the primary plant defense against infecting viruses. Thus when plants respond to a virus infection by RNAi, siRNAs (vsRNAs) covering the entire virus genome are produced. Then if a plant virus is engineered to contain an insect RNAi inducer sequence, this also will be targeted and yield specific siRNAs (vsRNAs). Several plant viruses can be engineered to contain and express desired sequences in plants, and this can be done fairly easily and quickly. This is a commonly used research strategy in plant- microbe interaction studies and is referred to as host induced gene silencing (HIGS). If abundant RNAi inducer siRNAs were generated from the engineered virus, perhaps these could be acquired by insects feeding on these plants and then induce RNAi effects.

Three midgut-expressed *McCP5s* mRNAs were targeted by RNAi activity in *M. sexta* through the use of recombinant *Tobacco rattle virus* engineered to produce RNAi inducers in *Nicotiana attenuata*.99 Almost all plant-infecting viruses infect and move systemically in plants via the phloem, and thus plant viruses might also be useful for targeting phloem-feeding insects. To test this, recombinant *Tobacco mosaic virus* (TMV) was used to target the phloem feeding hemipteran, *B. cockerelli*.51 When *B. cockerelli* were allowed to feed on tomato (*Solanum lycopersicum*), tomatillo (*Physalis philadelphica*) and tobacco (*N. tabacum*) plants infected with recombinant TMV harboring *B. cockerelli actin* and V-ATPase sequences, this resulted in a reduction of the corresponding *B. cockerelli* mRNAs.51 Tomatillo plants infected with recombinant TMV also showed a decrease in *B. cockerelli* progeny production.51 Similar approaches were applied in attempts to induce RNAi effects in *N. benthamiana* plants against the citrus mealybug (*Planococcus citri*), again by using recombinant TMV.50 The results obtained from that study showed mortality of adult insects and dramatic reduced fecundity of *P. citri* after feeding on plants infected by the corresponding recombinant TMV.50 These examples show experimentally the potential for using plant viruses to deliver RNAi inducers via non-transgenic plants, but obviously many plant viruses themselves are pathogens and this cure could be worse than the cause. However another plant virus has recently been used to assess whether this approach might have practical application. These authors engineered a common virus of citrus, *Citrus tristeza virus* (CTV) as a tool for expressing RNAs and proteins in citrus.100 But what is most important is that the engineered CTV causes essentially no symptoms in citrus plants. This engineered CTV was then used to target one of the most destructive pests affecting citrus industry in the United States, *D. citri*, which is the vector of *Candidatus Liberibacter asiaticus* (CLas), causal agent of Huanglongbing (citrus greening). *Citrus tristeza virus* (CTV) engineered with truncated abnormal wing disc (Awd) RNA sequence of *D. citri* triggered degradation of the *Awd* mRNA after *D. citri* nymphs fed on these plants.87 Furthermore, wing malformation and mortality of adult insects also was observed and attributed to RNAi.87 This result is a very promising result, suggesting that even in woody perennial plants like citrus, such an approach could have practical application potential.

**Delivery of RNAi Inducers-Plant Mediated RNAi**

Potential application of transgenic plant-based RNAi approaches for pest insect management in plants has been widely discussed,101,102 even leading to the suggestion of “insect-proof plants,”103 and is very relevant today. A recent issue of *Science* (16 August 2013) featured on the cover the suggestion of “smarter pest control.” A special section within that issue (pages 728 – 765) was dedicated to our “pesticide planet” and discussed several current topics, one entitled “A Lethal Dose of RNA,”104 and how new opportunities based on scientific research and our understanding of RNAi could help feed the world’s growing population and simultaneously contribute to better sustainability in agriculture by decreasing our dependence on pesticides for pest and pathogen control. Clearly, RNAi approaches offer great potential for insect pest control, and by understanding the specificity and activity our hope is to take advantage of the
opportunities to promote sustainable, environmentally sound applications of this technology.

Transgenic plant-mediated RNAi has proven to be successful against other plant pests and pathogens including nematodes, bacteria, and viruses. Transgenic RNAi-specific approaches are even used commercially in U. S. papayas, squash and recently in plums. The potential of transgenic plants targeting insects based on RNAi was first demonstrated in early studies against the western corn rootworm, D. virgifera. Transformed corn (Zea mays) plants expressing the V-ATPase subunit A dsRNA exhibited a significant reduction in D. virgifera feeding damage. Mao et al (2007) evaluated bollworm (Helicoverpa armigera) larvae growth on the N. benthamiana and Arabidopsis thaliana plants transformed with the P450 gene CYP6AE14 dsRNA. This mRNA is highly expressed in the H. armigera midgut and is important for larval growth and tolerance to cotton gossypol (a polyphenol compound playing an anti-herbivore role in cotton). The results showed that levels of CYP6AE14 mRNA were decreased and larval growth was reduced. They further targeted H. armigera larvae by feeding them on CYP6AE14-derived hpRNA transgenic cotton. Genetically modified cotton plants experienced less feeding damage and larval growth was significantly retarded but they were not killed. Recently, cotton plants showed greater protection from H. armigera when plants were engineered to express both dsCYP6AE14 and full length cotton cysteine protease. Plant cysteine proteases help to enhance uptake of dsRNA through increasing insect peritrophic matrix permeability. Zhu et al. also reported an improvement in pest resistance in engineered tobacco plants expressing dsRNA of edysyne receptor (EcR) from the cotton bollworm, H. armigera. EcR and ultrraspiracle (USP) are 2 components of a nuclear receptor complex of the steroid hormone, 20-hydroxyecdysone (20E), which controls molting and metamorphosis. Therefore, EcR and USP are required for insect development. Zhu et al. showed that not only was pest resistance improved after feeding of H. armigera larvae on the transgenic tobacco plants expressing EcR dsRNA, but also the level of the EcR mRNA was significantly decreased causing molting defects and eventually death. An interesting observation from that study was the resistance of the same transgenic tobacco plants to another pest, Spodoptera exigua. The reason behind that later resistance was the similarity of EcR gene sequence in S. exigua H. armigera. This is an example of off-target effects (more details in a separate section later).

Transgenic RNAi-based approaches also have shown good potential against some phloem-feeding hemipteran insects. N. lugens is an important phloem feeder pest of rice (Oryza sativa L) and is the vector for some important rice-infecting viruses. In N. lugens, the expression levels of 3 mRNAs of the hexose transporter gene NHHT1, the carboxypeptidase gene Nlcar, and the trypsin-like serine protease gene Nltry in the N. lugens midgut; were suppressed after N. lugens fed on transgenic rice plants; however, insect mortality was not observed. The green peach aphid, M. persicae, has one of the broadest host ranges among crop pests and is an important vector for many plant viruses. RNAi studies were performed targeting 2 different M. persicae mRNAs. Rack1 encodes an essential multi-functional scaffold protein and its mRNA is predominantly expressed in the gut, and the M. persicae C002 (MpC002), is highly expressed in the salivary glands. The expression of these 2 mRNAs was decreased by up to 60% after M. persicae fed on transgenic N. benthamiana and A. thaliana plants expressing dsRNAs corresponding to the targeted mRNAs, and M. persicae on these plants produced less progeny and some lethality was observed. Furthermore, Mao and Zeng, reported plant-mediated RNAi against the mRNA for the gap gene, hunchback (hb) in M. persicae. They demonstrated a reduction of the hb mRNA level in Myzus persicae nymphs after feeding on hb dsRNA-expressing tobacco affecting the insect fecundity.

In addition to using dsRNA in plant mediated RNAi targeting insects, amiRNAs are also being used as a strategy to induce RNAi. Recently, amiRNAs were used to confer aphid resistance in tobacco plants (N. tabacum cv. Xanthi). Nine genes of the aphid (Myzus persicae) were selected as silencing targets and their hpRNA-expressing vectors were constructed and transformed into tobacco plants. The results showed that amiRNA could be another approach to develop for conducting RNAi in insects.

**Potential for Off-target Effects**

The potential for unwanted off-target effects is an issue that has been discussed, particularly in regards to practical applications of RNAi. This issue is important and must be approached scientifically, particularly for RNAi-based approaches for insect control in crop plants. However, as RNAi activities occur naturally every day, everywhere, we must consider potential off-target effects against the background of naturally-occurring RNAi activities. Off-target effects can result if the interfering RNAs affect more than just the intended RNA target. This could be within the given insect, when the interfering RNA affects heterologous RNAs (Type I), or perhaps when the interfering RNA induces RNAi effects in non-target insects (or other organisms; Type II). The latter are generally thought to be of more concern from a risk point of view.

There are several potential ways that interfering RNAs might give rise to off-target effects. For example, if the mRNA to be targeted by RNAi activity is a highly conserved sequence among different species (e.g., actin), then the probability for potential off-target effects is likely to be higher than if the target mRNAs is unique, or if the specific region of the mRNA targeted is unique. Some small RNAs can have up to hundreds of potential target sequences in a genome and off-target effects can be triggered due to the partially complementary sequence of the mRNA 3'UTR. This mechanism of off-target effects has been suggested to be similar to that of miRNA silencing, and can be further complicated as some miRNAs can have 4 to 5 mismatched base pairs with their target mRNA. There are other features of siRNAs that can affect the binding with target mRNAs, such as compositional features, thermodynamic, and structural features. The loading of small RNAs into different Argonaute
proteins (AGO1, AGO3 and AGO4) besides AGO2 can also contribute toward different thermodynamic stability between the siRNA and its mRNA target, and target mRNA cellular abundance and turnover rate can influence siRNA efficacy.4,127

Some suggestions for designing dsRNA and siRNA/miRNA RNAi inducers have been proposed to help avoid potential off-target effects.128,129 Among them, several software are available online, such as SnapDragon, BLOCK-iT™ RNAi Designer, dsCheck, siDirect, and E-RNAi, etc. Some of the softwares are optimized for Drosophila melanogaster or other organisms. Each can be used for different purposes. For example, SnapDragon, dsCheck, and E-RNAi are web-based tools for the design of long dsRNAs for specific gene knockdown, and avoid the potential off-target effects; whereas, BLOCK-iT and siDirect are for selecting effective small RNA sequences with target specificity for multiple organism or a specific organism. Therefore, to have an efficient and specific RNAi, the correct program with the right algorithm for each siRNA design is important.

When the form of RNAi inducer used is dsRNA, then RNAi activity upon this dsRNA will occur yielding a range of siRNAs (Fig. 3), which might influence the potential for off target effects. Upon uptake by an insect, the dsRNAs are recognized as foreign and RNAi activity results. However, RNAi-based processing of dsRNAs does not occur at distinct, phased ~21 nt intervals. Rather the dsRNA sequence can be cleaved by Dicer beginning at essentially any nucleotide, thereby yielding a population of overlapping siRNAs ranging in size and sequence corresponding to the target sequence. This is further compounded by other size siRNAs generated by RNAi activity. The qualitative differences are small, sometimes just a single nucleotide, but this can be important and the population complexity is great.

When plants are engineered to produce dsRNAs, RNAi activity will result in the plant, thus yielding a population of siRNAs corresponding to those dsRNAs. The dsRNA per se, is not then the only RNAi inducer likely to be present in the plant. Furthermore, at least in plants RNAi activity includes RNA-dependent RNA polymerase-based amplification to yield secondary siRNAs (Fig. 3), resulting in skewed populations of siRNAs. In addition, the resulting siRNAs will not be equal in abundance, there are “hot spots” which amplify more, giving rise to specific siRNAs of much greater abundance for a given region. This has potential for greater homology of siRNAs/dsRNAs to different target mRNAs, possibly increasing the complexity for off-target effects. If the dsRNAs are directly acquired by the target insect, then RNAi activity within that insect will yield the population of siRNAs and this could give rise to potential Type I off-target effects. These may or may not be of real concern. However, Type II off-target effects could conceivably result if non-target insects acquire the dsRNAs. For example, transgenic maize plants engineered for RNAi-based resistance to D. virgifera also showed RNAi effects against several other beetle species in which the sequence conservation exists,98 supporting the arguments above.

Future Directions

We have covered some aspects of RNAi relative to insects, and even some possibilities and considerations relative to practical applications of RNAi approaches for insect pest control in plants. We also presented some examples of new approaches including using plant viruses as potential delivery vehicles in crop-mediated RNAi applications against insect pests. This strategy could be particularly attractive for use in woody plants such as grapes and/or citrus, where transgenic plant approaches would be very difficult due to the time required for developing all the needed plants and the great number of different plant genotypes. For example in overall citrus production there are many varieties (e.g. grapefruit, oranges, lemons, limes etc.) in a given area, and for old established orchards, or vineyards for grapes, there can be 100 year old plants that need to be protected (e.g., grapes Cabernet sauvignon). Plant transgenic approaches in such instances would be very difficult. But it might be possible to use recombinant plant viruses to deliver RNAi inducers in existing, non-transgenic plants. However, this
approach has its own challenges. Phloem sap feeding hemipteran insects acquire nutrients from phloem. Therefore, RNAi inducers must be available in the phloem, but evidence already shows that phloem feeding hemipterans can be targeted by this approach.26,51,87 And, if viruses are to be used in agriculture, they must not induce a negative phenotype (disease) in the plant, they must be engineered to not spread beyond the treated plants but must still be able to move in phloem of treated plants, and the recombinant virus must retain and express the RNAi inducing sequence while infecting the plant. Although this approach offers great potential, clearly more studies are needed.

But what new alternative approaches are there? Can insects be targeted directly, perhaps by using insect- specific viruses to induce RNAi in the target insects? Viruses are the most abundant microbes on our planet133 and various viruses are found in all types of organisms, including insects. Insects are known to be hosts to a variety of viruses belonging to different taxa including baculoviruses, picornaviruses, parvoviruses, entomopoxviruses, ascoviruses, iridoviruses and others,134 and probably many more remain undiscovered. We know that insects mount effective RNAi responses against infecting viruses, so if insect viruses could be engineered to contain specific target insect RNAi inducers, will the infected insect mount an RNAi response against this virus (and the insects’ own RNA)? There are some reports already suggesting that this strategy might have potential for at least some insects.135 Part of the problem, but also the beauty of this approach is that viruses have limited and specific host ranges. Therefore if the correct insect virus can be used, the potential for at least Type II off-target effects will be lessened, as a virus that might be used for an aphid such as R. padis, will not be useful for a psyllid such as D. citri.

Viruses naturally infect insects and thus are already present in the environment in non-recombinant forms, but there are important challenges for using recombinant viruses. For example, can insect viruses be engineered to contain, efficiently express and retain the inserted RNAi inducer sequence? Replication, packaging cycles often lead to recombination events that excise sequences not needed by the virus, thus the RNA inducer sequence might be lost over time. This is a potential problem for long term establishment of the recombinant virus in the population, but also means that the recombinant virus will evolve back to wild type and the recombinant virus is not permanently in the population, which might be desirable in some cases. These are questions/concerns which need to be addressed to see if insect viruses can be successful as gene delivery vehicles to induce RNAi and eventually control of crop pests as well as plant viruses. RNAi is clearly a very important topic for fundamental study but also with great practical potential. A variety of approaches are potentially available and we anticipate an even more rapid accumulation of new data and successes. We hope that RNAi approaches can be used for environmentally sound, efficacious insect control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

We would like to thank the California Department of Food and Agriculture Pierce’s Disease Research Program, the California Citrus Research Board, the Florida Citrus Research and Development Foundation and the U. S. Department of Agriculture, NIFA grants program for funding projects in our lab.

References

1. Ding SW. RNA-based antiviral immunity. Nat Rev Immunol 2010; 10:632–44; PMID:20706278; http://dx.doi.org/10.1038/nri2824
2. Ding SW, La R. Virus-derived siRNAs and piRNAs in immunity and pathogenesis. Curr Opin Virol 2011; 1:533–44; PMID:21247566; http://dx.doi.org/10.1016/j.coviro.2011.10.028
3. Pellechano V, Steinnert LM. Gene regulation by antisense transcription. Nat Rev Genet 2013; 14:880–93; PMID:24217735; http://dx.doi.org/10.1038/nrg3594
4. Sioni MC, Sato K, Peir D, Aravin AA. PIWI-interacting small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10:1006–16; PMID:1902513
5. Siomi H, Siomi MC. On the road to reading the RNA-interference code. Nature 2009; 457:396–404; http://dx.doi.org/10.1038/nature07442
6. Sioni MC, Cross MJ, Cross MJ. The role of the road to reading the RNA-interference code. Nature 2009; 457:396–404; PMID:19158785; http://dx.doi.org/10.1038/nature07754
7. Kim VN, Han J, Sioni MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10:126–39; PMID:19165215; http://dx.doi.org/10.1038/nrm2632
8. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002; 21:4663–70; PMID:12198168; http://dx.doi.org/10.1038/emboj.cdf867
9. Mallory AC, Elmayan T, Vaucheret H. MicroRNA maturation and action—the expanding roles of ARGONAUTEs. Curr Opin Plant Biol 2008; 11:560–6; PMID:18691933; http://dx.doi.org/10.1016/j.rpb.2008.06.008
10. Bohonack MT, Czaplinski K, Geiler D. Exportin 5 is a RanGTP-dependent dRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 2004; 10:185–91; PMID:14730017; http://dx.doi.org/10.1016/j.rna.2004.01.017
11. Lund E, Gutttinger S, Calado A, Dahlberg JE, Kurat U. Nuclear export of microRNA precursors. Science 2004; 303:95–8; PMID:14603148; http://dx.doi.org/10.1126/science.1090599
12. Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carroll RW. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 2004; 117:69–81; PMID:15066283; http://dx.doi.org/10.1016/S0092-8674(04)00261-2
13. Seitz H, Tushak J, Zannoni PD. A 5’-uridine amplifies miRNA/miRNA* asymmetry in Drosophila by promoting RNA-induced silencing complex formation. Silence 2011; 2:4; PMID:21649885; http://dx.doi.org/10.1186/2044-797X-2-4
14. Okamura K, Lai EC. Endogenous small interfering RNAs in animals. Nat Rev Mol Cell Biol 2005; 6:973–8; PMID:18719707; http://dx.doi.org/10.1038/nrm2479
15. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomas JJ, Song JI, Hammond SM, Joshua-Tor L, Hanono GJ. Argonaute2 is the catalytic engine of mammalian RNAi. Science 2004; 305:1337–41; PMID:15284456; http://dx.doi.org/10.1126/science.1102513
16. Miyoshi T, Takeuchi A, Somi H, Somi MC. A direct role for Hsp90 in pre-RISC formation in Drosophila. Nat Struct Mol Biol 2010; 17:1024–6; PMID:20639883; http://dx.doi.org/10.1038/nsmb.1875
17. Chapman EJ, Carrington J. Specialization and evolution of endogenous small RNA pathways. Nat Rev Genet 2007; 8:884–96; PMID:17943195; http://dx.doi.org/10.1038/nrg2179
18. Chung WJ, Okamura K, Martin R, Lai EC. Endogenous RNA interference provides a somatic defense against Drosophila transposons. Curr Biol 2008; 18:795–802; PMID:18501606; http://dx.doi.org/10.1016/j.cub.2008.05.006
19. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat Immunol 2006; 7:590–7; PMID:16554838; http://dx.doi.org/10.1038/nri1335
20. van Rij RP, Saleh MC, Berry B, Foo C, Houk A, Antoniewski C, Andino R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev 2006; 20:2985–95; PMID:17079687; http://dx.doi.org/10.1101/gad.1482006
21. Marques JT, Wang JP, Wang X, da Oliveira KP, Gao C, Aguilar ER, Jafari N, Carthew RW. Functional specialization of the small interfering RNA pathway in
74. Mutti NS, Park Y, Reese JC, Reeck GR. RNAi knockdown in the tomato fruit beetle, Solanum lycopersicum. Insect Biochem Mol Biol 2008; 38:1001-7; PMID:18568925; http://dx.doi.org/10.1016/j.ibmb.2007.04.006

80. El-Shesheny I, Hajeri S, El-Hawary I, Gowda S, Bolognesi R, Ramaseshadri P, Anderson J, Bachman M, McIver R, Turner CT, Davy MW, MacDiarmid RM, Ma EB, Zhang JZ. RNA interference to reveal roles of beta-N-acetylgalactosaminidase gene during molting in Locusta migratoria. Insect Sci 2013; 20:109-19; PMID:23955831; http://dx.doi.org/10.1111/j.1365-2497.2012.01375.x

84. Zhou X, Wheeler MM, Oi FM, Scharf ME. RNA interference-mediated knockdown of a cytochrome P450, CYP6BG1, from the diamondback moth, Plutella xylostella. Arch Insect Biochem Physiol 2009; 70:42-49; PMID:24572372; http://dx.doi.org/10.1002/ps.2014

85. Bautista MA, Miyata T, Miura K, Tanaka T. RNA interference of the salivary gland nitrophorin 2 in the triatomine bug, Rhodnius prolixus. Insect Biochem Mol Biol 2010; 40:666-71; PMID:20201031; http://dx.doi.org/10.1016/j.ibmb.2009.08.006

86. Zhang S, Shukle R, Mittapalli O, Zhu YC, Reese JC, Davis EL, Baum TJ, Hussey RS. Absence of transitive and systemic silencing through ingestion in the pea aphid (Acyrthosiphon pisum) associ- ated with knockdown of RNAi feeding and injection assay. Peptides 2014; 35:307-314; PMID:23494433

87. Hajeri S, Killiny N, El-Mohtar C, Dawson WO. Silencing abnormal wing disc gene of the honey bee. Proc Natl Acad Sci U S A 2013; 110:17989-94; PMID:24394433; http://dx.doi.org/10.1073/pnas.1306864110

102. Xue XY MY, Tao XY, Huang YP, Chen XY. New strategies for antagonistic RNA interference. In: Jockusch EL, ed. Advances in Insect Molecular and Cellular Biology. Pages 95-102. Dordrecht, The Netherlands: Springer; 2008; http://dx.doi.org/10.1007/978-1-4020-7991-0_7

103. Trachtenberg EJ, Chalfie M, Fire A. Imaging transgenic Caenorhabditis elegans using RNAi. Nature 2001; 410:522-525; PMID:11470038; http://dx.doi.org/10.1038/35068544.
plants by RNAi: silencing of a conserved and essential root-knot nematode parasitism gene. Proc Natl Acad Sci U S A 2006; 103:14302-6; PMID:16985000; http://dx.doi.org/10.1073/pnas.0604698103.

106. Steeves RM, Todd TC, Etting JS, Trick HR. Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress Helicoverpa zea. Plant Physiol 2011; 156:2050-4; PMID:21655219; http://dx.doi.org/10.1104/pp.111.193758.

107. Sinz M, Yoshii M, Wei T, Hirochika H, Oomura T. Silencing by RNAi of the gene for Pn12, a viropox virus matrix protein of Rice dwarf virus, results in strong resistance of transgenic rice plants to the virus. Plant Biotechnol J 2009; 7: 24-32; PMID:18761654; http://dx.doi.org/10.1111/j.1677-6672.2008.00366.x.

108. Simon-Mateo C, Garcia JA. Antiviral strategies in plants based on RNA silencing. Biochim Biophys Acta 2011; 1809:722-31; PMID:21652000; http://dx.doi.org/10.1016/j.bbagrm.2011.05.011

109. Fuchs M, Gonsalves D. Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. Ann Rev Phytopathol 2007; 45:173-202; PMID:17408355; http://dx.doi.org/10.1146/annurev.phyto.45.062806.094434.

110. Gonsalves D. Transgenic papaya: development, release, impact and challenges. Adv Virus Res 2006; 67:57-124; PMID:16682604; http://dx.doi.org/10.1016/S0065-3527(06)067009-7.

111. Lim LP, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20; PMID:15652477; http://dx.doi.org/10.1016/j.cell.2004.12.035.

112. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS. Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. RNA 2006; 12:179-87; PMID:16682560; http://dx.doi.org/10.1016/j.rna.2005.01.019.