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RNAi for Insect Control: Current Perspective and Future Challenges

Rajan Katoch · Amit Sethi · Neelam Thakur · Larry L. Murdock

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Abstract The research on the RNA interference (RNAi) for the control of insect pests has made significant growth in recent years. The availability of the genomic sequences of insects has further widened the horizons for the testing of this technology to various insect groups. Different modes of application of double-stranded RNA (dsRNA) have been tested; however, the practicability of delivery of dsRNA in insects still remains the biggest challenge. Till date, the oral delivery of dsRNA in insects is one of the efficient approaches for the practical application of this technique. The uptake of dsRNA from the insect gut is mediated either by SID-1/SID-2 transmembrane proteins or by endocytosis; however, the systemic RNAi machinery still remains to be revealed in insect species. The RNAi-mediated gene knockdown has shown striking results in different insect groups, pointing it to be the upcoming technique for insect control. However, before the successful application of this technique for insect control, some potential issues need to be resolved. This review presents the account of prospects and challenges for the use of this technology for insect control.

Keywords RNAi · Dicer · Gene knockdown · Insect control · dsRNA

Introduction

Crop losses caused by insects and insecticide usage to manage insects cost billions of dollars every year worldwide; this continuous usage of insecticides poses a potential threat of insecticide resistance development. The control of insect pests has become a priority in research planning as 3,000 million dollars were allocated for the protection of the top five most important agricultural crops with a mission to look for durable and cost-effective alternative pest control

R. Katoch (✉)
Biochemistry Laboratory, Department of Crop Improvement, College of Agriculture,
CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, HP 176062, India
e-mail: rajankatoch@yahoo.com

N. Thakur
Department of Zoology, PAU, Ludhiana 141004, India

A. Sethi · L. L. Murdock
Department of Entomology, Purdue University, West Lafayette, IN 47907, USA

strategies [86]. Out of the latest approaches of the twenty-first century developed in this direction, transgenics have emerged as a vital tool for the management of insect pests based on practical success shown by *Bacillus thuringiensis* (*Bt*) toxin in the protection of wide categories of crops and, to some extent, replacing chemical insecticides. However, many important insect pests are not covered under *Bt* protection, and also, there remains an imminent threat of at least some species developing *Bt* resistance [110, 111]. Therefore, there is a need to look for another sustainable and environmentally friendly approach to manage insect pests, and one such approach could be RNA interference.

RNA interference (RNAi) is a posttranscriptional gene-silencing mechanism which is initiated by the introduction of double-stranded RNA (dsRNA) into a cell [10, 46]. This technique is well known in plants as "posttranscriptional gene silencing." The process is closely related to posttranscriptional gene regulation by microRNAs (miRNAs), which involve inhibition of translation initiation [97]. RNAi was initially reported in plants [81], and thereafter, there were tremendous research outputs on different components of RNAi. Gene silencing using RNAi technology has been well studied in invertebrates, in particular *Caenorhabditis elegans* [31, 109, 116] and *Drosophila* S2 cells [14, 44]. This technique is usually accomplished by injection of double-stranded RNA or by a diet containing dsRNA. After the report of RNAi nearly 13 years ago by Fire et al. [31], it has been exploited for various applications ranging from study of functional genomics to gene knockdown effect in plants and insects. Gene knockdown by RNAi has now become a valuable tool to study the function of gene(s) in a variety of organisms. This technique has also led to the development of new methods of control of insect pests of agriculturally important crops by genetically engineering the plants to express dsRNA.

Eukaryotic organisms including insects have evolved a common mechanism for sequence-specific gene silencing, which is triggered due to the presence of dsRNA [50]. The idea of using RNAi to protect plants against insects by downregulating essential gene functions in the insect has been well conceived a long time ago, but the real application of the technique was considered unfeasible. The experimental results of RNAi are now well documented in some of the insects, such as *Drosophila melanogaster* [18, 77], *Tribolium castaneum* ([32, 61], *Bombyx mori* [47], *Reticulitermes flavipes* [145], *Rhodnius prolixus*, and *Glossina morsitans morsitans* [50].

Based on the advanced state of research on *C. elegans*, much insight on the uptake mechanisms of dsRNA and the machinery involved in RNAi has been already known. Nowadays, numerous studies involving RNAi are being conducted in different domains including plants, viruses, bacteria, fungi, nematodes, and insects. In insects, further progression of RNAi has now become possible as more insect genomes are becoming available day by day. However, this technique has yet to deliver its practicality in the field of plant defense against invading insect pests.

For effective insect control by RNAi, there should be a regular and an autonomous take-up of the dsRNA by the insect, which can be facilitated by feeding and digestion in the insect gut. Out of the three insect gut regions, the midgut is the best target for dsRNA uptake because it is the main site of absorption in insects [43]. Indeed, research in recent years gave new insights into dsRNA uptake mechanisms in insects with emphasis on uptake through midgut and body tissues, e.g., the transmembrane channel-mediated uptake and the "alternative" endocytosis-mediated uptake. RNAi is now being considered as a potential future approach for the control of insect pests due to its high specificity [37, 88].

In the present review, we discuss the recent developments in RNAi in insects and its application prospects in plants for insect pest management. We also discuss the mechanism of dsRNA uptake in insects, its current delivery approaches, and future problems for its future use in host plant resistance to control agriculturally important insect pests

RNAi Mechanism

RNAi is a specific downregulation or knockdown of gene expression by dsRNA involving degradation of a target messenger RNA (mRNA). In this technique, dsRNA can specifically lower the transcript abundance of a target gene when injected into an organism or introduced into cultured cells [31]. RNAi involves the cleavage of dsRNA precursors into small interfering RNA (siRNA) of approximately 21 to 23 nucleotides by the enzyme Dicer. These siRNAs are then incorporated into an RNA-induced silencing complex (RISC). Argonaute proteins, the catalytic components of RISC, use siRNA as a template to recognize and degrade the complementary mRNA [74]. RNAi can therefore be exploited to suppress gene expression through highly specific depletion of target transcripts. The functional RNAi machinery has two major components, (1) the core component inside the cells, which is comprised of Dicer enzymes, RNA-binding factors, and Argonaute protein, and (2) systemic component that amplifies the dsRNA signal and allows it to spread to other tissues within the animal [102].

In insects in general and *D. melanogaster* in particular, two RNA silencing pathways are known to exist which are mediated by siRNAs and miRNAs [118]. The miRNA pathway primarily uses endogenous products transcribed from the cell's genome with dsRNA structure to regulate developmental processes; the siRNA pathway is involved to primarily function as a defense response against exogenous dsRNAs. The specificity of RNAi is sequence based as it depends on the sequence of one strand of the dsRNA which corresponds to a part of or the entire gene transcript. The mRNA degradation is mediated by the production of siRNAs from the dsRNA, which is cleaved by dsRNA-specific RNase III-like endonucleases referred to as Dicers. In *D. melanogaster*, Dicer-1 is mainly used to produce miRNAs, whereas, Dicer-2 is responsible for the processing of long dsRNAs into siRNAs [66]. These siRNAs are generally 21-bp fragments of dsRNA and have two base extensions at the 3′ end of each strand. The mechanism involves assembly of one strand of the siRNA into an RISC in conjunction with the argonaute multidomain protein containing an RNaseH-like domain. After discarding the passenger strand, the RISC binds to an mRNA, cuts it, and thereby hinders translation [30]. An RNA-dependent RNA polymerase (RdRP) interacts with the RISC complex and generates a fresh lot of dsRNA which is based on the partially degraded target template. These RdRPs have been identified in plants and nematodes which amplify the RNAi effect by synthesis of endogenous dsRNA of target mRNAs [24, 100] (Fig. 1).

Efficient systemic RNAi also requires a machinery to amplify the initial dsRNA trigger and subsequently export it to the other tissues in the organism [50]. The first component relies on the function of RdRP enzymes, which are most extensively characterized in plants and the nematode *C. elegans* and which generate “secondary siRNAs” that can greatly sustain and amplify the RNAi response [22]. As compared with *C. elegans*, *Tribolium* lacks a robust systemic RNAi mechanism [120]. For example, *Tribolium* lacks a *C. elegans*-like RdRP; therefore, the signal amplification expected in *Tribolium* must be based on a different gene with a similar activity or possibly even a different mechanism. This concludes that, although dsRNA could inhibit gene expression in insects, it cannot act continuously as it does in nematodes [3, 120, 123]. Therefore, further investigations are required to define the function of the *sid-1* gene in insect RNAi. The absence of dsRNA amplification and RdRP in insects makes gene knockdown effects exhibited by feeding dsRNA to insects temporary. Therefore, RNAi effects achieved in the gut through feeding would require a continuous input of high levels of dsRNA for durability [88]. The amplification of the initial signal for RNAi takes place at three different steps in *C. elegans*. The first level of amplification could

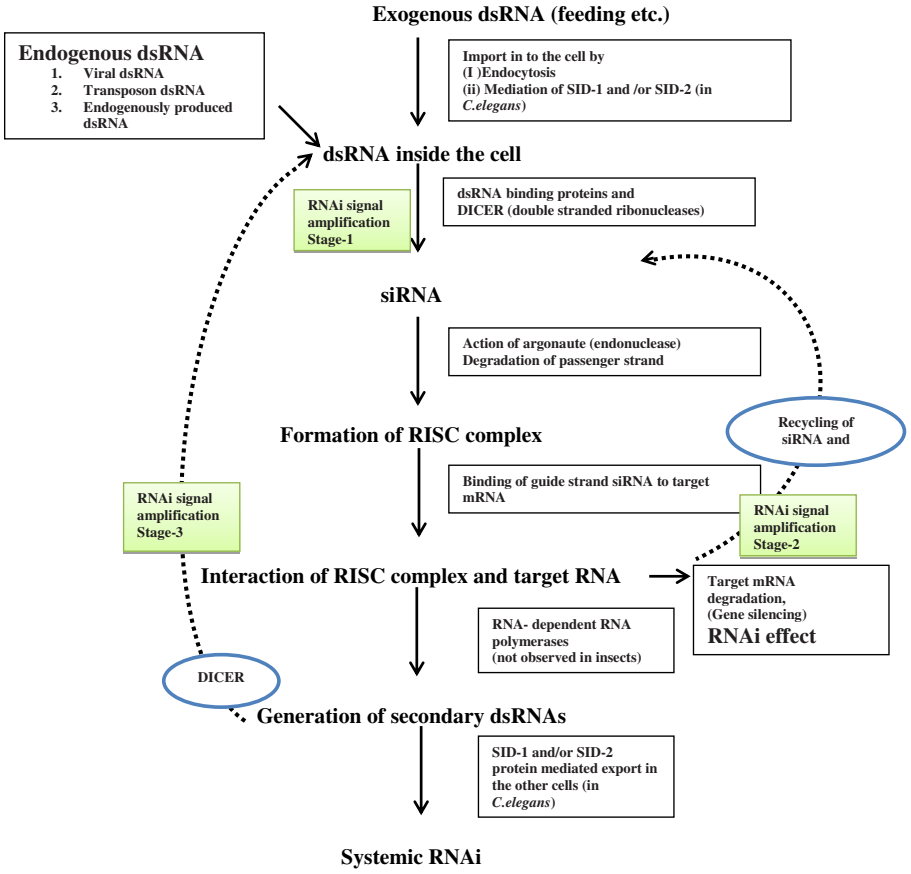

Fig. 1 Different stages of RNAi-mediated gene silencing. The mechanism of dsRNA transport varies in different organisms. dsRNA can induce the degradation of homologous mRNA transcripts when it is processed to 21–23-bp siRNAs by an RNase III-like enzyme known as Dicer. The resulting siRNAs function as sequence-specific guides to direct a RISC to degrade target mRNAs. The amplification of RNAi signals has been shown at three different steps

result from cleavage of the dsRNA trigger into siRNAs by Dicer. For a frequently used dsRNA, triggers of 500–1,000 bp would result in a 20- to 40-fold increase in molar ratio from target to trigger [100]. The second amplification of the initial signal generally results from recycling of siRNAs after the degradation of target mRNA. The third amplification process generally involves the activity of RdRPs for the production of a second generation of siRNA which drastically improves the effectiveness of RNAi [100]. For this, the antisense siRNAs with 3′ hydoxy group annealed to the ssRNA (target) elongates by RdRP activity resulting in longer stretches of dsRNA. The cleavage of RdRP elongated regions of dsRNA by RNase III, or Dicer activity produces numerous siRNA for the amplification of RNAi effect (Fig. 1). This mechanism of amplification stands for persistent RNAi effects. The gene encoding an RdRP homolog in insects and mammals has not been reported. Therefore, there is a possibility of involvement of a separate set of enzymes with RdRP-like activity in insects and mammals, or these groups of organisms may not require RdRP for this RNAi amplification [104].

RNAi Effects

The RNAi can be well distinguished into intracellular and extracellular RNAi. The intracellular RNAi involves the expression of hairpin RNAs in transgenes, introduction of dsRNA in cells using transfection or electroporation, and by injection directly into a cell. In extracellular RNAi, the dsRNA is delivered by soaking, feeding, or injection into the hemocoel. The latter requires uptake of dsRNA molecules by the cells [138]. The RNAi mechanism has been further categorized into cell-autonomous and noncell-autonomous systems.

Cell-Autonomous RNAi

In cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced or expressed. Here, the dsRNA is cleaved by an RNase III (Dicer) into 21–25-nt-long siRNA, and then, these siRNAs are incorporated in RISC, which mediates mRNA degradation. Cell-autonomous RNAi uses broadly conserved machinery, and similar strategies in a wide range of organisms and many initial studies were mainly focused on cell-autonomous RNAi [40, 130].

Noncell-Autonomous RNAi

The noncell-autonomous involves an RNAi effect at a site different from the application or production site of the dsRNA, and therefore, it happens exclusively in multicellular organisms. Noncell-autonomous RNAi was first noted in *C. elegans* by Fire et al. [31], when injection of dsRNA into the body cavity resulted in targeted gene silencing throughout the injected animal and its progeny. The environmental RNAi in *C. elegans* occurs in the following steps:

(a) dsRNA uptake by the intestinal cells
(b) Export of either the dsRNA or dsRNA-derived silencing signals from the intestinal cells
(c) Import of the silencing signals into other issues (e.g., muscle, epidermis, germline)
(d) Cell-autonomous RNAi-mediated gene silencing

Noncell-autonomous RNAi effects are of two types, such as environmental RNAi and systemic RNAi [126, 130]. Although, environmental and systemic RNAi are likely to share some overlapping machinery, the two processes are distinct because environmental RNAi has also been observed in unicellular organisms such as protozoans. With the exception of vertebrates, environmental RNAi has been observed in a wide range of species. It includes all the processes where dsRNA is taken up by a cell from the surrounding environment.

Noncell-autonomous RNAi has high applicability for the efficient application of RNAi in insect control. The treated insect needs to internalize the dsRNA of a target gene through feeding on plants sprayed with dsRNA or on transgenic plants expressing dsRNA. Further, the dsRNA must be taken up from the gut lumen into the gut cells involving environmental RNAi. If the target gene is expressed in a tissue outside the gut, the silencing signal will also have to spread via cells and tissues involving systemic RNAi. In vivo studies with *Spodoptera litura* provided an evidence for the role of aminopeptidase N (APN) as a *Bt* toxin receptor in the midgut of insects. After injecting dsRNA into the larvae, midgut columnar cells were able to pick up dsRNA molecules injected in the hemocoel, and the silencing of APN was observed in

the midgut columnar cells demonstrating that RNAi work in the whole larval body. Further, they suggested that the administration of dsRNA is heritable to the next generation [91].

The effect of environmental RNAi has also been studied with cell line experiments where dsRNA is applied via soaking of the cells in a dsRNA-enriched medium. These experiments revealed a link between Cry1Ac insecticidal protein and an aminopeptidase N (HaAPN1) in the gut of larvae of *Helicoverpa armigera*. Sf21 cells were modified to express HaAPN1. The expression of Haapn1 mRNA and its protein were reduced significantly as compared to the controls after 48 h of dsRNA soaking treatment, which ultimately resulted in decrease in the sensitivity of HaAPN1-expressing cells to Cry1Ac protein [103].

Systemic RNAi is prominent in multicellular organisms as the silencing signal travels from one cell to another or from one tissue to another [130]. The comparison of the genes involved in cell-autonomous and systemic RNAi between *T. astaneum* and *D. melanogaster* revealed that RNAi components are larger in *T. castaneum* than in *D. melanogaster*, thereby explaining the more sensitive response of the former to dsRNA compared to the latter. Contrary to this, there is little similarity between *C. elegans* and *T. castaneum* RNAi gene inventories, although both show efficient systemic RNAi [120].

dsRNA Uptake Mechanisms

The mechanism of dsRNA uptake via the gut is still not very clear. Studies in *C. elegans* and *D. melanogaster* revealed at least two pathways for dsRNA uptake [120, 132].

Transmembrane SID-1/SID-2 Channel Protein-Mediated Pathway

The RNAi effects inside the body of insects are determined by the spread of silencing RNA molecules known as systemic RNAi. In plants, nematode (*C. elegans*), and the planarian (*Schmidtea mediterranea*), the RNAi is systemic as the RNAi signal spreads throughout the entire biological system by traveling between cells [31, 83]. However, in insects, systemic RNAi is always not the case. For example, fly cells take up dsRNA, which cannot spread throughout the entire body [95]. A mutant nematode (*C. elegans*) was identified with impaired ability to show systemic RNAi response upon orally delivered dsRNA. The gene involved was identified as *systemic RNA interference deficient-1* (*sid-1*) which is essential to mediate systemic RNAi effect in *C. elegans* [29]. The study on RNAi-defective mutants (sid) have resulted in the description of two proteins involved in noncell-autonomous RNAi. SID-1 is a multispan transmembrane protein that is expressed on the cell surface, thereby mediating a systemic RNAi effect [132]. The protein is expressed on the cell surface and acts as a channel to allow passage of dsRNAs between cells and thereby enables uptake of dsRNAs. It probably functions as a multimer, in cooperation and/or coordination with SID-2, transporting dsRNA passively into the *C. elegans* cells. Homologs of *sid-1*-like genes have been identified in some insect species such as *T. castaneum*, *B. mori*, and *Apis mellifera* [136] and also in aphids [50]. *sid-1* mutant nematodes remain competent in cell-autonomous RNAi, and systemic RNAi does not perform in response to feeding, soaking, and injection of dsRNA or in vivo expression of dsRNA from transgenes. Therefore, *sid-1* mutants do not show environmental RNAi [133]. However, in the best characterized model insect, *D. melanogaster*, no *sid-1*-like gene has been found. It has not been demonstrated whether SID-1, which is also present in many insects, serves the same role [120]. Studies in *Drosophila* have shown that a systemic RNAi mechanism is lacking in this insect, which has been attributed to lack of an endogenous *SID-1* in the *Drosophila* genome [93]. However, expression of SID-1 protein in cultured *Drosophila* S2 cells

has been shown facilitating uptake of dsRNA [29]. A *SID-1* homolog has also been identified in *Schistocerca gregaria* [25] which had a significant sequence match within the honeybee (*A. mellifera*) genome. The partially assembled *B. mori* genome revealed several sequence similarities with *SID-1*. The possibility of SID-1 involvement in *Epiphyas postvittana* is also suggested due to the systemic effect observed during silencing of *EposPBP1* in adult antennae [123]. However, phylogenetic analysis and functional studies do not support a role for SID-1 homologs in the red flour beetle *T. castaneum* [120]. The pathway for spreading the RNA silencing signal still remains a matter of investigation for insects.

Another *C. elegans* mutant was identified, which was unable to mediate RNAi response when fed with bacteria expressing specific dsRNAs. This *sid-2* gene product (SID-2) was a gut-specific transmembrane protein of 311 amino acids with a single transmembrane region, facilitating environmental RNAi [133]. SID-2 functions exclusively in the uptake of dsRNA from the environment and is not required for the subsequent systemic spread of RNAi [133]. Three other membrane transport proteins (RSD-2, RSD-3, and RSD-6) have been identified in other insect tissues (e.g., gonads, fat bodies, salivary glands) as putative mediators of dsRNA uptake [55]. SID-2–GFP fusion protein localizes to intestinal cell apical membranes probably facilitating the import of ingested dsRNA from the intestinal lumen. However, SID-2 alone is not sufficient for dsRNA import from the intestinal lumen as *sid-1* mutants are also unable to uptake environmental dsRNA. Therefore, SID-2 might modify or compliment SID-1 at the lumen or might function in series with SID-1 to internalize dsRNA for SID-1 transport across the membrane [133]. Different correlations are proposed for interrelationship between the two proteins [130]:

(a) SID-2 modifies SID-1 protein to activate the transport.
(b) SID-2 binds dsRNA from the environment and delivers it to SID-1.
(c) SID-2 protein induces the endocytosis pathway of dsRNA, where SID-1 delivers dsRNA to the cytoplasm.

Endocytic Pathway of dsRNA Uptake

In *D. melanogaster* S2 cells, dsRNA is taken up via an active receptor-mediated endocytosis. Pharmacological inhibition of endocytic pathways disrupts exogenous dsRNA entry and thereby inhibits gene silencing. Studies involving vacuolar H^+ ATPase have further strengthened the hypothesis of endocytic uptake of dsRNA. In *D. melanogaster* and other insect species which apparently lack SID-1 ortholog, endocytosis appears to facilitate cell uptake [131]. Apart from inhibiting RNAi effects, the pharmacological inhibition of endocytosis in *C. elegans* also results in knockdown of endocytotic pathway components and produces worms with a "loss-of-RNAi-function" phenotype. This implies that dsRNA uptake into cultured *Drosophila* S2 cells does not involve a *sid-1*-based mechanism and goes by more common receptor-mediated endocytosis [95]. It seems like the receptor-mediated endocytosis is a widespread mechanism of dsRNA uptake and could be affirmative to the other classes of insects. If receptor-mediated endocytosis is worked out as a common mechanism of dsRNA uptake, then knockdown of important genes for the survival of insects of economic interest could be effectively targeted by oral delivery of dsRNA. Understanding the molecular mechanisms of determining how RNA spreads systemically inside the insect body would facilitate the application of RNAi technology for pest control. In recent years, several reviews have systematically summarized research results and limitations of RNAi technology in the field of entomology [50, 88, 113]. In the study by Terenius et al. [113], who collected detailed data from more than 150 experiments, it was

proposed that RNAi is particularly successful in the Saturniidae family. In comparison, gene expression in epidermal tissues seems to be most difficult to silence, and gene silencing by feeding dsRNA required high concentrations for success. This review also points to the need for further investigations on the mechanism of RNAi in lepidopteran insects and its possible connection to the innate immune response [113].

dsRNA Delivery into Insects

The overall success of RNAi is dependent on the mode of delivery of dsRNA; therefore, it remains a major consideration for planning any strategy to use RNAi in insect control. Various delivery systems have been studied in different groups of organisms. As gene silencing is only limited to cells that are infected, the main challenge is the selection of the delivery system [113]. The main dsRNA delivery methods tested so far include injection, feeding, soaking, and transgenic plants expressing dsRNA.

Delivery of dsRNA Through Injection

During initial stages of research on this technology, the direct injection of dsRNA into target tissues and life stages of insects was used as a promising approach for initiating RNAi effects [15, 90]. The dsRNA is synthesized according to the target gene in the insect in vitro and then injected into the insect hemocoel [26]. RNAi effects were also achieved via injection in *Acyrthosiphon pisum* [80]. The major advantage of injecting dsRNA into the insect body is high efficiency of inhibiting gene expression. The direct injection of dsRNA has the advantage of delivery of dsRNA immediately to the target tissue or into the hemolymph, and the exact amount of dsRNA brought into an organism is known, in contrast to delivery by soaking or in some cases by feeding [138]. However, this method has disadvantages of being delicate and time consuming. The dsRNA injection into the insect hemocoel often compromises many investigations because of the stress related to wounding caused by injection. Cuticular damage caused during injection stimulates immune function which can further complicate the interpretation of the results [45, 138].

Oral Delivery/Ingestion of dsRNA

Oral delivery of dsRNA was first demonstrated in *C. elegans* [117], and thereafter, it has been tested in a number of insect species. RNAi in *C. elegans* was also observed when worms were fed on the bacteria engineered to express large quantities of dsRNA. RNAi in *R. flavipes* and *Diatraea sacchralis* was also successful via feeding [137, 145]. The dsRNA feeding method is comparatively attractive as it is convenient, easy to manipulate, causes less damage to the insect, and a more natural method of introducing dsRNA into insect body [23]. It also has its merits in small insects that are more difficult to manipulate using microinjection. The dsRNA could also be fed by either expressing dsRNA in bacteria or by in vitro synthesis. Early insect RNAi feeding studies did not give the desired knockdown effects as the injection of dsRNA effectively silenced the aminopeptidase gene *slapn*, which is expressed in the midgut of *Spodoptera littoralis*, but feeding with dsRNA did not achieve RNAi [91]. However, further studies on feeding dsRNA revealed effective gene knockdown effects in many insects, including insects of the orders Hemiptera, Coleoptera, and Lepidoptera [69] (Table 1). Feeding dsRNA to *E. postvittana* larvae has been shown to inhibit the expression of the carboxylesterase gene *EposCXE1* in the larval midgut and also inhibit the expression of the pheromone-binding

Table 1 Successful examples of RNAi-mediated gene knockdown in different insects

Target insect	Mode of delivery	Concentration of RNA	Target gene	Reference
Anopheles gambiae	Feeding nanoparticles	–	*AgCHS1 AgCHS2*	[142]
Apis mellifera	Abdominal injection	1 μl	Vitellogenin	[2]
Apis mellifera	Mixed with natural diet	0.5	Vitellogenin	[84]
Apis mellifera	Soaking	1.26	Toll-related receptor	[5]
Diabrotica virgifera virgifera	Artificial diet	5.4 ng/cm^2	Vacuolar ATPase subunit A	[11]
Diabrotica virgifera virgifera	Feeding	1,000 ng	DvSnf7	[19]
Epiphyas postvittana	Droplet feeding	1.0 μg	Gut carboxylase	[123]
Glossina morsitans morsitans	Blood meal	10 μg	Midgut protein TsetseEP	[127]
Helicoverpa armigera	Feeding	25–50 nM siRNA	Acetylcholine esterase	[64]
Helicoverpa armigera	Transgenic plant	–	CytochromeP450 (CYP6AE14)	[69]
Menduca sexata	Injection	100 ng	Moricin	[33]
Nilaparvata lugens	Feeding	0.02, 0.1, and 0.5 mg/ml	Trehalose phosphate synthase (TPS)	[23]
Phyllotreta striolata	Injection	1 mg/ml	PsOr1	[143]
Phyllotreta striolata	Feeding	0.05–3.2 ng/ml	Arginine kinase	[144]
Reticulitermes flavipes	Artificial diet	13 μg	Cellulase	[145]
Schistocerca gregaria	Injection	5 μg dsRNA	Vitellogenin	[8]
Schistocerca gregaria	Injection	5 μg	gapdh	[134]
Spodoptera frugiperda	Droplet feeding	0.4 mg	Cytochrome P450 (CYP6BF1v4)	[38]
Spodoptera litura	Soaking and in artificial diet		Aminopeptidase N	[91]
Spodoptera litura	Injection	1.5 or 2.5 mg/ml	Vitellogenin receptor (VgR)	[99]
Tribolium castaneum	Injection	1 μg	eGFP	[78]

protein *EposPBP1* in adult antennae [123]. The feeding of dsRNA also inhibited the expression of the nitrophorin 2 (*NP2*) gene in the salivary gland of *R. prolixus*, leading to a shortened coagulation time of plasma [3].

The RNAi pathway is a well-conserved mechanism in insects and holds high potential as an insect control technology as many insects have been found to be susceptible to orally ingested dsRNA [13]. The RNAi effects with dsRNA via oral ingestion are widespread in insects; however, the effect is not universal, and there are variations in sensitivities across taxa [130]. Another way of oral dsRNA delivery is dsRNA droplet feeding as described by Turner et al. [123], where 48-h starved third instar larvae of *E. postvittana* were fed on dsRNAs derived from the larval gut carboxylesterase gene (*Epos*CXE1) and the adult antennae-expressed pheromone-binding protein (*Epos*PBP1) gene. The silencing of *Epos*PBP1 indicated the existence of systemic RNAi in *E. postvittana* and persistence of dsRNA across the life stage from larva to adult. Oral delivery of dsRNA into insects has the advantage of cost-effective, labor and time saving, and it is easy to perform [115]. The method is a less-invasive and more practical method

for small insects such as aphids and first and second instar larvae or nymphs [115, 127]. The optimization of the used concentration of dsRNA to trigger RNAi is important for oral delivery [123]. Feeding of dsRNA seems to be the most practical method due to its ease, cost-effectiveness, time saving, less invasiveness, and above all, its natural route of entry. This method also fits well with the field application of this technology for the control of insect pests.

One of the promising methods having merits over direct feeding with dsRNA is the use of transgenic plants to produce dsRNA [11, 69]. Following the development of *Bt* toxin transgenic plants, lepidopteran pests have been effectively controlled [82]; however, phloem sap-sucking insects, such as aphids, whiteflies, planthoppers, and plant bugs, have evolved from minor pests to major pests, because there is no *Bt* toxin with adequate insecticidal effects on these kinds of pests [35]. This points out an urgent need to develop this technique for application in insects for which no effective *Bt* toxins are known. These hemipterans are highly destructive agricultural pests worldwide, causing millions of dollars' worth of yield loss and control costs [7]. Transgenic plants producing dsRNAs directed against genes function in Lepidoptera, Coleoptera, and Hemiptera pests are becoming more common [69]. The advantage of this method is the generation of continuous and stable dsRNA material. Mao et al. [70] generated dsRNA-expressing cotton (*Gossypium hirsutum*) plants, and the bollworm larvae (*H. armigera*) reared on these plants exhibited drastically retarded growth; the transgenic plants were less damaged by bollworms than the control. Quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that the CYP6AE14 expression level was reduced in the larvae as early as 4 h after feeding on the transgenic plants. Chen et al. [23] reported the successful feeding of trehalose-6-phosphate synthase (TPS for the synthesis of trehalose, main sugar reserve in the hemolymph) dsRNA solutions to silence this gene thus proposing it as a useful pest control agent.

The brown plant hopper, *Nilaparvata lugens* St°al, is the most destructive insect pest for rice crops which feed on the rice stems by sucking phloem sap through their stylet mouthparts, causing nutritional or physiological damage to rice. Zha et al. [140] used plant-mediated RNAi strategy for controlling this sucking pest. Three genes (a hexose transporter gene *NlHT1*, a carboxypeptidase gene *Nlcar*, and a trypsin-like serine protease gene *Nltry*) that are highly expressed in the *N. lugens* midgut were isolated and used to develop dsRNA constructs for transforming rice. When the insect's nymphs were fed on rice plants expressing dsRNA, the transcription levels of the target genes were reduced in the midgut. These reports confirmed that when planthoppers were feeding on rice expressing dsRNAs, target gene RNAi was triggered, and the gene transcript levels were suppressed. This study is important in view of designing strategies for the control of sucking insects.

In transgenic plants expressing hairpin RNAs, the rate of production needs to be faster than they can be diced. As to this fact, the several attempts involving similar approaches failed because they generated transgenic plants with insufficient hairpin RNA transcription. Mao et al. [69] proposed that RNAi was effective against P450 and V-ATPase genes because they were mainly expressed in the midgut, and the insect gut cells received a continuous supply of dsRNA. Hairpin RNA was diced into siRNAs in the gut cells and spread to the surrounding cells and tissues via intercellular dsRNA transport SID proteins. In insects, there is dsRNA uptake from the hemolymph for systemic RNAi and from the gut content for environmental RNAi. However, experiments using tissue culture cells have shown that uptake of dsRNA does not always trigger an RNAi response. For example, in silkmoth (*B. mori*)-derived Bm5 cells, fluorescently labeled dsRNA added to the culture medium can be internalized by the tissue culture cells, but the dsRNA does not trigger gene silencing [107, 113]. It gives the impression that for RNAi effects internalization of dsRNA should be linked with efficient transfer to the RNAi machinery.

Ingestion of dsRNA is less effective for inducing RNAi in *C. elegans* [49] and *R. prolixus* [3] than injection. The ingested dsRNAs targeting a gut-specific aminopeptidase N failed to induce RNAi in *S. litura* [91] suggesting that the oral delivery of dsRNA may not be suitable for all species.

The efficiency of RNAi by ingestion of dsRNA varies between different species. After oral delivery of dsRNA, it is hard to determine the amount of dsRNA brought inside the insect through ingestion [105]. Another complication with this dsRNA delivery method is the requirement of greater amount of material for delivery [23]. This phenomenon has been observed after ingestion of *CELL-1* dsRNA by the termite *R. flavipes* [145], *TPS* dsRNA in *N. lugens* nymphae [23], and *Nitrophorin 2* dsRNA by *R. prolixus* [3]. Moreover, different species of insects have different sensitivities to RNAi molecules when delivered orally, for example, *G. morsitans* fed with dsRNA may effectively inhibit the expression of *TsetseEP* in the midgut, but cannot inhibit the expression of the transferrin gene *2A192* in fat bodies due to lack of transfer capacity between tissues [127]. The mechanisms associated with the transfer of gene expression through feeding delivery method still need further investigations. The delivery mode of dsRNA in insects could be modified by coating the dsRNA to enhance its uptake in the gut and ultimately could increase efficiency of the silencing as this method was tried in the delivery of siRNA to mammalian cells and specific tissues [65]. Coating of dsRNA is also very important for its protection from endogenous nucleases of insects. In addition, coating can also be helpful in protecting dsRNA designed for spray formulations on aerial plant parts.

Delivery of dsRNA Through Soaking

The soaking of the organism into a dsRNA solution with extracellular RNAi is a convenient method for triggering RNAi response. The first experiment of soaking of nematodes (*C. elegans*) in dsRNA solution for RNAi was reported by Tabara et al. [108]. It was reported to work with similar efficiency as feeding in *C. elegans*. This technique was subsequently used in this organism for analysis of gene function [68]. However, delivery of dsRNA by this method is more applicable for insect cells than for whole insect bodies, due to certain barriers such as the insect cuticle. Soaking *D. melanogaster* S2 cells in *CycE* and *ago* dsRNA solutions has been shown to effectively inhibit the expression of these two genes for cell cycle and elevating levels of protein synthesis [71]. The soaking method is suitable only for certain insect cells and tissues as well as for specific insects of developmental stages that readily absorb dsRNA from the solution, and therefore, it is rarely used. Soaking of *Drosophila* embryos [27, 117] in dsRNA solutions has also been reported with RNAi effects.

Direct spray of dsRNA on newly hatched *Ostrinia furnalalis* larvae has been reported by Wang et al. [129]. The studies have shown that after spraying dsRNAs (50 ng/ml) of the DS10 and DS28 genes on the newly hatched larvae placed on the filter paper, the larval mortalities were around 40–50 %, whereas, after dsRNAs of ten genes were sprayed on the larvae along with artificial diet, the mortalities were significantly higher to the extent of 73–100 %. It was proposed through these results that in a lepidopteron insect, dsRNAs are able to penetrate the integument and could retread larval developmental ultimately leading to death. Although, the study did not elaborate on tissues or cells where these genes were silenced and the mechanism of the dsRNA uptake leading to RNAi effect, yet these findings may be helpful in the future for designing RNAi-based technology against insect damage. One of the studies had provided evidence that topically applied *AaeIAP1* dsRNA products in acetone dilution was able to kill adult female mosquito *Aedes aegypti* [89].

New Strategies for dsRNA Delivery

Recently, nanoparticle-mediated RNAi has been reported as a novel way of delivering dsRNA [138]. The nanoparticles stabilize the dsRNA molecules through the delivery process resulting in enhanced efficiency of RNAi. dsRNAs were entrapped by the polymer chitosan with electrostatic forces to form a chitosan/dsRNA nanoparticle, which were delivered into the insect by ingestion [142].

Use of biolistics has been also shown to be effective at delivering dsRNA into *Drosophila* embryos [139], but this technique has not yet been used for any other species. Other techniques such as electroporation and hairpin RNA expression have been demonstrated in certain species. Recombinant viruses have also been used to deliver the dsRNA [122]. In *Spodoptera exigua*, gene silencing effects have been shown after mixing bacteria expressing target dsRNAs in their food [115]. Wang and colleagues [129] analyzed the experimental results of delivery method of dsRNA and observed that the efficiency of RNAi by dsRNA delivery by injection into early predifferentiated embryos solely depends on the availability of the core RNAi machinery. Expression of hairpin RNAs by transgenes was also efficient as it happens inside the cells. The analysis of results of different experiments revealed that embryo injection, transfection of cell lines, or expression of hairpin RNAs by transgenes was successful [129]. Wang et al. [129] report RNAi-induced lethality by direct delivery of ds RNA via integument in the Asian corn borer (*O. furnalalis*). They adopted second-generation sequencing technology to screen RNAi targets.

Genetically engineered dsRNA-producing yeast strains have also been developed to feed *D. melanogaster*, but gene silencing was not successful [41]. However, dsRNA produced in bacteria is effective in *C. elegans* [117]. Therefore, the use of bacteria, especially insecticidal microorganisms to produce dsRNA for insect RNAi, also needs further investigations.

RNAi Effects in Different Insect Orders

RNAi has been shown successful in different insect orders. The successful RNAi effects in insects have also been reviewed by Katoch and Thakur [57].

1. Diptera [58, 79, 121].
   The knockdown the immunoresponsive midgut-expressed gene *TsetseEP* was demonstrated in *G. morsitans* [127]. This was also the first report of a gene knockdown in a blood-sucking insect by including dsRNA in the bloodmeal.

2. Lepidoptera [15, 91, 137]. Application of dsRNA in an artificial diet resulted in knockdown of targeted genes in *E. postvittana* [123] and *Plutella xylostella* [12]. Mao et al. [69] genetically engineered *Nicotiana tabacum* and *Arabidopsis thaliana* plants with the cytochrome P450 gene from *H. armigera*. The level of cytochrome P450 was reduced, and larval growth was retarded when the larvae were fed on transgenic leaves. The cotton bollworm uses an antidote against the cotton plant's natural insecticide called gossypol. RNAi of cytochrome P450 monooxygenase triggered by the ingested dsRNA reduces the insect's tolerance to gossypol and exposes the insect to a full force of gossypol. In the permethrin-resistant *P. xylostella* strain, RNAi-mediated silencing of cytochrome P450 gene CYP6BG1 after consumption of a droplet of dsRNA solution resulted in an increased sensitivity of the insect to pyrethroid insecticide [12].
   RNAi effects against carboxyestrases were studied in *E. postvittana* larvae via feeding them on an artificial diet supplemented with dsRNA. The repression of carboxyestrase gene *EposCXE1* was observed after 2 days of feeding, and maximal

repression was observed after 7 days [123]. In addition, RNAi was also studied the pheromone-binding protein gene (*EposPBP1*) in adult antennae of *E. postvittana* through feeding EposPBP1 dsRNA to the larvae. Knockdown of *Epos-PBP1* transcripts was observed for the first 2 days after adult eclosion but recovered to wild-type levels at 4 days of posteclosion [123]. In *S. litura*, vitellogenin receptor was shown as a critical component for binding vitellogenin and transporting it into the oocytes [99]. Therefore, an alteration of insect reproductive system is another approach for designing RNAi-mediated insect control strategies. The heritable RNAi effects on the embryos of the next generation were reported in *Hyalophora cecropia* following injection of dsRNA into pupae, suggesting that the injected dsRNA had entered into the gonads of the developing pupae [15].

3. Coleoptera [21, 119].

   The success of RNAi in *Diabrotica virgifera virgifera* has been shown by feeding plant material expressing hairpin dsRNA. Transgenic plants were genetically engineered to produce hairpin dsRNAs in vivo [11, 69]. Baum et al. [11] conducted feeding assays where larvae were fed on an artificial diet supplemented with specific dsRNAs for screening a large number of essential insect genes. Fourteen candidate genes for knockdown were identified with different levels of dsRNA which killed *D. virgifera virgifera* larvae. Corn root damage was significantly reduced when the larvae were fed on transgenic maize plants producing vacuolar H^+ ATPase dsRNA to target a subunit of vacuolar ATPase in the midgut. This protection against *D. virgifera virgifera* infestation was comparable to that provided by a *Bt* transgene. After knockdown of the *PsOr1* gene from *Phyllotreta striolata* through microinjection, the adult beetles were unable to sense the attractant or repellent odor stimulus. Silencing *PsOr1* significantly impaired the host plant preferences of the beetles for cruciferous vegetables. The modulation of this unique receptor family by using RNAi blocked host plant-seeking behavior. Therefore, RNAi to target *PsOr1* and its orthologues might be an effective strategy to block host plant-seeking behaviors in diverse insect pests [143]. A striking RNAi effect was also demonstrated in *P. striolata*, where silencing of the invertebrate-specific phosphotransferase arginine kinase was done via feeding. The development of the beetle was severely impaired due to disruption of cellular energy homeostasis [144].

   RNAi has also been shown effective in *D. virgifera virgifera* larvae via oral delivery of synthetic dsRNA both in artificial diet bioassay and by ingestion of transgenic corn plant tissues engineered to express dsRNA [19]. The Snf7 ortholog (DvSnf7) was selected as the target mRNA, which is a component of the ESCRT-III complex (endosomal sorting complex required for transport), which is involved in vital processes including sorting of cell membrane receptors [60, 106, 125]. Even, the low concentration of dsRNA leads to mortality due to the vital functions of gene in WCR. The study revealed that dsRNAs greater than or equal to approximately 60 bp as requirement for biological activity in artificial diet bioassays. The 240-bp dsRNAs containing a single 21-bp match to the target sequence were also effective in RNAi; however, 21-bp short interfering RNAs matching the target sequence were not effective for DvSnf7 knockdown. The gene suppression was shown to spread to tissues beyond the midgut within 24 h after dsRNA ingestion. The results of oral delivery of DvSnf7 dsRNA to WCR pointed the possibility of using DvSnf7 as a potential RNAi target for the control of WCR through transgenic approaches ([19]).

4. Hymenoptera [2, 16, 34].

   A noninvasive method of gene knockdown was shown in *A. mellifera* by feeding dsRNA for vitellogenin (dsVg-RNA) in the natural diet of second instar larvae. Around

90 % of vitellogenin transcripts were silenced in the workers as compared with the control. The knockdown of honeybee vitellogenin mRNA suggested that the method is also applicable to other target genes during postembryonic development [84]. The whole study favors the success of feeding method as compared to the other methods, which could have adverse effects on the developmental processes.

5. Hemiptera [3, 67, 80, 87, 98, 124].

Insects in the order Hemiptera feed exclusively on plant sap, which diverts plant nutrients essential for plant growth and reproduction and thereby influencing global agriculture. The plant-feeding Hemiptera may be specialized to feed on phloem, xylem or mesophyll, or to feed on a combination of these tissues. In hemipteran insects, mouthparts are formed into beak-like structures that are used to pierce plant tissues and suck the sap from the phloem or the xylem. Besides causing injury to plants and concomitant opportunities for infections by secondary insects or fungal pathogens, hemipteran insects are also vectors of many plant viruses. More than 275 plant viruses and around 50 % of all insect-borne plant viruses are vectored by aphids [76]. Among these aphids cause major economic losses on almost all crops, and account for a large part of the 13 % of agricultural output estimated to be lost to insect pests [28]. This group of insects is therefore are one of the biggest challenges for insect pest management. RNAi has been successfully used for investigation of gene function in aphid. The application of dsRNA in an artificial diet resulted in knockdown of targeted genes in *R. prolixus* [3]. The feeding of dsRNA resulted in a systemic effect as the gene knockdown was also observed in other tissues. In *R. prolixus*, RNAi response after feeding of dsRNA has also been reported where a salivary gland transcript encoding nitroporin 2 (NP2) was targeted both by oral delivery and microinjection of dsRNA. Both treatments resulted in downregulation of NP2 expression. It was observed that microinjection of dsRNA was more effective (75 % reduction in gene expression) than the feeding of dsRNA (42 % reduction) [3]. The feeding of dsRNA solutions resulted in a strong decline in the expression of *TPS* in *N. lugens* larvae and ultimately reduced the insect survival rate [23].

The plant-mediated RNAi method effectively silenced genes of brown planthopper, a hemipteran species [11]. As RNAi in aphids has been reported feasible, thereby, gene knockdown via delivery of dsRNA from plants would be useful for control aphid pests in crop production. Hemipterans are sensitive to systemic RNAi with dsRNA injection [48]. Knockdown of c002 mRNA, corresponding to the most abundant transcript in the salivary glands of the pea aphid, resulted in a dramatic reduction in lifespan, apparently by disruption of feeding efforts through an unknown mechanism ([80]08). On the other hand, knockdown of a calcium binding protein or a cathepsin L of the gut did not result in a clear phenotype in this species [53]. In the whitefly, RNAi resulted in a lethal phenotype for one out of four target genes tested, although in all cases silencing was observed [36]. Gene silencing through feeding of dsRNA was successfully attempted in different studies. The aquaporin protein is involved in osmoregulation and feeding of dsRNA resulted in elevated osmotic pressure in the hemolymph [98]. The studies by Shakesby and colleagues [98] on *A. pisum* fed on an artificial diet containing dsRNA corresponding to the aquaporin transcript showed regulation by more than 2-fold within 24 h. In an another study feeding of dsRNA targeting vATPase transcripts from an artificial diet resulted in 30 % decrease in transcript levels in *A. pisum* and a significant increase in aphid mortality [131]. MpC002 gene is predominantly expressed in the aphid salivary gland, whereas, Rack-1 is predominantly expressed in the aphid gut. Plant-mediated RNAi was tested on green peach aphid *Myzus persicae* using transgenic *Nicotiana benthamiana* and *A. thaliana* with two *M.*

persicae genes, MpC002 and receptor of activated kinase C (Rack-1) as targets. The expression of both MpC002 and Rack-1 was knocked down when *M. persicae* were fed from transgenic plants corresponding to MpC002 and Rack-1. The silenced aphids have reduced progeny production. The expression of MpC002 which is predominantly expressed in the salivary glands is knocked down by up to 60 % proving that the silencing signal is also present and spread through the aphid [54, 59]. These results illustrate that RNAi through feeding can be used to control hemipteran pests.

A major challenge for the control of hemipterans, however, is the delivery of dsRNA to the insect, considering its mode of feeding through sap-sucking from the phloem or xylem. In the plants expressing dsRNA, systemic RNAi signals triggered by RNA hairpin transgenes can spread over long distances in plants through the phloem and may consist of ~25-nucleotide ssRNAs complexed to small RNA-binding proteins [135]. It remains to be established whether these can trigger an RNAi response in the (phloem) sap-sucking hemipteran. On the other hand, no study till date has clearly revealed whether dsRNAs somehow can be introduced into and transported by the xylem vascular system of the plants to provide protection against xylem feeders.

6. Isoptera [62, 96, 145]. The application of dsRNA in an artificial diet has also resulted in knockdown of targeted genes in *R. flavipes*. Feeding of cellulose disks supplemented with dsRNA to *R. flavipes* resulted in silencing of a cellulase and a caste regulatory hexamerin storage protein. The silencing of the genes reduced termite fitness and increased its mortality [145]. The results are very important in exploring the possibilities of the development of RNAi-based termiticides.

7. Orthoptera [73, 75].

Badisco et al. [8] used RNAi to investigate a possible involvement of insulin-related peptides (IRP) and neuroparsins (NPs) in *S. gregaria* control. An RNAi-mediated knockdown of either Scg-NP or Scg-IRP transcript levels was induced in the females. Knockdown of Scg-NPs or Scg-IRP affected vitellogenin transcript levels and oocyte growth, which provided an indication of the role of Scg-NPs and Scg-IRP in vitellogenin synthesis.

Challenges for RNAi as Successful Insect Control Strategy

While the prospects of using RNAi as an insect control strategy look promising, we still need to study several aspects before this becomes a practical reality. Although there are logical considerations for success in some of the experiments, the long-term effectiveness of the approach will need to be established after field experimentation. The dsRNAs either administered in the diet or from transgenic plants would first make entry inside the insect midgut. The body of an insect is covered by a chitin exoskeleton, while the midgut of most insects is lined by the peritrophic membrane (PM) or the perimicrovillar membrane (PMM) in Hemipterans [101]. The cells of the midgut, which are responsible for nutrient absorption from the gut lumen are capable of taking up dsRNA [128]. Therefore, the midgut region is the only part for exchange between the hemolymph and the gut contents with exposed cells affecting nutrient absorption. All the factors including efficiency of RNAi, such as expression levels of the basic RNAi machinery, uptake of dsRNAs/siRNAs from the extracellular medium and absence/presence of dsRNA-degrading enzymes in midgut are important for RNAi. The dsRNA are presumably mediated by the midgut surfaces through exposure of cells of the midgut epithelium and the

Malpighian tubules to dsRNA in the gut contents. The plant material with hairpin RNA ingested by the insect may not provide sufficient levels of intact dsRNA to trigger potent RNAi. Further, it is not necessary that dsDNA should always be present in large quantity to have an effective RNAi, but several factors are involved in determining gene silencing effects [50]. The possibilities of using RNAi in crop protection are subject to some potential challenges:

Insect Nucleases

That dsRNA catabolism could influence the efficiency of RNAi when administered orally. The presence of nucleic acid degrading enzymes in the gut content that are capable of degrading dsRNA is another consideration for RNAi effects [4]. Double-stranded RNA is quite stable, but it could be digested by a variety of ribonucleases that are specific for double-stranded RNA [56]. In some insects, dsRNA-degrading activity in the gut is lowest during the molt and can be further reduced by starvation [92]. Very little is known about the digestion mechanism of nucleic acids found inside the gut after the ingestion of plant material. So far there is no such report of dsRNA digestion inside the gut, but the nucleases could be an integral part of the digestive cocktail found inside the insect gut, which has not been examined thoroughly till date. The dsRNA in the insect gut may be a direct substrate for RNases in the insect gut. Considering the above facts, research on insect nucleases is a novel area of research for refinement of this technology. In the study conducted. Gene knockdown through microinjection of dsRNA into the hemocoel of the tarnished plant bug, *Lygus lineolaris*, was achieved; however, delivering dsRNA to insects by feeding were repeatedly unsuccessful in gene knockdown, which was attributed to dsRNA digestion and degradation by the insect prior to contact with the insect cells. The saliva of *L. lineolaris* was found to rapidly digest double-stranded RNA. The study revealed that the prospects of using dsRNA and RNAi as pest control strategy could be complicated by the presence of dsRNAase activity. Another study on the persistence of dsRNA in insect hemolymph in two insects, the tobacco hornworm, *Manduca sexta*, and the German cockroach, *Blattella germanica*, the ex vivo assay by Garbutt et al. [33] revealed that dsRNA was rapidly degraded by an enzyme in *M. sexta* hemolymph plasma, whilst dsRNA persisted much longer in *B. germanica* plasma. The quantitative reverse transcription PCR-based assay revealed that dsRNA disappeared rapidly from *M. sexta* hemolymph in vivo. The study proposed that the rate of persistence of dsRNA in insect hemolymph which is mediated by the action of one or more nucleases could be an important factor in determining the susceptibility of insect species to RNAi.

Gut pH

Gut pH is quite variable among insect orders ranging from very acidic (typical for Coleoptera) to strong alkaline (even up to pH 10.5 in some species of Lepidoptera). In addition, pH also changes among different gut regions and also with a distance from the gut epithelium within one region. The lepidopterans have a strong alkaline pH, which provides a hostile environment for dsRNA. Stability of ingested dsRNA in the gut could be affected by both chemical and enzymatic hydrolysis [88]. Therefore, the integrity of fed dsRNA poses many questions that need to be answered. However, despite such conditions, ingested dsRNA have been able to initiate RNAi [114] suggesting that the ingested dsRNA must have remained sufficiently intact in the gut [114]. The dsRNA uptake machinery involves different proteins (SID-1, SID-2, and other endocytotic proteins). However, a clear picture of these proteins as a sole mechanism of dsRNA uptake is yet to confirm.

dsRNA Length

The length of dsRNA has also been observed as one of the potential factor for RNAi mechanisms. In *Drosophila* S2-cells, the minimum length of uptake of dsRNA was 211 bp [95] whereas, in *H. armigera* silencing P450 with transgenic *Arabidopsis* plants producing dsRNA, Dicer mutants showed long, unprocessed dsRNA fragments to be more effective than the siRNA products of Dicer activity [69]. The selection of the target gene and target region within the gene is also important for successful RNAi. Recently, the development of high-throughput sequencing has facilitated the target gene selection. The length of dsRNA should be considered as by different sizes of dsRNA resulted in different efficacies [131]. Most of the feeding studies have given good results with the sequence length ranges between 300 and 600 bp. Length of dsRNA influences the uptake and silencing efficiency both in the insect cell lines [95] as well as in whole insects [69].

Delivery Method

A method to deliver dsRNA ultimately ensures the availability of dsRNA for RNAi effects. Feeding and injection delivery methods vary in their results when compared with each other. For example, injection of dsRNA was successful in silencing of targets than feeding experiments [91], which could be attributed to the uptake mechanism of dsRNA.

The introduction of dsRNA by feeding is interesting and comparatively simpler but some researchers cast aspersions on this method believing that the induction of gene silencing by dsRNA feeding is less effective than that by dsRNA injection as each individual consumes different amounts of food and consequently ingest a variable dosage of dsRNA [3]. The feeding of dsRNA seems somewhat less efficient than the other delivery methods. Rajagopal et al. [91] pointed out that the midgut aminopeptidase N gene was efficiently downregulated by micro-injection of dsRNA into hemoceol of *S. litura* larvae. However, the feeding of dsRNA was unsuccessful in generating an RNAi response suggesting a delivery through feeding may not be suitable for all insect species. In lepidopteran species, the results of over more than 150 RNAi experiments have been reviewed by Terenius et al. [113]. Through their analysis they have revealed that RNAi is successful in the family Saturniidae and in genes involved in immunity; however, gene expression in epidermal tissues are difficult to silence. Systemic RNAi has been demonstrated in some species, such as *H. cecropia* and *B. mori*, in which injection of dsRNA into the pupa can result in phenotypic effects in developing embryos, indicating dsRNA uptake by the developing oocytes of the pupa. However, a great variation of sensitivity to systemic RNAi has been revealed among different lepidopteran species leading to high or even no silencing at very different concentrations of dsRNA. The study put forth the further need to investigate the mechanism of RNAi in lepidopteran insects and its possible connection to the innate immune response [113].

Off-Target Effects

One of the major concerns regarding the use of RNAi in insect pest control includes the specificity of action and off-target effects [6]. For a gene knockdown in insects, dsRNA is designed to target one particular gene, but off-target effects may occur if siRNAs have sequence homology with genes (especially, 3′ untranslated regions of genes) not intended for RNAi targeting [17, 63]. The ingestion as opposed to injection of dsRNA is the probable solution, which limit the dsRNA spread to a localized area and therefore reducing the probability of encountering more genes with sequence homology. The specificity of the

RNAi effect, whether it targets a single species or a group of species of a wider taxonomic group, could be achieved by careful selection of the gene region for the production of dsRNA. The unique sequences might target efficiently only a single species whereas more conserved sequences could achieve more broad specificity [94]. Zhang et al. [142] has reported that two genes with high sequence similarities can be silenced by the same dsRNA.

RNA Concentration

Miller and colleagues [78] suggested that the size and concentration of dsRNA are critical to the effectiveness of the RNAi response. The longer dsRNA are more effective with respect to initial knockdown and duration of the RNAi effect. The 60-bp dsRNA induced a moderate level of knock down (70 % reduction), while the 30-bp dsRNA was least effective (30 % reduction). These data provide quantitative evidence for the dsRNA size dependency in systemic RNAi in *Tribolium*, with a longer dsRNA being more efficient to trigger systemic RNAi. They further provided the evidence that dsRNA cellular uptake is the major step affected by dsRNA length in *Tribolium*. Their studies also revealed that when multiple dsRNAs are injected, competition between dsRNAs can occur, resulting in a less effective RNAi response. An optimal concentration of dsRNA is required for inducing the efficient silencing of the targeted gene, and it also depends on the nature of the targeted gene or target insect [98]. The silencing of vacuolar H^+ ATPase after feeding dsRNA to *Leptinotarsa decemlineata* also silenced the ortholog gene in *D. virgifera virgifera*, though the silencing effect was concentration dependent in both organisms [11]. Recent research has shown that oversaturation of the RNAi machinery can occur when multiple dsRNA or siRNAs are delivered [9, 85]. As the miRNA and RNAi pathways share components, oversaturation of these components during the RNAi response can result in unintentional inhibition of the miRNA pathway resulting in phenotypes related to a loss of miRNA function. Because miRNAs are essential for growth, development, and tissue homeostasis, this inhibition may result in lethality [39, 120]. Moreover, having a mixture of dsRNA can result in competition between the dsRNAs for RNAi machinery components and transport components resulting in competitive inhibition.

dsRNA Availability

Upon finding a successful approach for the production of large quantities of dsRNA, there will be a further limitation in terms of availability of dsRNA throughout all the plant parts from roots to leaves. This will be a case of concern in case of pests which are foliage as well root feeders. Spray of dsRNA on the leaves may not be sufficient to protect the plant from an economic damage as roots will remain unprotected and vulnerable to attack by root feeders [144]. Another in case of transgenic plants, expression of dsRNA can be plant-part specific. If the spray treatment of dsRNA turns out to be effective, then cost-effectiveness of producing commercial formulations of dsRNAs will turn out to be a strong limitation. Further, targeting different insect groups using this technology would be difficult as the plants experience a variety of insect pests.

Target Gene

The selection of gene to be silenced can significantly affect the outcome of an RNAi effects in the insects. Terenius and colleagues [113] after analysis of the RNAi experimental results of lepidopterans, observed that out of 130 genes used for the analysis, only 38 % were silenced at

high levels while 48 % and 14 % of the genes failed to be silenced or they were silenced at low levels. It was also observed that the immunity-related genes were silenced more effectively by RNAi. In the same tissue, a particular gene may be resistant to RNAi, whereas the other genes in the same tissue may gave better results for RNAi [113]. It has been shown that the 5′ end of the dsRNA can influence the effectiveness of RNAi; a phosphorylated 5′ end exhibits better gene silencing rate than does a hydroxylated 5′ end [20].

dsRNA Resistance

Similar to chemical insecticides and *Bt* plants, it is possible that some insects may acquire a resistance to one particular dsRNA sequence due to mutations in the target mRNA. However, if this technique develops a resistance then it will be easy and safe to select another dsRNA which either targets another portion of the same gene or targets a new gene. Further, with the apprehension of development of mutations to RNAi machinery, resistance can be subsided as these proteins are essential for the normal processing of insects' endogenous double-stranded RNAs and microRNAs, which are in turn essential for regulating gene expression during development [22].

Sequence Polymorphism

The sequence polymorphism in insect pests is another thought-provoking point for the applicability of RNAi in insect control. Will the insect develop resistance through acquisition of point mutations in the target genes? It is also possible that a viral infection in the transgenic crops could significantly decrease the efficacy of this strategy. Thus, there remain some researchable thoughts before this technique takes some final shape.

Variation in RNAi Effects

The RNAi-mediated gene knockdown effect is variable in different insect orders, and it has been observed difficult to achieve in moths and butterflies (Lepidoptera) [113]. Different studies have shown variation in RNAi effects in different species, in which some of them have high resistance to application of dsRNA. In *Bicyclus anynana*, *Chrysodeixis includes* and *S. littoralis*, even high doses of dsRNA (more than 1 mg/mg of tissue) did not result in any silencing effects [52, 72, 113].

Functional gene analysis and RNAi mechanism studies mainly focus on model insects, for example, *Drosophila*, *Bombyx*, and *Tribolium*. However, most agricultural insect pest species lack the genome sequence and gene annotation information; once the homologous gene has some sequence differences between species, the function of the homologous gene will be difficult to understand or explain. Similarly, the mechanism of RNAi in insects varies among different species; for instance, lepidopteral insects are very different from coleopteral insects. Sometimes, an effective RNAi target in one species may not play the same or similar role in another species. Terenius et al. [113] systematically summarized the relationship of RNAi efficiency with insect species and gene function in a recent review [113].

Life Stage of the Target Organism

In insects, RNAi effect is more prominent in the early stages as compared to the late stages. Silencing of nitropin 2 was 42 % in second instars of *R. prolixus* as compared to none in the fourth instars even though treated with the same concentration of dsRNA [3]. In *Spodoptera*

frugiperda, a higher gene silencing was observed in the fifth instar larvae as compared to adult moths [38]. It has been recently shown that when dsRNA is injected at the last larval stage, the RNAi effect can last for many months and could extend to the entire lifespan of the individual [78]. In parental RNAi, where the female pupae or adults are injected with dsRNA, the effect is seen in the offspring for several months [21]. However, this parental RNAi is less efficient when last instar larvae are injected with dsRNA. One of the possible reasons for this is that the female reproductive organs do not complete formation until the pupal stage. For the oocytes to efficiently uptake dsRNA, they must be formed at the time of dsRNA introduction to the body [21].

Prospects of RNAi in Insect Control

Control of insect pests using RNAi is a promising approach. Once the potential researchable issues related to this technology are dealt, it will allow us a wide range of potential targets for the suppression of gene expression in insects. Utilization of multiple gene targets would further strengthen the application of this technology in controlling insect pests. The success of RNAi as a commercial product for controlling insect pests will depend upon identification of target genes that potently kill the pests or could inhibit toxin resistance of insect. The most appealing approach that validates the field application of this technology is ability to genetically engineer plants to express dsRNA for knockdown of crucial genes required for insect survival. One of the encouraging part of applying dsRNA as a pesticide for insect control is its feasibility to design specifically against a single insect species or a group of related species, which poses a little chances of threat to other organisms. Different approaches are now being studied to avoid the off-target effects, which are assumed as one of the limiting factors for this technique. A chemical modification of 2′-*O*-methyl ribosyl at position 2 in the guide strand reduces silencing of most off-target transcripts [51]. The much debated likelihood of resistance of insect pests could be counteracted by the design of dsRNAs for a different region of the same gene or a different gene [138].

Transgenic plants expressing dsRNA would be very cost effective as they will provide a continuous supply of RNAi inducers during the entire life of the plant. With spray technology, a particular life stage of the plant that is vulnerable to insect damage can be sprayed with dsRNA. In addition, different types of insects which appear in succession can be controlled by spraying of different dsRNAs. Spray consisting of crude preparations of dsRNA is efficient at silencing genes of plant viruses and dsRNA stably exists on leaves for several days and confer resistance to viral infection [112]. For root feeders, soil treatment with dsRNA solution can be done, but the stability of dsRNA in the soil will still remain under question. Once we identify a best vector–host combination capable of producing a high amount of dsRNA, it would be possible to overcome the limitation associated with the cost-effectiveness of dsRNAi spray. At this point, production of RNAi transgenic plants seems to be a better approach as it will have the dsRNA in the plant itself without any contact with the environment. RNAi effects via feeding are quite variable as each individual of the test population may consume different amount of dsRNA. Therefore, there will be a need to establish a threshold dose of dsRNA that the transgenic plants should produce considering damaging stage of the insect. Gene pyramiding (multiple target genes) approach is another option for controlling some insects as silencing of a single gene may not result in killing of the insect. In future, RNAi-mediated control of stored grain insect pests, such as *Callosobruchus maculatus* might be possible via coating of grains with dsRNA. However, other safety issues would need a due consideration before its application. At present, it

appears to be imaginary and a costly affair, but its logic lies in production of dsRNA through bacteria as bacteria could be engineered to mass produce dsRNAs [117].

A number of target genes for RNAi in insects have been identified and tested. The major among these include, for example, P450s, glutathione *S*-transferases, α-tubulin, β-tubulin, carboxylesterases, vacuolar ATPase, ribosomal protein S4 and actin among others. The research on the identification of other potential genes for RNAi effects is in progress. Thus, plant-derived silencing of insect-detoxifying genes has a potential to become a powerful practice for controlling insect pests. With advanced technologies like next-generation sequencing (NGS) and RNAi target sequencing (RIT-seq), there is acute advantage in the precise selection of target gene [1, 42]. NSG allows de novo reconstruction of transcriptome for nonmodel organism and has provided opportunities for expression profiling in organisms lacking any genome or transcriptome sequence information [141]. The sequencing of *D. melanogaster* genome in 2000 accumulated research on functional genomics for this dipteran insect [138]. Later, the genomes of other insects have been sequenced, including the honeybee *A. mellifera* (Honeybee Genome Sequencing Consortium, 2006) and the red flour beetle *T. castaneum* (*Tribolium* Genome Sequencing Consortium, 2008). These sequences of these organisms are crucial in further RNAi research for practical applications. Although RNAi technology cannot be compared with *Bt*-based insect control strategies at this juncture, but in the future, it will most likely stand beside or move in conjunction with *Bt*-based products. RNAi may work better where *Bt* toxins have failed or have not given the desired results. For example, it could be a powerful approach against termite and mosquito control, which are common menace in some parts of the world and the available methods have not been so successful or other associated environmental issues.

Conclusion

RNAi has a huge potential to become a successful approach for insect pest management. However, several research and ethical issues need to be addressed before this technology can be applied on a commercial level. Till date, *Bt* transgenic is the major insect biocontrol method, however the pace with RNAi being researched reveals that RNAi will play a significant role in controlling insect pests in near future. Transgenic plants producing *Bt* toxins have been proven to be successful in controlling insect pests of many crop plants, however there have been sporadic reports of resistance development against *Bt* toxin [110]. Therefore, there is an utmost need to look for an alternative sustainable technology. Progress made so far in RNAi technology provides an ample evidence for RNAi to become a successful alternate for insect pest management. Exploration of RNAi to use in insect pest management has begun, and one can easily believe that it has a potential to become the most powerful pest management strategy. Despite various doubts associated with this technology, the days are not too far when one would see RNAi would stand alongside *Bt* technology in insect pest management programs. In the future, RNAi is bound to start a new era in insect pest management.

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