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# Insecticidal RNA Interference, Thinking Beyond Long dsRNA

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# Insecticidal RNA interference, thinking beyond long dsRNA

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## Abstract

Over twenty years ago double-stranded RNA (dsRNA) was described as the trigger of RNAi interference (RNAi)-based gene silencing. This paradigm has held since, especially for insect biopesticide technologies where dsRNAs, similar to those described in 1998, are used to inhibit gene expression. In the intervening years, investigation of RNAi pathways has revealed the small RNA effectors of RNAi are diverse and rapidly evolving. The rich biology of insect small RNAs suggests potential to use multiple RNAi modes for manipulating gene expression. By exploiting different RNAi pathways, the menu of options for pest control can be expanded and could lead to better tailored solutions. Fortunately, basic delivery strategies used for dsRNA such as direct application or transgenic expression will translate well between RNAs transiting different RNAi pathways. Importantly, further engineering of RNAi-based biopesticides may provide an opportunity to address dsRNA insensitivity seen in some pests. Characterization of RNAi pathways unique to target species will be indispensable to this end and may require thinking beyond long dsRNA.

RNA interference (RNAi) technology is a candidate next generation biopesticide that promises unparalleled species specificity and biocompatibility (1). RNAi exploits animal biology where 19–30nt small RNAs base-pair with cellular RNAs, which leads to destruction or translational inhibition of the bound target RNA (2). RNAi is used as a ubiquitous genetic tool to effectively silence gene expression in animals, plants, and fungi. As recent as 2017 RNAi-based, insecticidal transgenes received regulatory approval, which was followed shortly by the commercial corn product SmartStax PRO (3). This successful commercialization suggests that agriculture is on the cusp of a biotechnology gold rush. However, many challenges remain before the dawn of an RNAi-everywhere world, such as mass production of RNAs, engineering of chemical modifications to promote stability or uptake, and methods to deliver RNAs to target cells. This review will focus on a different, fundamental consideration: design of the RNA molecule itself, highlighting options that don’t involve long dsRNA. Validated delivery methods for long dsRNA will work with these alternatives, making them compatible with exciting delivery breakthrough approaches that could make RNAi a market-ready pest control technology (4).

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## Endogenous insect RNAi pathways

Perhaps the greatest attribute of RNAi is the simplicity of the concept that dsRNA elicits gene silencing. However, as with most things in biology, simplicity is elusive. In insects three distinct RNAi pathways are recognized: small-interfering RNA (siRNA), microRNA (miRNA), and Piwi-interacting RNA (piRNA) (2). Each has distinct biogenesis, cellular functions, and conservation patterns with a shared feature of mature small RNAs loading into Argonaute/PIWI (Ago/PIWI) protein family members. Small RNAs when in complex with Ago/PIWI proteins bind target RNAs. Some Ago/PIWI proteins have "slicer" ribonuclease (RNase) activity that cleaves a target RNA at the base bound to the 10th base of the small RNA (5).

### siRNA biology

Insect RNAi is typically designed to exploit the siRNA pathway. siRNAs are products of Dicer cleavage, an RNase III enzyme, excised from long dsRNA (Fig 1A). dsRNA can be in the form of a long intramolecular hairpin or an extended duplex RNA. The insect siRNA pathway is not found in other types of animals such as all vertebrates, and is mediated by arthropod specific Dicer (Dicer 2) and Argonaute (Argonaute 2) proteins (6). Dicer 2 (Dcr2) processing of dsRNA yields ~21nt RNA duplexes that exhibit 2nt 3' single-stranded overhangs. Both strands of the duplex are loaded into Argonaute 2 (Ago2) after which the Hen1 methyltransferase methylates terminal 2' hydroxyls. Ago2 has slicer activity that cleaves siRNA target transcripts. Destruction of targets by Ago2 slicing is critical to silencing by siRNAs. Disruption of slicer by mismatches at $10^{th}$ base of the siRNA and target RNA leads to loss of gene silencing. Thus, siRNA-mediated silencing requires near-perfect pairing between the target RNA and siRNA.

### miRNA biology

miRNAs are ~22nt RNAs excised from short imperfect hairpin folds of heterogeneous origin (Fig 1B) (7). Unlike insect siRNAs they are found in nearly all animals. In insects, miRNA biogenesis is typically carried out by the sequential action of Drosha and Dicer 1 (Dcr1) followed by loading into Argonaute 1 (Ago1). Drosha "crops" hairpins in the nucleus from nascent transcripts, which are then diced by Dcr1 in the cytoplasm. Both Drosha and Dcr1 are RNase III enzymes and leave 3' 2nt overhangs on miRNA duplexes. An important feature of miRNA duplexes is a central mismatch, which leads sorting into Ago1 and not Ago2. Another mismatch is usually found in the lower stem as a signal for Drosha recruitment. One strand of the miRNA duplex is loaded into Ago1. Unlike siRNAs, miRNAs are not methylated. While insect Ago1 has slicer activity, miRNAs typically do not slice target mRNAs. Instead, miRNAs pair to targets with approximately eight bases at their 5' end, which leads to translational repression of the target RNA (8). The pairing region is called the miRNA seed. Through reduced pairing requirements miRNAs can regulate expression of hundreds of transcripts.

### piRNA biology

The last class of small RNAs is piRNAs, which are Dicer independent. piRNA are derived from fragments of transcripts sliced by existing piRNA-Piwi protein complexes (Fig 1C).

Unlike siRNAs and miRNAs suppression of targets and biogenesis of piRNAs is intimately coupled. piRNAs are 27–30nt which is clearly longer than siRNAs and miRNAs. Two modes of piRNA production have been described. The first is the “ping pong” cycle where partner Piwi proteins (Aub and Ago3 in *Drosophila*) cleave complementary transcripts which are then converted to new piRNAs (9). A similar principle is at work for the second mode: phasing piRNA biogenesis. Phasing is initiated when a Piwi protein cleaves a single stranded transcript that then becomes processively converted into new piRNAs in a head to tail fashion by the Zuc ribonuclease (10). A characteristic of piRNAs is a 5’ 1U residue, which is particularly apparent on phased piRNAs. piRNAs are also 2’Omethylated by Hen1. Recognitions of targets by piRNAs requires perfect complementarity (11). As piRNAs are longer than siRNAs and miRNAs this means they have the greatest target specificity of all small RNA classes.

## Variability in insect RNAi biology

The three classes of small RNA (siRNAs, miRNAs, and piRNAs) are only shared between insects at a basic level. With the exception of ~34 miRNA families, small RNAs sequences are not conserved (12). In insects the siRNA pathway has anti-viral activity, however, there are many cryptic endogenous-siRNAs (endo-siRNAs) that have species specific functions such as suppressing recently evolved deleterious genes and transposable elements (13, 14). This contrasts with miRNAs, which regulate gene expression in a similar fashion regardless of insect species. piRNA function was initially thought to be germline specific with an essential role in transposable element suppression (15). Recent studies have found piRNAs in the somatic tissues of most insects (16). Somatic piRNAs are predicted to participate in gene regulatory networks in addition to suppressing transposons. The variation in RNAi function suggests that there could also be variation in the most effective RNAi pathway to exploit for gene silencing technology-based pest control.

To highlight this reality a meta-analysis was performed using public datasets from five major plant pests that belong to different insect orders (Fig 2) (see meta-analysis approach section). The animals examined are *Bactrocera dorsalis* (oriental fruit fly), *Spodoptera frugiperda* (Fall Army Worm), *Diabrotica virgifera virgifera* (Western Corn Root Worm), *Acyrthosiphon pisum* (Green Pea Aphid), and *Locusta migratoria* (Migratory Locust) (Fig 2A). These animals belong to some of the most successful and diverse insect orders: Diptera, Lepidoptera, Coleoptera, Hemiptera, and Orthoptera, respectively. The five pests represent over 350 million years of evolution. Orthologs of Ago1, Ago2 and Aub were curated for each species and inputted into the PhyML program to calculate pairwise dN/dS ratios (Fig 2B,C) (17). dN/dS ratios are a measure of evolution with higher values indicating functional divergence. The ratio is determined by comparing nonsynonymous (dN) to synonymous (dS) amino acid coding differences as a measure of constraints on sequence divergence. Ago2 shows the highest average dN/dS ratio, followed by Aub, and then a significantly lower value for Ago1. This shows that siRNA pathway machinery is evolving on average 10 times faster than miRNA pathway machinery, and the piRNA pathway 8 times faster than the miRNA pathway.

To further demonstrate divergence of siRNA and piRNA pathways, small RNA public sequencing datasets were re-analyzed from the five species above. Understanding RNAi pathways has been greatly facilitated by high throughput sequencing technology even moreso than other RNA species. Unlike mRNA sequencing which involves fragmentation, small RNAs are cloned end to end, which captures sites of cleavage created during biogenesis. The size of small RNAs is also well-suited to short read, high throughput sequencing platforms. Popular library protocols use sequential ligation of adapters to small RNAs followed by reverse transcription and addition of flow cell adapters through PCR.

Four analyses were preformed to assess siRNA and piRNA small RNA. First the size distribution of small RNA in each dataset was determined to establish the relative abundance of Dicer products and piRNAs (Fig 2D). miRNAs and siRNAs are 19–23 nt RNAs, and piRNAs are 26–30nt. Next, the abundance of Dicer products was assessed by identification of small RNA reads that exhibit Dicer processing based on genome alignments. Following alignment of small RNA libraries from the five species to their respective genome, small RNA read sense/antisense pairs were identified that overlap by less than 2nt of their total length. This method identifies the 2nt 3’ overhangs left by Dicer cleavage of opposing strand transcription products that hybridize to form intermolecular dsRNA, such as cis-NAT RNAs (Fig 2E). Perfect, invert repeated, long hairpin structures processed by Dicer are also found with the method. Strand mapping artifacts will occur in an invert repeat, leading to alignments on both strands yielding a Dicer signature. Loci with Dicer signature read pairs are compared to the total number of loci that show a bias toward 19–23nt reads (Fig 2E). A greater proportion of loci with the Dicer signature suggests a more active siRNA pathway. The last two analyses are used to characterize piRNA processing (Fig 2F). One calculates the abundance of ping pong piRNAs through identification of sequencing reads with the 10nt overlap ping pong signature. The other assesses phasing piRNA biogenesis by calculating the distance of trailing 3’ 1U reads in sequencing datasets. Phasing is characterized by only 2–4 nucleotides of separation between 1U reads.

From this metanalysis it is clear that small RNA biology varies significantly between the five species. For *B. Dorsalis* small RNA libraries from adult flies were used. The cloned small RNAs are highly biased towards smaller sizes typical of miRNAs and siRNAs. Dicer loci were found in *B. Dorsalis*, but they are a minority of the overall small RNA generating regions. Surprisingly, in *B. Dorsalis* very little evidence of piRNA biogenesis was observed. Both the signature of ping pong and phasing was absent. *B. dorsalis* is reported to be sensitive to dsRNA feeding, however, this analysis shows there might be abundant alternative small RNA types that could be used for gene silencing (18). *S. frugiperda* libraries from larva were used in the metanalysis. Reads showed greater bias toward siRNAs and miRNAs with many of the small RNA loci showing the Dicer processing signature. Both ping pong and phasing piRNA processing was also clear. *S. frugiperda* mounts a strong RNAi to cytoplasmic dsRNA, however, barriers to uptake exist that make feeding dsRNA ineffective (19). In contrast to *S. frugiperda, D. virgifera* had significantly more piRNA sized-reads. Here libraries from larva were also used. While germline tissue is present in larva the sheer amount of piRNA reads indicates that western corn root worm has abundant somatic piRNAs. Many of the small RNA expressing loci in *D. virgifera* have the Dicer signature, consistent with effective RNAi by feeding in this animal (20). Adult *A. pisum*

libraries show evidence of siRNAs and piRNAs with many Dicer processing loci and strong ping pong and phasing piRNA signatures. Aphids are susceptible to RNAi by topical application, which is reflected in sequencing data here with the abundance of Dicer loci (21). For the last pest, *L. migratoria*, adult libraries showed a higher bias towards siRNAs and miRNAs, however, with relatively few Dicer loci. Interestingly, while ping pong processing was evident while phasing was not. *L. migratoria* will mount an RNAi response but only through dsRNA injection due to the delivery barriers like gut dsRNases (22).

Analyses presented here show the comparative abundance of the different small RNA pathways (miRNA, siRNA, or piRNA). Options for RNAi technology come into clearer focus. For example, *D. virgifera* would be an excellent candidate for gene silencing approaches that exploit piRNAs, while *B. Dorsalis* would not. Also, from the metanalysis it is clear that the biology of small RNAs, particularly siRNAs and piRNAs, is divergent between insect orders. Profiling of small RNAs becomes valuable for identifying these differences. Further, sequencing data can also be used to monitor the fate of exogenous RNAs (23). Following assessment of a pest's RNAi biology a small RNA pathway can be selected for gene silencing. All three classes of RNAi (siRNA, miRNA, piRNA) have been effectively used to inhibit gene expression in one or more pest, suggesting the full menu of RNAi pathways could be used for commercial biopesticides. Rationale design will be greatly assisted by small RNA sequencing data.

## Design of RNAi technology

### Engineering siRNAs

siRNAs are the intended small RNA class most insect RNAi biopesticides seek to exploit (1, 4). Typically, this involves long dsRNA that is 200–520 nt long (24). While some animals such as *D. virgirfera* undergo gene silencing in response to dsRNA feeding, others insects like *S. frugiperda and M. locusta* do not (25, 26). Barriers to uptake are the major cause of RNAi insensitivity. However, some insensitivity to RNAi could be due to the biology of the siRNA pathway. For example, *S. frugiperda* has significantly more endogenous siRNA generating loci compared to *L. migratoria,* suggesting efficient siRNA production is more fundamental to *S. frugiperda* physiology. Thus, additional engineering of the RNA maybe able to further enhance RNAi biopesticides.

A significant consideration is that the status of siRNAs are not equivalent. There is a difference in insect siRNAs based on whether they originate from viral RNAs or from endogenous transcripts. In *D. melanogaster* this can be seen in the requirement of the Dcr2 partner protein loqs-PD, which is essential for endo-siRNAs but not viral siRNAs (27). Exogenous dsRNA when injected into embryos follows the endo-siRNA biogenesis pathway suggesting the anti-viral mode of siRNAs may not underlie existing pest control technologies (28). Exploiting viral siRNA pathways could lead to an alternate pest control, which may be more potent in some species if endo-siRNAs are not a robust part of an insect's overall biology, as seen in *L. migratoria*. As shown above, the siRNA pathway is the most rapidly evolving RNAi mode and endo-siRNA roles are not conserved between species (29). For example, while many types of endo-siRNAs are documented in *D. melanogaster*, Dcr2 and Ago2 mutants show almost no reduced viability with only a modest increase in

somatic transposition (30). This contrasts with *D. simulans*, the sister species of *D. melanogaster,* where mutations of homologous proteins results in collapse of male gametogenesis (14). In *D. virgifera,* strains have emerged that are resistant to RNAi biopesticides. Genetic changes affect take-up of dsRNA, and not the core RNAi pathway (Dcr2/Ago2) (31). Lesions in D. virgifera Dcr2 or Ago2 would be expected to occur if these proteins had the importance described in *D. melanogaster*. It would appear that siRNAs are more essential to *D. virgifera* physiology, consistent with the abundance of Dicer loci in this beetle (Fig 2E).

Recent investigation of Dcr2 enzymology provides guidelines for gating exogenous dsRNA into either siRNA or viral RNA pathways (Fig 3A) (32). dsRNA termini can be engineered to have either a 3’ overhang for endo-siRNAs, or a blunt end or 5’ overhang for viral siRNAs. Viral dsRNAs are typically cleaved in a processive manner where multiple siRNAs are produced before the enzyme dissociates. It is an ATP dependent process that involves helicase-mediated threading of the RNA. Processing of endogenous siRNAs occurs through a distributive activity to generate siRNAs, disengaging substrates after each cut. The 2nt 3’ overhangs are characteristics of RNase III cleavage products such as those seen on the termini of miRNA precursor hairpins. When Dcr2 recognizes this arrangement, distributive cleavage occurs. Other types of termini, either blunt or with a 5’ overhang invokes helicase activity and designates the dsRNA as likely viral in origin. Whether this work in *D. melanogaster* translates to other insects is an outstanding question considering the divergence of siRNA pathway enzymes. For example, despite conservation of the helicase domain of human Dicer it does not appear to thread substrates in an ATP dependent fashion. This suggests that simple conservation of Dcr2 helicase domains in other insects might not be sufficient to predict if this rule from *D. melanogaster* applies more broadly. Despite being able to divert exogenous RNAs into endo-siRNA or viral-siRNA biogenesis it is unclear which would be most advantageous for gene silencing. Some studies suggest that viral siRNAs are not strongly *trans*-acting, which is evident from poor loading into Ago2 (33). Primarily this may be a consequence of the act of dicing itself being anti-viral. Dicing destroys viral replication intermediates and the production of siRNAs is secondary to suppressing infection. Again, the answer to which is the optimal mode (endo-siRNA or viral-siRNA) will likely be species specific. Together this paints a challenging picture for choosing a design and suggests that investigation of target animal small RNA biology is needed as predictions based on protein homology are not sufficient.

Another potential limitation of siRNA-based RNAi is how active the RNAi pathway is in a given animal or cell type. In *D. melanogaster* somatic tissues when long dsRNA is used to silence genes it is advantageous to overexpress Dcr2 via transgenes to potentiate the knockdown. This suggests that under normal physiological conditions siRNA machinery may be low expressed and needs to become activated to achieve optimal gene silencing. One stimulus that seems to promote greater expression is viral infection. Such a response is seen in species like *D. melanogaster, G. pallidipes, A gambiae, S. furcifera, L striatellus* (34–37). A similar phenomenon is seen with injection of high concentrations of dsRNA, however it is not clear if this occurs in a feeding setting (38, 39). In contrast, in *B. Dorsalis* prior exposure to dsRNA decreases RNAi efficiency (40). Again, siRNA biology varies by species more than other RNAi modes.

Additional siRNA engineering is possible by altering the size of the dsRNA. Studies in *D. virgifera* suggest that 200–500 bp dsRNA are the optimal length, with a minimum length around 60 bp (41). Presumably, longer dsRNA would be inefficient due to exclusion from the size of clathrin pits, or other endocytic routes (42). The issue with less than 60nt dsRNAs is they are not well-retained in the gut. If delivery to cells was successful dsRNAs could be even shorter. Short-hairpin RNAs similar in size to miRNAs can enter the siRNA pathway (43). The distinguishing feature of a hairpin that undergoes siRNA biogenesis vs miRNA biogenesis is an unpaired base or G-U pair at the $10^{th}$ nucleotide of the hairpin (Fig 3B). By changing RNA duplexes or hairpin RNAs to include an unmatched $10^{th}$ base nucleotide leads to sorting into the miRNA pathway. Indeed, there are situations where miRNAs would be more effective. This is seen in the *Drosophila* female germline where RNAi reagents require sorting into the miRNA pathway to induce gene silencing (44).

## Engineering miRNAs

The greatest benefit of targeting the miRNA pathway for gene silencing is that it is deeply conserved as shown by the dN/dS rates of Ago1 orthologs (Fig 2A). Moreover, miRNAs are found in all tissues of animals. Using miRNAs for biopesticides will avoid species specific effects like the inability of long dsRNA to silence genes in *D. melanogaster* ovaries. The other benefit of targeting miRNA pathways is increased promiscuity of targeting. miRNAs only require 8 bases of complementary to elicit silencing (Fig 1B). Many miRNAs target hundreds of protein-coding genes. Using this activity, a pesticidal miRNA could be designed that targets multiple mRNAs thereby increasing toxicity through a combinatorial effect. However, this decreases the species specificity due to off-target effects that could occur in animals exposed to miRNA-based RNAi molecules. Despite this, miRNA biopesticides will be more specific than chemical pesticides (45).

Reciprocal trans-kingdom miRNA interactions have been reported to occur between plants and insects (46). One example is the role for miR-162a in caste differentiation in *A. mellifera* (47). miR-162a is a plant miRNA present in pollen. When miR-162a is ingested by bees it regulates amTOR expression leading to development of the worker bee caste. Exploiting such an arrangement, artificial miRNAs (amiRNA) have been reported to elicit gene silencing in multiple pest species: *H. armigera, M.persicae*, and *C. suppressalis* (48–51). However, it is unclear if these amiRNAs traffic specifically through the miRNA pathway to load into Ago1. Reports in *H. armigera* and *M. persicae* used amiRNAs based on plant miRNAs. Unfortunately, the longer dsRNA structure of plant miRNAs resemble animal hairpin RNAs (hpRNAs) that are processed into endo-siRNAs. These gene silencing constructs likely exploit siRNA and not miRNA biology. Other structures tested were based on insect miRNAs, however, a central bulge was not included in the design. These shorter hairpins likewise may be processed into siRNAs. miRNA-type and siRNA-type small RNAs can be distinguished by 2’O methylation which only occurs for siRNAs. Detecting this modification might be needed to verify the intended small RNA pathway is being transited by an amiRNA. These results do suggest that miRNA-sized hairpins can be taken up by insect gut cells in some organisms despite being below the size of 60nt dsRNA. The third study, however, in *C. suppressalis* designed an amiRNA that did include a central bulge and would be expected to be Dcr1 processed and Ago1 loaded. The ecdysone receptor gene of *C.*

*suppressalis* was down regulated by 40%. Transgenic rice expressing the amiRNA were protected from the pest.

Better design of amiRNAs could include known motifs that promote miRNA processing. Nuclear processing of miRNAs is promoted by an apical UGU motif in the hairpin precursor loop. miRNAs also have multiple elements in hairpin base such as a 5' UG motif at the base, a mismatched GHG motif within the lower stem of the hairpin and a CNNC motif (52, 53). Inclusion of these motifs in an artificial miRNA would promote recognition by Drosha, which would be important if a hairpin is embedded in a longer transcript. The hairpin loop is also a site of regulation. Multiple RNA binding proteins (RNAbp) have been found to influence miRNA processing though binding single-stranded loop sequences (54). For example, the RNAbp KSRP promotes the entrance into the Dicer processing complex, inclusion of such elements could improve the potency of gene silencing (55).

A major issue with introducing miRNAs is recognition of hairpins as suitable substrate by Dcr1. This requires a 3' 2nt overhang to be recognized by the PAZ-platform structure of Dcr1 (56). If a miRNA hairpin RNA is introduced exogenously it would be unlikely to encounter Drosha for generating the 3' overhang. Fortunately, there are many routes for maturation, which could be used to build RNA molecules that harbor an anti-pest hairpin. While most miRNAs are produced by sequential Drosha and Dicer cleavage there are a large cohort that are produced through alternative means (57). miRNAs are found that circumvent both Dicer and Drosha, though most non-canonical miRNAs are Drosha independent. miRNAs have been found in abundant non-coding RNAs such as tRNAs, rRNAs, and snoRNAs (58–60). These miRNAs are processed by enzymes involved in maturation of non-coding RNA and not Drosha. By exploiting the lifecycles of these RNAs it might be possible to have synthetic RNAs traffic to the nucleus for processing (61). A specific example could be designed around tRNA mimics. A major surveillance mechanism for tRNAs is retrograde nuclear trafficking to reprocess or modify a defective tRNA. An RNA molecule could be introduced into insect gut cells that have features of a defective tRNA that also harbors an amiRNA pesticidal hairpin. Such an RNA would be imported into the nucleus for formation of the 3' overhang required for Dicer recognition. Further engineering could take advantage of viral tRNA mimic behaviors to drive efficient processing of amiRNAs (62).

## Engineering piRNAs

piRNAs are likely the most elaborate variety of small RNA. There are several biogenesis modes, with mysteries remaining regarding their biology (63). Initially, piRNAs were thought to be only a feature of the germline. Like many insights into animal molecular biology, model organism genetics have been essential to elucidating piRNA mechanics. In *D. melanogaster*, *C. elegans*, and vertebrates, piRNAs are extremely abundant in germline with limited evidence that piRNAs are in somatic tissues. This contrasts with most arthropods that have abundant somatic piRNAs with the exception of pill bugs, bumble bees (but not honeybees), and the bury beetle (but not other beetles like D. virgifera or T. castaneum) (16). The widespread presence of somatic piRNAs in invertebrates is a recent revelation and represents a novel opportunity for gene silencing technology. Building upon

ectopic generation of piRNAs using transgenes in *D. melanogaster* and mice, somatic piRNAs have been used to silencing gene expression in *B. tabaci* (23).

The primary principle of piRNA biogenesis is that RNAs cleaved by existing piRNA-Piwi protein complexes are recruited into piRNA pathways (Fig 3C). In mice and *D. melanogaster,* transgenes with non-piRNA sequences have been inserted into piRNA loci and led to ectopic piRNAs (64). Using this strategy, segments of piRNA loci can be fused to target gene sequences that would be recognized and converted into piRNAs (Fig 1C). This could lead to on-target piRNAs that would silence genes. Due to the developing field of piRNA biology the exact sequence fused to the target gene sequence requires greater exploration.

In *D. melanogaster* there are several types of piRNA loci. The first recognized were large dual-strand and uni-strand loci. These are >100Kb long loci that produce millions of unique piRNAs, which function to suppress transposable elements (65). This is reflected by the main phenotype caused by loss of piRNAs in *D. melanogaster*–catastrophic mobilization of transposable elements. The other type of documented locus is piRNAs produced from 3' UTRs, which appear to be gene regulatory with a reported role in gonadal development (66). Multi-species surveys have found that ping pong and phasing biogenesis are present in most insects, including those with somatic piRNAs (10). This suggests that the principles for ectopic piRNA production in soma is likely similar to what has been observed in gonad.

Using these principles of piRNA production synthetic piRNA triggers were generated to silence genes in the whitefly *B. tabaci* (23). Synthetic RNAs against two genes using two different piRNA sequences were fed to adult whiteflies. piRNA sequences were identified using small RNA expression data to identify piRNA biogenesis patterns. *B. tabaci* has abundant piRNA loci which show evidence of ping pong and phasing processing. The piRNA trigger molecules were tested in two different formats, dsRNA and single-stranded RNA (ssRNA). Both configurations of piRNA triggers were effective for gene silencing. Target mRNAs showed an 80% reduction in expression. The level of suppression was equivalent to conventional dsRNA molecules. Sequencing of the animals fed the piRNA triggers found that, as expected, phasing and ping pong piRNAs were produced. The dsRNA version of the piRNA triggers were to also generate siRNAs, demonstrating a gene silencing molecule that simultaneously exploits two RNAi pathways.

There are multiple benefits associated with piRNA-based gene silencing that is not available with siRNAs or miRNAs. One benefit would be that structured RNAs could be avoided. dsRNA folds into A-shaped helices, which due to deep helical grooves makes the molecules structurally homogenous. The common structure of dsRNAs is recognized and eliminated by gut dsRNase regardless of sequence content (67). piRNA triggers only require a specific sequence that is independent of RNA structure. This allows formulation of RNA molecules that could be designed around any structural motif. Another benefit is the trigger would be even more species specific compared to siRNAs. They would exploit the endogenous piRNA sequences of the target species, which would probably not be present in other species. The other beneficial aspect of piRNAs is that they are 27–30 nt in length compared to shorter ~21nt siRNAs/miRNAs. The probability of a 30nt piRNA sequence occurring in a given

transcriptome is several orders of magnitude lower compared to a 21nt siRNA. However, the problem with developing a piRNA approach is the need for annotation of loci expressing piRNAs in the target pest. This can be achieved through the transcriptomics approach used for the metanalysis presented in this article.

## Conclusion

RNAi holds great promise for biopesticide approaches through high species-specificity and environmental friendliness. Moreover, RNAi is suitable for both transgenic and synthetic applications. The major barrier to widespread use of RNAi biopesticides is insensitivity in some pests. To date a single mode of RNAi, the insect siRNA pathway, has been developed into a commercial pest control product (3). The two other insect RNAi pathways, miRNAs and piRNAs, have also been used to silence pest genes through feeding RNAs suggesting they could also be the basis of RNAi biopesticides. Insensitivity to gene silencing by siRNAs could potentially be addressed by a different mode of RNAi. The piRNA pathway in particular offers unique opportunities to design RNAi triggers that are not dependent on an RNA structure. This could lead to novel packaging of RNAi molecules that avoid known blocks to RNAi such as gut dsRNases. Further engineering of RNAs may be key to realizing the promise of RNAi-based pest control and may require thinking beyond long dsRNA.

## Meta-analysis Approach

Small RNA metabolism in five insect pests was assessed using public datasets (Table 1). Genomic and high throughput sequencing datasets were accessed through NCBI genbank, https://www.ncbi.nlm.nih.gov/genbank/, ensemble metazoan and small Read archive databases https://www.ncbi.nlm.nih.gov/sra. The pests subjected to analysis are: *Bactrocera dorsalis* (oriental fruit fly), *Spodoptera frugiperda* (Fall Army Worm), *Diabrotica virgifera virgifera* (Western Corn Root Worm), *Acyrthosiphon pisum* (Green Pea Aphid), and *Locusta migratoria* (Migratory Locust) (Fig 2A).

Orthologs of *Drosophila melanogaster* small RNA machinery (Ago1, Ago2, and Aub) in the five pests were identified using either NCBI blastp or ensemble metazoa blast webservers. Amino acid sequences and their corresponding nucleic acid open reading frame sequences were acquired from these databases. Multiple sequence alignments from the CLUSTAL omega program were used to confirm orthology of protein sequences (68). Evolutionary rates were calculated by the dN/dS method using the PhyML program module codeML (17). Pairwise comparisons made by codeML used CLUSTAL omega multiple sequence alignments and associated tree data. Data was visualized using pheatmap and pirateplot R modules (69, 70).

Small RNA populations from the five species were analyzed using published methods (23, 71). Public small RNA high throughput sequencing datasets were first processed with the fastx toolkit to remove adapter sequences followed by alignment with bowtie with -a -m100 options (72). Subsetting of sequencing reads and evaluation of sizes was accomplished with awk. Read overlap/overhang patterns to identify ping pong or Dicer processing were evaluated by published python-based algorithms (73). For Dicer processing, small RNA

pairs were identified that overlap by less than two nucleotides of their entire length. The ping pong signature is found in small RNA pairs that overlap by 10 nucleotides. piRNA phasing patterns were assessed using code snippets from the piPipes small RNA analysis suite (74). This method calculates the bias of reads to align head to tail along on genomic strand. Phasing is defined as small RNAs that are separated by <4 nucleotides of distance from a single-stranded precursor RNA.

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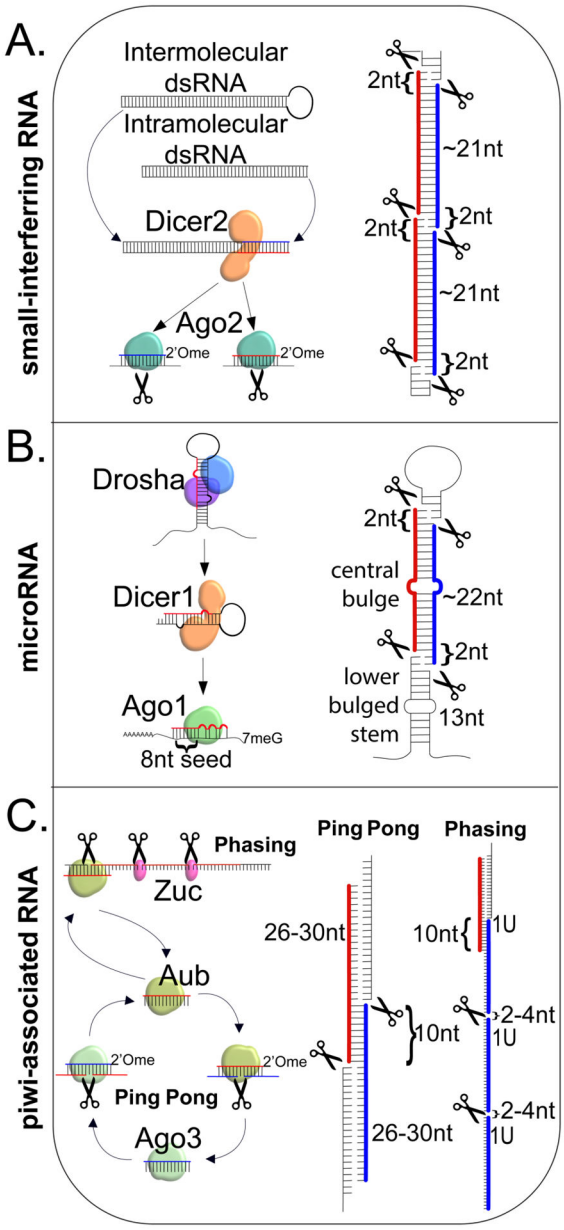


**Figure 1.**

Endogenous insect RNAi pathways. **A)** The small-interfering RNA pathway (siRNA). Diagram on left shows the stepwise production of siRNAs. Either long intermolecular or intramolecular RNAs are processed by the enzyme Dicer2. Small RNA duplexes are then unwound, and individual strands loaded in Ago2 where they bind and destroy target RNAs via slicer activity. Right Diagram shows the register of long dsRNA processing by Dicer. Small RNA duplexes with ~21nt strands are produced that have staggered 2nt 3' overhangs. **B)** The microRNA (miRNA) pathway. Left shows the maturation of miRNAs through the sequential cleavage of Drosha and Dicer1. The resulting ~22nt miRNA is loaded into Ago1. Targets are recognizeb by ~8 nt of base pairing involving the 5' end of the miRNA strand. This is referred to as the miRNA seed. Right shows the relative position of Drosha and Dicer

cleavages on a miRNA hairpin. Several structure elements important for entrance to the miRNA pathway such as a central bulge and bulged lower loop are depicted. **C)** the piwi-associated RNA (piRNA) pathway. Left shows the two biogenesis modes of piRNAs. The ping pong cycle where partner Piwi proteins (Aub and Ago3) work in tandem to cleave and recruit sliced RNAs. The phasing mode is initiated by Piwi protein cleavage followed by head to tail processing of the RNA by the nuclease Zuc. On right are two diagrams showing the RNA processing events associated with ping pong and Phasing. Characteristics such as 10nt overlaps and 2–4nt distances between phasing reads are indicated.

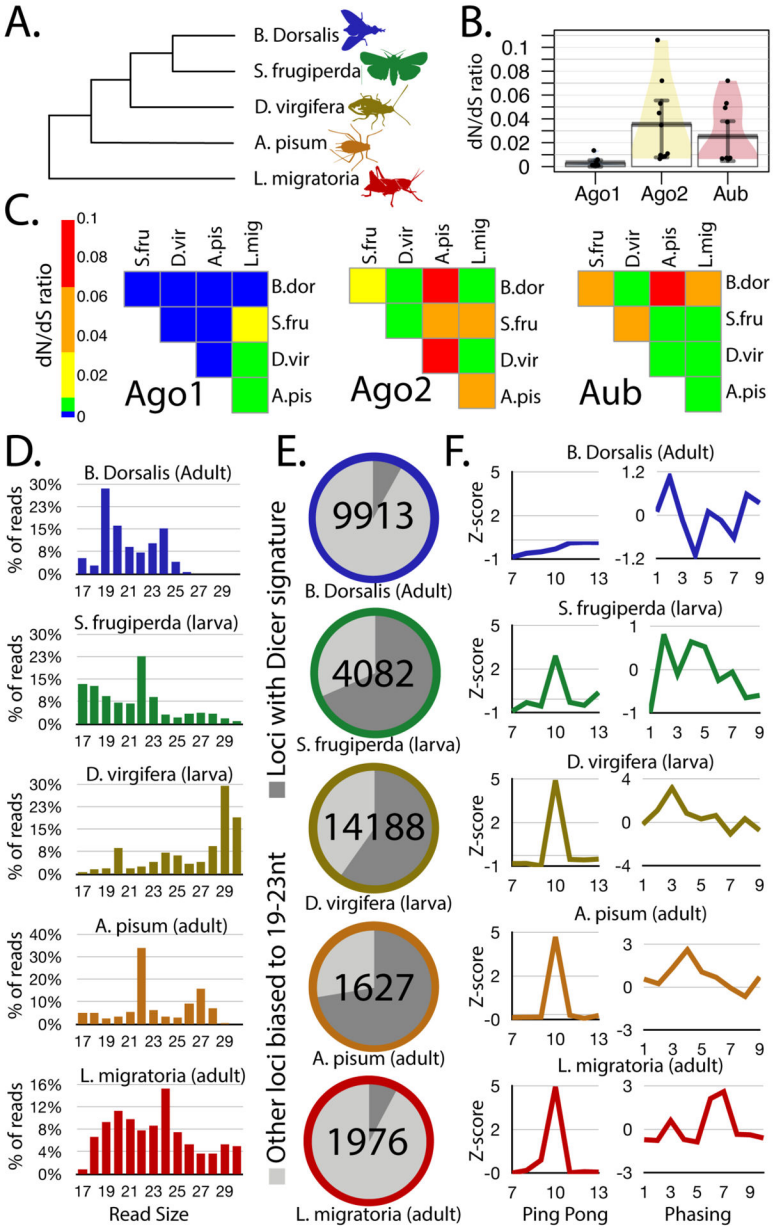


**Figure 2.**

Metanalysis of RNAi machinery and small RNA populations in five pests. **A)** Phylogenetic relationship between *Bactrocera dorsalis* (oriental fruit fly), *Spodoptera frugiperda* (Fall Army Worm), *Diabrotica virgifera virgifera* (Western Corn Root Worm), *Acyrthosiphon pisum* (Green Pea Aphid), and *Locusta migratoria* (Migratory Locust). **B)** HDI plot showing the average pairwise dN/dS ratios for Ago1, Ago2, and Aub orthologs in the five pests. Error bars show standard deviation, violin plots data density, and dots values for each ortholog. **C)** Heatmaps showing the pairwise dN/dS ratios plotted in part B. Color key on left. **D)** Size distribution of small RNA reads mapping to their respective genome. Y-axis is percentage of reads. X-axis is size of reads. **E)** Abundance of small RNA expressing loci with a bias towards 19–23nt reads determined by a coverage depth of 10 reads and a minimum length of

200 bases. Number in middle of circle is the total number of loci annotated. Dark portion of the pie chart shows the portion with the Dicer 2nt 3' overhang signature. **F)** piRNA processing signatures. On left is the ping pong signature where a signal at 10nts represents overhangs left by Piwi slicer. Y-axis indicates Z-score, and X-axis overhang lengths in nucleotides. Right is a measure of phasing biogenesis. The phasing signature is a bias toward close, 1–4nt, trailing 1U reads. Y-axis is Z-score and X-axis is the distance to the trailing 1U reads.

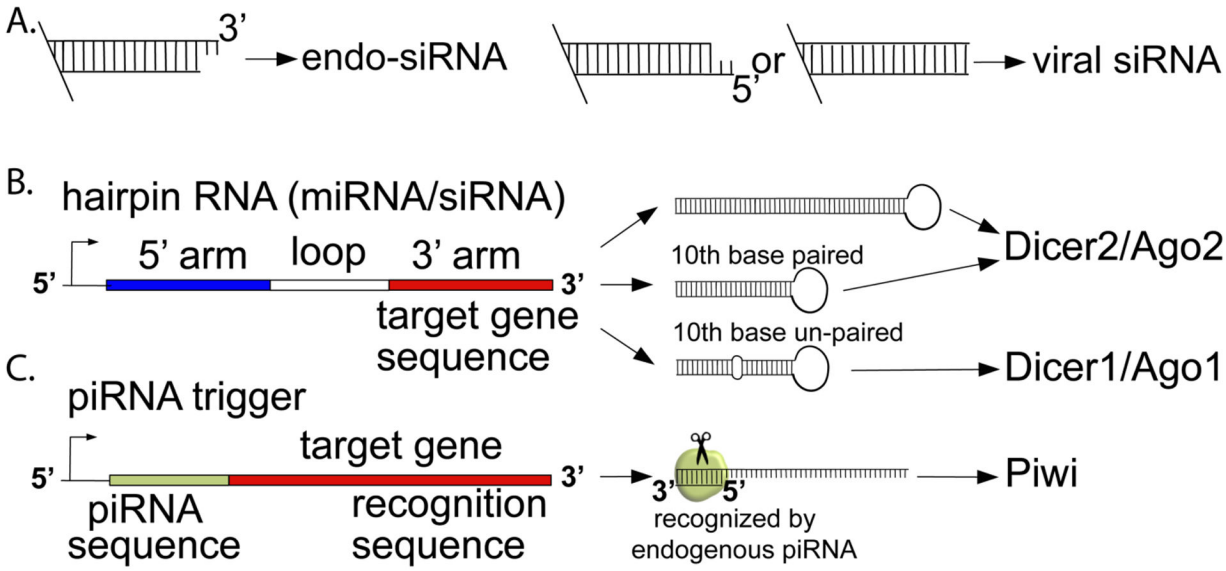


**Figure 3.**

Design principles for directing RNAs into each of the RNAi pathways. (A) Configuration of dsRNA ends that determine if siRNAs are endo-siRNA-like or viral-siRNA-like. A 3′ 2nt overhang specifies endo-siRNA type. A blunt or 5′ overhang designates viral-type siRNAs. (B) Design of hairpin RNAs that leads to siRNA or miRNA biogenesis. A longer hairpin or a shorter hairpin with a perfectly paired stem is processed into siRNAs. A central bulge is needed to generate miRNA class RNAs. (C) Generation of ectopic piRNAs. The sequence of endogenous piRNA targeted transcripts is fused to a target gene. This recruits piRNA-Piwi complexes to the synthetic RNA for cleavage, which converts fragments of the cleaved RNA into ectopic piRNAs. Endogenous genes complementary to the target recognition sequence will be targeted and silenced by the cognate ectopic piRNAs.

**Table1.**

Public datasets used in meta-analysis.

| Pest | Ago1 | Ago2 | Aub | sRNA sequencing |
|---|---|---|---|---|
| *B. dorsalis* | XP_011201160.1 | XP_019846276.1 | XP_011209691.1 | PRJNA319219 |
| *S. frugiperda* | XP_022821691.1 | AVK59454.1 | XP_035434685.1 | PRJNA432886 |
| *D. virgifera* | XP_028146527.1 | XP_028131557 | XP_028150966.1 | PRJNA588643 |
| *A. pisum* | ACYPI007150* | XP_001944852.2 | XP_001949497.1 | PRJNA445568 |
| *L. migratoria* | BAW35367.1 | BAW35368.1 | QLQ34856.1 | PRJNA112823 |

*denotes an ensembl metazoan accession number. All other are NCBI accession numbers. The NCBI accession numbers for Ago1, Ago2 and Aub are from Genbank. Small RNA (sRNA) sequencing data are SRA bioproject numbers.