Review

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Current scenario of RNAi-based hemipteran control

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Abstract

RNA interference (RNAi) is an homology-dependent gene silencing mechanism that is a feasible and sustainable avenue for the management of hemipteran pests. Commercial implementation of RNAi-based control strategies is impeded by limited knowledge about the mechanism of double-stranded RNA (dsRNA) uptake, the function of core RNAi genes and systemic RNAi mechanisms in hemipteran insects. This review briefly summarizes recent progress in RNAi-based studies aimed to reduce insect populations, viral transmission and insecticide resistance focusing on hemipteran pests. This review explores RNAi-mediated management of hemipteran insects and offers potential solutions, including *in silico* approaches coupled with laboratory-based toxicity assays to circumvent potential off-target effects against beneficial organisms. We further explore ways to mitigate degradation of dsRNA in the environment and the insect such as stacking and formulation of dsRNA effectors. Finally, we conclude by considering nontransformative RNAi approaches, concatomerization of RNAi sequences and pyramiding RNAi with active constituents to reduce dsRNA production and application cost, and to improve broad-spectrum hemipteran pest control.

Supporting information may be found in the online version of this article.

Keywords: RNAi; dsRNA; Hemiptera; non-transformative RNAi; hemipteran pest control

1 INTRODUCTION

Hemiptera is one of the most significant orders of insects encompassing aphids, whiteflies, planthoppers, leafhoppers, psyllids, plant bugs, stink bugs and shield bugs. Their invasive nature and efficiency as vectors for numerous bacterial and viral pathogens, results in substantial economic losses to agricultural industries and pose the biggest challenge for current pest management practises.[1] Insecticide application is effective for hemipteran pest control but the evolution of insecticide resistance, detrimental impact on beneficial insects and environment poses limitations on the utility of insecticides.[2] Traditional breeding of plant cultivars that are resistant to hemipteran pests had shown limited success due to a lack of endogenous resistance genes in plants. Although genetically modified plants expressing *Bacillus thuringiensis* (*Bt*) toxins have been successful against lepidopteran and coleopteran pests, *Bt* is not generally effective against hemipterans – although exceptions have been reported[3] – and they have emerged as primary pests of some *Bt* crops.[4,5] As a result, concerted efforts to develop alternative, insect-specific, environmentally friendly and easy-to-adopt technologies for hemipteran pest management is underway.

RNA interference (RNAi) is a conserved, nucleic acid metabolism mechanism able to be initiated by exogenously applied or endogenously expressed double-stranded RNAs (dsRNA).[6] A comprehensive review of the RNAi mechanism and its many facets is beyond the scope of this review; the basic mechanism of RNAi is described in Fig. 1. RNAi-based transgenic and nontransgenic pest management strategies offer potential benefits over traditional chemical insecticides owing to the flexibility, environmentally benign and species-selective nature of RNAi effectors. This flexibility allows the targeting of specific genes, species and orders of insects. Consequently, growing interest in RNAi technology is driving increased efforts to develop RNAi-based products that are expected to reach global markets in the coming decade.[8]

RNAi technology is emerging as a powerful crop protection tool for controlling hemipteran pests, including aphids, whiteflies, planthoppers and psyllids (Table S1 in Appendix S1). Here, we outline the current knowledge on the mechanism of dsRNA uptake in hemipteran insects, and explore critical studies in hemipteran pest control, limitations of RNAi and potential future solutions for the application of RNAi for hemipteran pest control.

2 RNAI IN HEMIPTERAN INSECTS

For RNAi-mediated gene silencing, dsRNA must first enter into the target cell of the insect. Laboratory-based dsRNA delivery methods include microinjection, artifical diet feeding, detached leaf/petiole dip or topical application to either the insect or plant, with field delivery methods to plants/trees including spraying, trunk injection and/or root drench. Each method has advantages and disadvantages with respect to the required experimental logistics. For a discussion of traditionally used delivery methods, readers are directed to a

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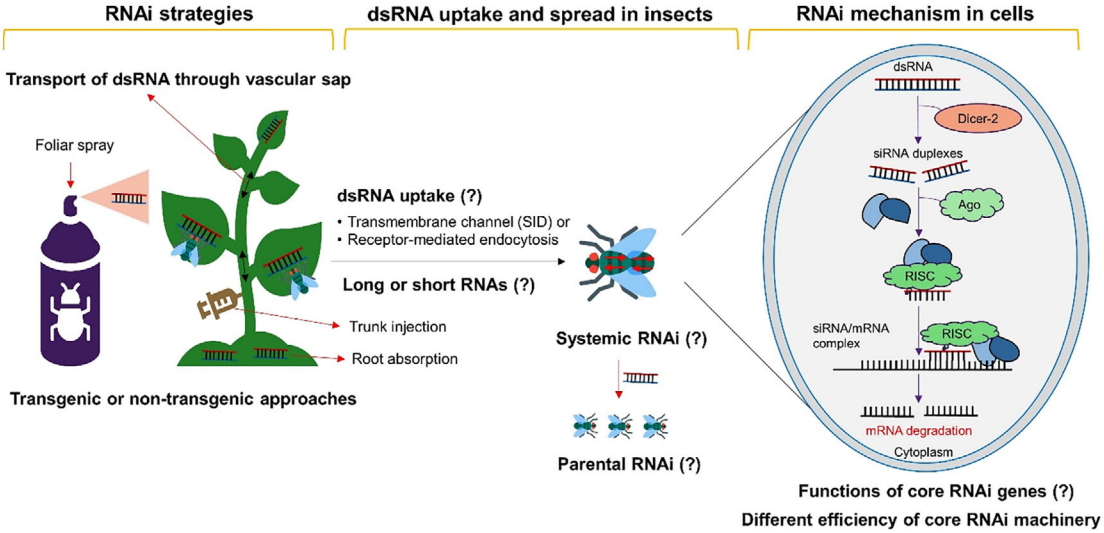

Figure 1. The current state of knowledge on RNAi in hemipteran pest control. The left panel shows the transgenic and nontransgenic RNAi delivery strategies: these methods deliver dsRNA to the vascular system, allowing dsRNA to be transported throughout the plant, critical for sap-feeding insects. The middle panel demonstrates research gaps in the dsRNA uptake and spreading mechanisms in hemipteran insects that need critical investigation. The right panel illustrates the cellular RNAi mechanism of gene silencing in insects. In the cell, the molecular mechanism of RNAi initiated with the cleavage of dsRNA by the Dicer 2 (Dcr2) enzyme into short 21–24 nucleotide small interfering RNA (siRNA) duplexes. Subsequently, siRNAs are recruited by Argonaute 2 (Ago2) proteins that assemble the siRNA duplexes into a multiprotein-siRNA structure known as the RNA-induced silencing complex (RISC). Dissociation of the two strands of the siRNA, into the passenger strand (which is degraded) and guide strand occurs. The RISC then mediates the cleavage of mRNA transcripts complementary to incorporated guide strand, thus preventing protein translation and effectively silencing target gene.[7] There is a variety of forms sRNAs can take depending on the source of the RNA and include small interfering RNA (siRNA) and microRNA which are differentiated by their source. MicroRNA are sourced from endogenous genes and mostly effect endogenous gene expression via degradation of messenger RNA (mRNA), whereas siRNA is mostly derived from dsRNA molecules exogenously applied, derived from replicative intermediates of viruses, or expressed transgenically. In this review we are concerned with the generation of siRNA from exogenous sources.

recently published review.[9] Upon entry into the insect, one of the first requirements for initiating the RNAi mechanism is the presence of a functional RNAi machinery (Fig. 1).[10] In hemipteran insects, the basic pathway is utilized with several core genes, including Dicer-2 and Argonaute, endonucleases and helicases involved in the RNAi mechanism (Table S2) putatively identified; however, their biological function at the cellular level and the extent to which they are involved in the RNAi mechanism remain largely unknown. Furthermore, the variability in RNAi efficiency in different tissues and life stages, as well as within and between insect species is highly variable.[11] Much conjecture surrounds the presence of dsRNA uptake mechanisms[12] as well as proteins such as the RNA-dependent RNA polymerases (RdRp) (Table S1), that enable systemic propogation and translocation of RNAi signals distal to the site of application – supposibly key requirements of a systemic RNAi mechanism – are absent in Hemiptera.

For the success of RNAi in the hemipteran insects, both cell-autonomous, where the RNA silencing response is confined to the cell, and noncell-autonomous responses, where the RNAi silencing effect can occur in cells or tissues distal to the site of application of dsRNA, are critical and depend on four hurdles. Primarily, (i) the pest must ingest appropriate amounts of target dsRNA or siRNA through feeding (environmental RNAi), (ii) the dsRNA or siRNA must traverse the gut lumen into cells to affect gene silencing, (iii) the dsRNA or processed siRNA signal should propagate systemically to cells or tissues expressing the target gene (systemic RNAi), and (iv) target specific gene silencing (cell-autonomous RNAi) needs to occur. However, current knowledge about these steps is limited and further research is critical to define whether siRNA or longer dsRNA is required for efficient gene silencing, the mechanism and pathways involved in the transport of dsRNA across the midgut cells, and whether systemic RNAi actually exists in hemipteran insects.

3 DSRNA UPTAKE AND SPREAD IN HEMIPTERAN INSECTS

3.1 Transmembrane protein-mediated dsRNA uptake

The mechanism of dsRNA uptake in hemipteran insects is still unclear. An approach to further investigate the mechanism and elucidate those genes involved in Hemiptera is to explore similar studies performed on the nematode *Caenorhabditis elegans*.[7,13] Genetic analysis on systemically RNAi-deficient worms confirmed the involvement of two multimeric transmembrane proteins, systemic RNA interference deficient-1 (SID-1) and SID-2, in the dsRNA-mediated uptake and the systemic RNAi response.[14] However, no homologues of *sid-2* have been identified to date in insects, and the presence of SID-1 and its function in dsRNA uptake (or systemic spread) of dsRNA in insects remains unclear. The question then arises, Does the presence of *sid-1* homologue in some species of Hemiptera (Table S2) demonstrate a functioning systemic RNAi mechanism?

Homologues of *sid-1* have been identified in several hemipteran insects such as *Aphis glycines*, *Nilaparvata lugens* and *Bemisia tabaci* (Table S2), suggesting the potential for a systemic RNAi mechanism in these species. However, *sid-1* homologues are not present in all hemipteran species,[12] and a definitive mechanism for systemic RNAi and genes involved in the transfer of the RNAi signal has not been elucidated. Several successful gene silencing events following administration of nonmidgut-specific fluorescent-labelled dsRNA in the brown planthopper, *N. lugens*, and cotton aphid, *A. gossypii*, alude to a systemic RNAi mechanism

(Table S1A,D). These studies lacked validation of the fluorescence signal bound to dsRNA, yet fluorescence-based visualization techniques are extremely promising and easy to conduct, but one must be careful to track systemic movement in insects using real-time PCR and confirmed ideally with RNA sequencing.

In order to further explore potential candidate genes involved in systemic RNAi, genomic information from *C. elegans* and the beetle *Tribolium castaneum*, or the hemipteran *Euschistus heros*[12] can be used to identify, compare and contrast homologues in other hemipteran insects. Artificial diet screening assays using an 'RNAi of RNAi' approach[15] to contrast gene knockdown between dsRNAs targeting nonmidgut genes against dsRNAs specific to systemic RNAi homologues should indicate whether systemic RNAi exists in any hemipteran insect under study. The identification of candidate genes involved, if any, in translocation of the RNAi signal to the site required (RNAi spreading) is critical for a pest control perspective.

3.2 Uptake of dsRNA by endocytic pathways

A second mechanism of dsRNA uptake in hemipteran insects, and likely the most important in those lacking SID-1 orthologues or systemic RNAi, is the scavenger receptor-mediated clathrin-dependent endocytic mechanism as observed in coleopterans, dipterans and orthopterans.[16] Pharmacological inhibition of the endocytic pathway of *Drosophila* S2 cells has been shown to surpress dsRNA entry, resulting in no RNAi response.[17,18] However, individual knockdown of 19 genes encoding for scavenger receptors did not significantly inhibit the RNAi response, which suggests redundancy or that alternative functional molecules of the dsRNA uptake mechanism are present.[17] A receptor-mediated dsRNA uptake mechanism has been reported in *T. castaneum*[19] and *Schistocerca gregaria*[20], with both species demonstrating a significant RNAi response despite silencing of the endocytic pathway-related genes or pharmacological inhibition of scavenger receptors and suggests, dsRNA uptake mechanisms may be conserved in different orders of insects. However, the mechanism of interaction between dsRNA and scavenger receptors, the number of scavenger receptors involved in dsRNA uptake still requires basic functional investigation. Also, there is much conjecture regarding whether a receptor-mediated uptake mechanism is dominant in hemipteran insects, although evidence that insects use primarily clathrin-mediated endocytosis as an uptake mechanism does exist.[21] This could be investigated by selecting scavenger receptor genes from *D. melanogaster* and *T. castaneum* and identifying potential homologues from transcriptomic or genomic data of hemipteran insects as done in a similar way in *E. heros*.[12] Subsequently, the effects of RNAi-mediated silencing of individual or multiple homologues on RNAi efficiency primarily needs to be tested using injection and/or artificial diet feeding of dsRNAs, thus allowing bolus dosages to effect RNAi in specific tissues (e.g. organs), the whole insect by direct delivery to the haemolymph or by direct ingestion of RNAi effectors to affect the alimentary tract. If the clathrin-dependent dsRNA uptake mechanism exists in hemipteran insects, hydrogen peroxide or arachidonic acid could be exploited to enhance the endocytosis-mediated dsRNA uptake, as was demonstrated in the fruitfly *Bactrocera dorsalis*.[22,23]

4 RNAI-BASED STRATEGIES FOR HEMIPTERA PEST MANAGEMENT

RNAi has been investigated in approximately 38 Hemipteran species belonging to 14 families. Most studies have reported RNAi-induced insect mortality, mitigation of pathogen transmission, or reduction of insecticidal resistance. Studies that were documented in the different groups of hemipteran insects are presented in Table S1 and briefly summarized below.

4.1 Aphids

RNAi has been utilized to research potential control of several species of phloem-feeding aphids by individually targeting critical genes involved in feeding, moulting, development and fecundity (Table S1A). RNAi-mediated silencing of the *gap* gene *Hunchback*, chitin synthase *CHS*, salivary protein *C002* gene, aquaporin *ApAQP1*, and angiotensin-converting enzymes *ACE1* and *ACE2* in reducing fecundity, mRNA expression and survival of the *Acyrthosiphon pisum* are encouraging examples of the application of RNAi for aphid management.

Although we have highlighted successful RNAi studies conducted by either injection or feeding on transgenic plants, there is marked variation in the success of RNAi studies regarding *A. pisum*. This can be attributed to natural genotypic variations within populations that either induce total susceptibility or complete resistance when challenged with *ApAQP1* knockdown.[24] The implications are potentially significant regarding the effectiveness of using RNAi only treatments against aphids and further definition of resistance induced by genotypic variation in other aphid and hemipteran species is warranted.

RNAi also has been exploited to enhance the susceptibility of aphids to insecticides with the aim of reducing insecticide use and overcoming pesticide-resistant evolved populations. This idea combines insecticides with RNAi silencing of aphid genes responsible for insecticide detoxification. For example, silencing individually GATA-binding gene (*GAT*), multidrug resistance-associated gene (*MRA*) or takeout-like precursor gene (*TLP*) using artificial diet-mediated RNAi was shown to enhance, by 3.6-, 2.5- and 2.7-fold, respectively, the susceptibility of greenbug, *Schizaphis graminum*, to concentrations lethal to 50% of tested animals (LC_{50}) dosages of imidacloprid (Table S1A).

Some novel applications of RNAi have been discovered when exploring parental RNAi and transgenerational RNAi in aphids. Aphids are able to reproduce parthogenically and are viviparous. Transgenerational effects in birthed young from a parent exposed to RNAi have been reported in several hemipteran species such as *Sitobion avenae* (Table S1A), *Rhodnius prolixus* (Table S1I) and *Myzus persicae* (Table S1A). For instance, investigations of the effect of RNAi over four generations of *S. avenae* showed that first-generation aphids reared on zinc finger protein (*SaZFP*) transgenic wheat plants experienced a 50–80% decline in aphid population growth and a significant reduction in target mRNA expression over successive generations (Table S1A). These studies indicate that a transgenerational effect of RNAi delivered transgenically to aphids could facilitate the development of RNAi-based strategies for their control. However, no studies presently have reported transgenerational RNAi in aphids via nontransgenic approaches and, thus, research investigating transgenerational or parental RNAi in aphids through foliar spray, root drench and/or stem injection-based insectary trials is required.

4.2 Whiteflies

RNAi technology has shown potential as a control for whitefly, *Bemisia tabaci*, by silencing genes associated with osmoregulation, sugar transport, reproduction, development, signalling pathways, insecticide resistance and virus transmission.[25] Whitefly fed on transgenic tomato expressing *cyclophilin B* and heat shock protein *HSP70* dsRNAs led to 82–86% mortality and impaired the

ability of *B. tabaci* to act as virus vectors (Table S1B). This study indicates a potential application of RNAi for exploring insect–virus interaction and the phytopathogen transmission ability of whitefly. RNAi-mediated silencing of the ABC transporter gene *ABCG3* significantly enhanced the lethality of imidacloprid-exposed laboratory and field species of *B. tabaci* Q (Table S1B), demonstrating that the combination of RNAi-based products and insecticides could be exploited for improved whitefly control measures. Notably, the entomopathogenic fungus *Isaria fumosorosea* was used to deliver dsRNA targeting the immunity-related Toll-like receptor 7 (*TLR7*) gene of *B. tabaci* resulting in 90% nymph mortality (Table S1B). In a similar study, when RNAi-mediated *TLR7*-silenced whitefly adults were challenged with a mycotoxin from the entomopathogen (destruxin A (DA)), the mortality of whiteflies increased by 71% compared to DA treatment only (Table S1B). Still, large-scale field application of entomopathogens for delivery of RNAi may raise serious environmental and regulatory concerns, and future investigations need to explore the fate of entomopathogens on nontarget and/or beneficial organisms before considering its application for RNAi-based management of whitefly.

4.3 Leafhoppers and planthoppers

The adoption of RNAi technology has allowed the understanding of critical gene functions in hoppers and demonstrate its practical application in controlling several species of hoppers by silencing crucial genes related to ingestion, digestion, immune response, ovulation and behaviour (Table S1C). Microinjection of dsRNA targeting the salivary protein gene *NcSP75* into third instar nymphs and adults of green rice leafhopper, *Nephotettix cincticeps*, impaired the developmental growth of insects, resulting in 90% mortality when reared on rice plants (Table S1C). However, no significant mortality of *NcSP75*-silenced adults was observed when fed on an artificial diet, highlighting the role of *NcSP75* for phloem ingestion. Recently, silencing cytochrome P450 genes *CYP4G76* and *CYP4G115* via microinjection into brown planthopper *N. lugens* nymphs enhanced the rate of penetration of insecticides, exploring the idea of using RNAi for improving insecticide uptake in planthoppers (Table S1C). This could be further explored in different species of hoppers and orders of insects whose wax and cement layers of cuticle block the invasion of insecticides.

4.4 Psyllids

The Asian citrus psyllid, *Diaphorina citri*, is a pest responsible for citrus greening disease.[26] Psyllids possess functional RNAi machinery and several studies showing the application of RNAi for targeting *D. citri* genes responsible for pathogen transmission, wing development and reproduction have been published (Table S1E). RNAi silencing of the tropomyosin-X1 gene *DcTm1-X1* via artificial diet feeding of dsRNA to fifth instar *D. citri* nymphs led to 31% mortality (Table S1E), indicating the potential role of *DcTm1-X1* in pathogen transmission. RNAi has been used in a functional sense to identify genes involved in the metabolism of insecticides in *D. citri*. For example, suppressing the expression of the detoxifying enzymes, cytochrome P450 esterase and glutathione S-transferase via leaf dip assay showed a 47–72% reduction in the respective target mRNA level with a corresponding 66% and 83% mortality after two days (Table S1E). Further, RNAi also can be used as a topical insecticide, where dsRNA targeting esterases FE4 (*EstFE4*) and acetylcholinesterase (*ACh*e) applied topically to fourth and fifth instar nymphs resulted in >50% mortality (Table S1E). These studies demonstrate that RNAi may potentially be exploited for reducing insecticide application to control *D. citri*, further addressing insecticide resistance issues and providing evidence of using RNAi as a potential biopesticide to control the economically significant and industry devastating disease, citrus greening.

4.5 Plants bugs

RNAi studies in plant bugs are limited to few species, despite being key agricultural pests (Table S1F). Although microinjection-delivered dsRNA targeting the inhibitor of apoptosis (*IAP*) gene of *Lygus lineolaris* resulted in significant mortality, the lack of RNAi response by oral feeding-based RNAi (due to the salivary nucleases) limits the application of RNAi in high-throughput artificial diet assays.[27] However, silencing of the eye pigment-related genes via dsRNA injection into fifth instar *L. hesperus* led to visible RNAi phenotypes of red banding or uniform brown discoloration with cuticular and behavioural deficiencies (Table S1F). Furthermore, injection of dsRNA targeting the *CYP307B1* Halloween gene into fifth instar *L. hesperus* impeded adult eclosion resulting in 85% mortality (Table S1F). This study supported the assertion that *CYP307B1* Halloween gene is essential for insect development and targeting *CYP307B1* via RNAi highlights potential targets for *L. hesperus* control. Surprisingly, the feeding of transgenic maize and soybean expressing *v-ATPase E* dsRNA to the green plant bug, *Apolygus lucorum*, resulted in 75% mortality, indicating that oral RNAi might be effective against some species of plant bugs (Table S1F). Taken together, these studies provide valuable insights into the development of bug-resistant transgenic plants through a plant-mediated RNAi approach.

4.6 Stink bugs

The stink bug complex represents major pests of cotton and soybean in the USA and Brazil. RNAi offers a potential to control this complex because RNAi against the brown marmorated stink bug, *Halyomorpha halys (Stål)*, via feeding and/or injection of *IAP*, *PP1*, *ATPase N2B* juvenile hormone acid methyltransferase (*JHAMT*) or *vitellogenin* dsRNAs (Table S1G), demonstrates the existence of functional RNAi machinery in these insects. It is noteworthy that ingestion of liposome-encapsulated and ethylenediaminetetraacetic acid (EDTA)-formulated dsRNA targeting *v-ATPase A* and *muscle actin* could prevent the potential degradation of dsRNA by salivary nucleases in the neotropical stink bug, *Euschistus heros* (Table S1G). Here, an *ex vivo* study of dsRNA stability in insect saliva showed strong dsRNase activity, which supports the idea of using formulated dsRNA for the control of stink bugs and other insects.

4.7 Mealybugs

Mealybugs are extremely difficult to control with insecticides as a consequence of their waxy covering, mealy secretions and their habit of hiding in confined plant places.[28] In an effort to effectively control mealybugs, virus-based RNAi delivery technology has been researched as a potential control strategy. Here, infection of tobacco plants with recombinant Potato virus-X expressing chitin synthase 1 (*CHS1*), *bursicon* or *v-ATPase* dsRNA (Table S1H) of the cotton mealybug, *Phenacoccus solenopsis*, showed predation on virus-infected *Nicotiana tabacum* resulted in physical deformities with a 21–45% mortality rate in adults and their offspring. Likewise, silencing of *actin*, *v-ATPase* and *chitin synthase 1* via feeding on *Nicotiana benthamiana* plants systemically infected with recombinant Tobacco mosaic virus exhibited

lower fecundity and mortality in the citrus mealybug, *Planococcus citri* (Table S1H). Taken together, the application of recombinant viruses to deliver RNAi represents a potential alternative approach for mealybug control, although it is envisaged that significant regulatory hurdles would need to be overcome to use this RNAi delivery control method in agricultural systems.

5 DEVELOPMENT OF RNAI-BASED PRODUCTS FOR HEMIPTERAN PEST CONTROL

RNAi has been largely successful, in a research context (Table S1), and the studies outlined above highlight several critical aspects (Fig. 2) that need to be addressed before the development of RNAi-based products against hemipteran pests can be considered. Here, we provide an overview of the considerations required in the application of RNAi in Hemiptera and address issues that presently limit the commercial and environmental viability of RNAi for hemipteran pest control.

5.1 Off-target effects and dsRNA design

The application of RNAi for hemipteran insect control primarily rests on the selectivity and specificity of the dsRNA molecule to display minimum to no off-target effects with respect to the host plant, nontarget and benificial insects, animals and humans. Off-target effects are caused by significant sequence homology of the applied dsRNAs or siRNAs to a nontarget mRNA sequence. For example, when dsRNAs specific to Western corn rootworm, *Diabrotica virgifera virgifera*, were fed to larvae of a related coleopteran, a significant increase in mortality occurred compared to controls.[29] Therefore, the best approach for minimizing potential off-target RNAi responses is to select highly conserved species-specific genes or unique regions within mRNA sequences, ideally identified from transcriptomic or genomic data. Further assessment of identified sequences using web-based software such as ERNAi (https://www.dkfz.de/signaling/e-rnai3/) and/or dsCheck (http://dscheck.rnai.jp/) and by BLAST analysis against the transcriptomic datasets of human and selected beneficial insects are the initial considerations required to minimise potential off-target effects. Further, laboratory feeding-based toxicity studies against closely related hemipteran pest and common nontarget species (honeybees, predators and parasitoids), and assessment of any potential immune system activation cascades[30] can functionally validate sequences chosen for a target-specific RNAi-based biopesticide. Currently, this approach is being applied against the colorado potato beetle (*Leptinotarsa decemlineata*) (http://opendata.syngenta.agroknow.com/dataset/beneficials-description) and demonstrates the strategies to determine potential off-target effects against nontarget insects and related species when genomic data are not available. However, field-based longitudinal studies that are comprehensively controlled are the best and most realistic approach to determine the potential effects of RNAi-based products on biological networks, ecosystem and food chain functioning.

Earlier studies involving the delivery of dsRNA to hemipteran insects by microinjection or artifical diet have preferentially employed long dsRNAs ranging from 143 to 675 bp by contrast to sRNAs to induce RNAi (Table S1). In general, greater success has been achieved with dsRNAs >200 bp (Table S1) with higher silencing efficiencies observed with dsRNA of lengths of 211 and 592 bp in *D. melanogaster* S2 cells.[17] In the few hemipteran RNAi studies to date, suppression of target expression has been

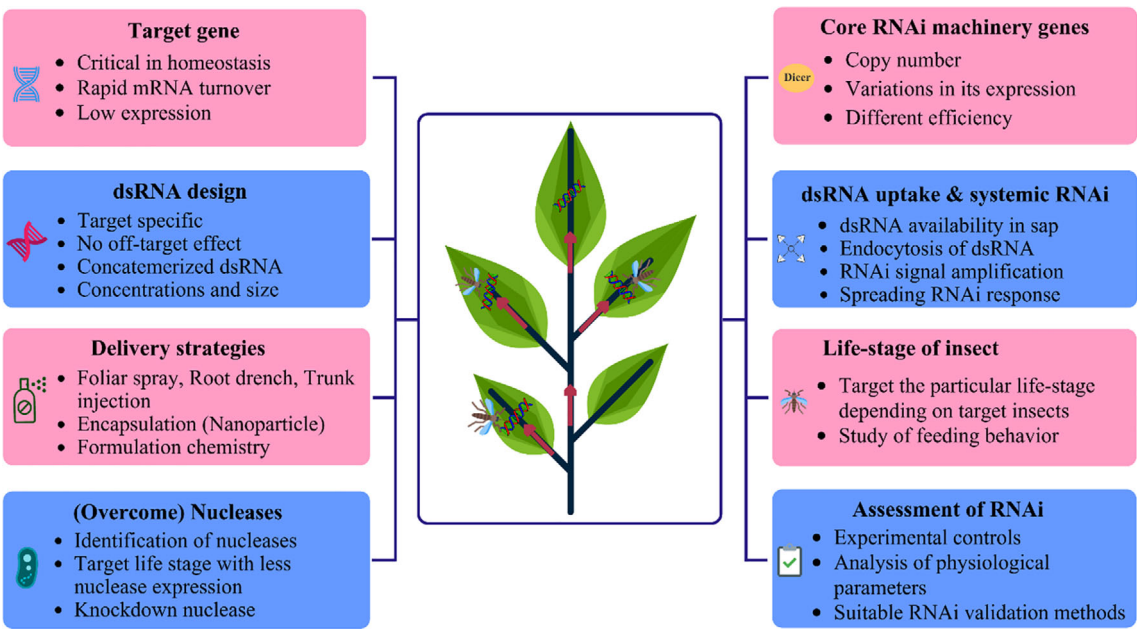

Figure 2. Variables affecting RNAi efficiency and potential avenues for improving the RNAi-mediated management of hemipteran insects. The first parameter for RNAi-mediated gene silencing is the selection of a target gene which plays an essential role in a biological pathway of the insect. Then, a highly conserved region of the gene must be identified using *in silico* approaches to minimize off-target effects on nontarget organisms. The following step is to select a suitable RNAi delivery strategy (foliar spray, root drench or trunk injection) which can deliver dsRNA to the vascular system of plants. The target life-stage of the insect must take up the RNAi molecule which can be protected from gut nucleases using nanoparticles and/or formulation strategies. Then, target dsRNA must be recognized and processed by the RNAi machinery of the insect into siRNAs. A systemic and transgenerational RNAi has been demonstrated in few hemipteran insects; however, the mechanisms of RNAi spreading still need further investigation. Lastly, RNAi silencing in insects must be confirmed by phenotypic observations as well as reliable molecular techniques such as qPCR and Northern blot.

obtained with siRNAs (21–22 bp) in the *A. pisum* and *B. tabaci* (Table S1A,B). Interestingly, using dsRNA 756 bp and 1068 bp in *Spodoptera litura*[31] and *Helicoverpa armigera*,[32] members of the Lepidopteran order notoriously refractile to RNAi, resulted in gene silencing, suggesting that longer dsRNAs (>700 bp) also can be effective in insects. Although dsRNAs >200 nucleotides long offer the advantage of resulting in a high number of processed siRNAs and thus enhancing RNAi effects,[33] an essential consideration is that longer dsRNAs may increase the potential for off-target effects on beneficial organisms resulting from the potentially large siRNA pools generated. The optimal length of dsRNA required for maximal RNAi response in hemipterans is a topic of debate and is an issue that needs to be addressed, namely by testing multiple dsRNAs of varying lengths targeting different regions of a single gene of interest in the specific pest via appropriate feeding experiments. As a starting point, by contrast with siRNA molecules (~20–25 nt), researchers should consider dsRNA lengths of >200 to <800 bp with the shortest dsRNA molecule chosen that provides the maximal effect. This approach can significantly minimize any potential off-target effects on the nontarget species. Therefore, determining the optimal length of target-specific RNAi effectors in conjuction with effective siRNA analysis will improve the development of RNAi-based biopesticides.

There are several reports describing the knockdown of transcript expression of a single gene in hemipteran insects using RNAi causing mortality (Table S1). However, in our opinion, to be truly effective as a crop protection application and taking into account the complex metabolic pathways of insects, a single gene knockdown albeit effective in killing the insect provides no redundancy and simultaneous multigene knockdown in the hemipterian under study should be explored.[34] Here, the design of RNAi constructs using gene pyramiding (multiple target genes) and/or concatomerization (repeats of target dsRNA) and /or multiple targets within a single gene are approaches to focusing on multiple sites simultaneously to potentially achieve broad-spectrum control of hemipteran insects. For instance, simultaneous knockdown of *trehalase-1* and *trehalase-2* genes in *N. lugens* via injection resulted in increased moulting deformities compared to individually targeted genes (Table S1D). Likewise, feeding of concatomerised dsRNA targeting three different regions of *acetylcholinesterase* through cabbage leaf-disc induced higher mortality of *Plutella xylostella* compared to nonconcatemerized long dsRNA.[35] These studies show the feasibility of targeting multiple genes or regions of a gene for improving RNAi effects; however, care must be taken in construct design to avoid off-target effects and/or a loss of RNAi efficiency resulting from competition between dsRNAs for binding with core RNAi enzymes as observed in a similar way in *C. elegans*[36] and *T. castaneum*.[37]

5.2 Concentration and delivery of RNAi

The optimal concentration or dosage of dsRNA required to trigger an RNAi response in hemipteran insects differs with the insect targeted, developmental stage, the target mRNA abundance, delivery method, dsRNA stability and processing. Studies on *N. lugens* and *A. pisum* (Table S1D,A) showed that the interference response was directly proportional to the dsRNA concentration. Conversely, increasing the dsRNA dosage does not always result in increased gene knockdown, but can reduce the time to insect death,[38] and higher concentrations of RNAi effectors or delivery of multiple dsRNAs/siRNAs can oversaturate the RNAi machinery of the insect.[11] Thus, the optimal dosage of dsRNA ideally needs to be determined using dose-range experiments with consideration of tissues targeted and expression level of the gene and, potentially, feeding behaviour. A defining feature of the order Hemiptera is the presence of specialized mouthparts able to pierce and suck sap from plants (or, in the case of predatory Hemiptera, bodily fluids). With respect to phytophagous Hemipteran insects, sap can be drawn from phloem (e.g. aphids), xylem or mesophyll tissues (e.g. plant/leaf hoppers). The mode and site of feeding may impact on the amount and thus the efficiency of which RNAi affects insects predating on treated plants is a consideration that should be explored.

Homologues of lepidopteran extracellular endonucleases have been reported in several hemipteran insects, such as *Nezara viridula*, *B. tabaci* and *H. halys* (Table S2). DsRNAses reduce the concentration-dependent RNAi response as it challenges the amount of intact dsRNA that is taken up and/or transported to the target cell. dsRNase-mediated dsRNA degradation is responsible for reduced RNAi responses in ten hemipteran species.[39] Extracellular dsRNase, homologues to a *Bombyx mori* endonucleases in the midgut of *Myzus persicae*, likely contributes to the low efficacy of orally delivered dsRNA targeting the salivary gene *C002* (Table S2). Four approaches could be used to overcome the enzymatic degradation of dsRNA in insect body: (i) selecting developmental stages of the insects with the lowest expression of nucleases (dsRNase); (ii) stacking or mixing dsRNase-specific dsRNA with target gene-specific dsRNA; (iii) dsRNA formulation using liposome/ribonucleoprotein[40] encapsulation or guanylated polymer[41] to protect dsRNA and enhance cellular uptake; and (iv) continuous exposure of insect to dsRNA targeting the gene of interest ensuring persistent knockdown of the target genes.

For plant-mediated RNAi control of hemipteran insects, delivery of dsRNA is a major hurdle as RNAi effectors must reach the vasculature of the plant. Previous studies have shown successful delivery of RNAi molecules to hemipteran pests by plant-incorporated protectants through transgenic plants, or by nontransgenic approaches through root and trunk injection (Table S1). Despite successful studies involving transgenic plants, this approach has not been adopted universally in all field crops owing to technical limtations, the refractile nature of many crop plants to stable transformation, long development periods,[42] high development costs, significant regulatory hurdles, and community aversion to transgenic fruit and fibre.[8] Therefore, research is warranted to develop nontransgenic dsRNA delivery approaches that can trigger RNAi response in sap-sucking pests without making changes to the plant genome.

For insects feeding on stems, leaves or fruits, foliar spray of RNA molecules may offer an alternative nontransgenic method of delivery to plants. One exciting study on foliar application of RNA molecules against the Colorado potato beetle, *L. decemlineata*, was performed using dsRNA targeting the *actin* gene, resulting in >90% mortality.[43] A similar approach has been tested against the psyllid *D. citri*, the leafhopper *Homalodisca vitripennis* and the root weevil *Diaprepes abbreviates*, on citrus plants.[44] Here, dsRNA targeting arginine kinase, delivered to citrus trees via trunk injection or root drench (2 g dsRNA/15 L water) showed systemic distribution and persistence in trees for 57 days with, presumably, plant-processed siRNAs, able to be detected for up to three months. Surprisingly, persistence of RNAi effectors in *D. citri* and *H. vitripennis* was detectable up to eight days post-ingestion from treated plants.[44] Trees were directly inoculated with dsRNA but the persistance and longevity of the signal highlights the potential of a foliar spray to persist and potentially control hemipteran insects. Recently, exogenously applied dsRNA on tomato leaves

dusted with carborundum showed systemic translocation within 20 min from local leaf application to an adjacent leaf, and was able to be taken up by aphids, whiteflies and mites.[45] However, RNAi-mediated control of hemipteran insects fed on plants that have been topically sprayed with dsRNA is yet to be reported. This mode of delivery could be challenging against sap-feeders owing to potentially insufficient uptake and transport of exogenously applied dsRNA to the vascular sap of the plant.

The addition of surfactants/penetrants or carriers such as nanoparticles may have to be used for dsRNA to traverse rigid plant cell walls and be internalized in the cell, which poses the primary barrier for foliar application of dsRNA.[46] DNA nanostructures with programmed sizes and shapes have been synthesised to deliver siRNA to mature *N. benthamiana* plants without any external aid or mechanical inoculation/abrasion.[46] These DNA nanostructures were internalized into plants cells and delivered siRNAs which efficiently silence endogenous plant genes. Likewise, DNA nanostructures can be synthesized and programmed for exogenous delivery of dsRNA into plants and, subsequently, foliar spray-based bioassays can be conducted to assess the efficacy of nanostructure-delivered RNAi-mediated gene silencing against hemipteran pests. A comprehensive study with these parameters would elucidate the potential of programmable nanomaterial-based delivery of dsRNA for controlling hemipteran pests.

5.3 dsRNA production costs and stability

Field deployment of RNAi-based products for controlling hemipteran pests depends on the economical production of dsRNA. Large-scale *in vivo* (using bacteria) or *in vitro* (using enzymes) dsRNA production allow g- to kg-scale dsRNA synthesis with economy of scale reducing costs.[8] In the near future, dsRNA production cost is expected to decrease rapidly with commercial companies investing in dsRNA production capacity.[33,47] The amount of dsRNA required for practical field application of RNAi will need to be determined empirically, with consideration of the insects' life stage, dsRNA purity required, quantity required and agricultural production system (i.e. broadacre *versus* protected cropping or hydroponic). Also, the necessity of multiple/periodical/seasonal applications of RNA-based biopesticides throughout the growth cycle of the plant to maintain the RNAi signal implies an increase in cost to farmers. Thus, future research is warranted to quantify the intensity of dsRNA-based biopesticide used in the field. One such model is using an appropriately modified treatment frequency index as applied in case studies on insecticides.[48] In addition, dsRNA formulation strategies will reduce costs and boost the use of RNAi-based strategies for hemipteran pest control.

The short half-life of dsRNA on plants or in the soil is a major limitation to its commercial application.[49] DsRNA formulation using nanoparticles is a promising approach to increase stability and improve the efficacy of gene silencing in hemipteran pests, allowing crops/plants to be protected for a longer duration. Pioneering work on the topical application of dsRNA complexed with layered double hydroxide (LDH) nanosheets, termed BioClay,[50] protected dsRNA from nucleases and demonstrated its ability to prevent degradation of RNA on the leaf surface. Functionally, BioClay afforded virus protection to both sprayed and unsprayed systemic leaves, suggesting that dsRNA can enter the leaf and be transported to other parts of the plant most likely through the vascular system. To date, this innovative clay-based RNAi delivery system is yet to be tested against hemipteran insects.

5.4 Potential development of resistance

Numerous studies have shown a trend of increasing insect resistance against conventional chemical insecticides.[51,52] Presently, no studies have been published regarding the evolution of resistance in hemipteran pests toward RNAi; however, evolution of resistance to dsRNA in the coleopteran insect, Western corn rootworm, *D. virgifera virgifera*, recently has been shown experimentally.[53]

Concerning hemipteran insects, three potential mechanisms of resistance are envisaged that can affect RNAi efficiency in sap-feeding pests: (i) target modification; inducing resistance due to variations and/or mismatches between dsRNA and the endogenous target sequence[54]; (ii) mutations in the core RNAi machinery of the insect (Table S2) that could render the interference response null[55], and (iii) physiological mutations the target pest evolves resistance through mutations that affect the stability and uptake of dsRNA in the midgut.[56] Physiological mutation has been induced in the laboratory using RNAi studies on field populations of *D. virgifera virgifera* which demonstrated a differential RNAi response without a difference in the targeted gene sequence,[57] suggesting that field insect populations may respond rapidly to RNAi pressures owing to inherent genetic and physiological variations. This has also been experimentally defined recently in the aphid *A. pisum*,[24] suggesting that this mechanism may already be inherent in hemipteran populations.

Although RNAi delivered transgenically can be exploited for hemipteran pest control, the constant supply of dsRNA from plants may enhance selection pressure and induce resistance evolution in these insects. Thus, nontransgenic delivery approaches such as foliar spray and soil drench[33] may mitigate constant exposure of hemipteran insects to dsRNA treatments thereby reducing the evolution of resistance in the target insects. Further, if RNAi is able to reduce the population in a crop to below economically damaging infestation levels and/or enhance the effectiveness of applied pesticides by inducing increased suseptiblity or reduced application rate, a significant milestone would be reached in using RNAi as a crop protection product. However, it is foreseeable that RNAi resistance will evolve and the research field would benefit from deeper understanding of the potential mechanism of RNAi resistance as explored in *L. decemlineata* and *T. castaneum* by identifying key proteins responsible for RNAi resistance.[58] Potential candidate gene homologues involved in RNAi resistance and the potential to target these genes would be useful for the management of RNAi resistance. Also, several strategies to subvert field-evolved RNAi resistance in hemipteran insects can be mitigated by refuge plantings,[53] concatomerization/pyramiding of multiple dsRNAs in a single expression construct, or combining dsRNA with active substances having different modes of action.

6 CONCLUSIONS AND FUTURE DIRECTIONS OF RNAI IN HEMIPTERAN PEST MANAGEMENT

Although considerable progress has been achieved in RNA-mediated management of hemipteran pests, it is still in its infancy and the field has several significant hurdles that will require substantial research focus to cross. First, RNAi efficiency in hemipteran insects can be extremely variable due to dsRNA degradation by nucleases, lack of functional RNAi machinery, and limited knowledge regarding dsRNA uptake, transport and systemic propagation mechanisms. Thus, basic research to

determine the role of enzymes that degrade dsRNA upon ingestion, RNAi machinery including core RNAi genes responsible for dicing dsRNA molecules, ideally from different tissue extracts, delineation of the dsRNA uptake mechanism and endosome trafficking in model- and species-specific cell lines compared with whole insect experiments, while taking into account genotypic variation, would define why such differences are observed. Recent studies on a transmissible RNA pathway in the honeybee[59] and the development of small RNA sequencing methods to confirm systemic RNAi-based immunity in *Drosophila*[60] can be applied to facilitate future studies exploring the mechanism of RNAi translocation/spreading in hemipteran insects. Furthermore, insight into the interaction between insect RNAi core machinery and virus infection to tease out details of the interactions between the insect RNAi mechanism regarding dsRNA uptake, molecule processing, loading, RNAi sequestration and other regulatory processes such as recruitment and interaction with insect-encoded microRNAs as studied in a similar way to *Drosophila* in the context of antiviral response by RNAi-suppressor-defective mutant viruses provide alternative approaches to define the functions and limitations of hemipteran RNAi machinery.[61]

The potential benefits of deploying RNAi as a crop-protection strategy include insect-specificity, low toxicity compared to existing pesticides and minimal environmental impact. RNAi strategies need to be explored further to bolster community acceptance and to avoid regulatory delays. The short persistence of dsRNA in the environment and its uptake and systemic transport in the plant presumably limits its application for effective management of any insect pest, not just hemipterans. This issue must be addressed by experimental assessment of the molecular and cellular basis of dsRNA uptake in the plant, and any potential limits addressed by using formulation/encapsulation chemistry or nanoparticles to improve the persistence, penetration and transport of RNAi molecules in the plant or insect. RNAi strategies such as stacking or pyramiding dsRNA molecules in combination with sublethal-dose insecticides, although not ideal, can be adopted to enhance RNAi efficiency, check population control and to minimize the evolution of resistance in hemipteran insects. If development is conducted diligently and thoroughly encompasses bioinformatic identification, *in silico* best-design of RNAi effectors, and laboratory- and field-based RNAi toxicity studies, RNAi-based biopesticides have tremendous potential to revolutionize hemipteran pest management in a safe, specific and effective manner.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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