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RNAi technologies for insect control in crop protection

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ARTICLE INFO

Article history:

Received 21 January 2025

Revised 10 May 2025

Accepted 17 May 2025

Available online 11 June 2025

Keywords:

Crop protection

Double-stranded RNA (dsRNA)

Insect control

RNA interference (RNAi)

ABSTRACT

RNA interference (RNAi) has been used for agricultural insect pest control based on silencing of targeted insect genes. However, the effectiveness of RNAi and its applications in insect pest control remain challenging. Here we review factors that may affect the effectiveness of RNAi application, including the variability in RNAi efficacy among different insect species, a limited understanding of double-stranded RNA (dsRNA) uptake and systemic RNAi mechanisms, and the effective delivery of dsRNA in field conditions. Furthermore, we summarize recent progress in RNAi strategies for crop protection, discuss the advantages and disadvantages of RNAi-based insect control, and propose potential strategies to increase the effectiveness of RNAi in insect control.

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1. Introduction

Insect pests cause crop yield losses, competing with humans for food and fiber resources and spreading plant viruses [1]. Chemical pesticide applications exacerbate environmental pollution [2,3], promote rapid evolution of pest resistance, and harm beneficial pest predators [4]. RNA interference (RNAi)-based technology has emerged as a promising technology for insect pest control, offering a potentially species-specific, low-risk, effective, and environmentally friendly strategy for crop protection [5].

RNAi is an RNA-mediated regulatory mechanism present in most eukaryotic organisms [6]. It was first discovered in the nematode *Caenorhabditis elegans* in 1998, demonstrating potent and sequence-specific interference that knock down the expression of targeted genes [7]. The potential RNAi mechanisms are highly conserved in eukaryotic cells, using various small RNA (sRNA) groups to regulate messenger RNA (mRNA) degradation/translation or heterochromatin establishment in a sequence-specific mode [8]. In general, endogenously synthesized or exogenously supplied dsRNAs are processed into sRNA duplexes typically ranging from 21 to 24 nucleotides (nt) in length by ribonuclease III enzymes known as Dicer (Dcr), which was initially identified in *Drosophila melanogaster* [9]. The sRNA duplexes are then loaded into members of the Argonaute (Ago) protein family, either in the cytoplasm or within the nucleus, to form an RNA-induced silencing complex (RISC) [10]. The guide strand of sRNA typically mediates the binding of RISC to complementary mRNA in the cytoplasm, thereby inducing RNAi. It directs the RISC complex to a DNA target after RISC has entered the nucleus, leading to transcriptional gene silencing through DNA methylation [11]. In insects and other animals, three types of sRNAs are known to activate corresponding RNAi pathways, including small interfering RNAs (siRNAs), which mediate the siRNA pathway; microRNAs (miRNAs), which mediate the miRNA pathway; and piwi-interacting RNAs (piRNAs), which

mediate the piRNA pathway [12] (Fig. 1). In general, the siRNA pathway defends against viral infections and suppresses transposable elements [13,14], whereas the miRNA pathway regulates gene expression [15], and the piRNA pathway suppresses germline transposon expression [13].

2. Factors influencing RNAi efficacy in insects

2.1. Differential response of insects to RNAi

Not all insect species respond equally to ingested dsRNA and RNAi. Among these insects, those belonging to Coleoptera are typically highly susceptible to RNAi, with both injected and ingested dsRNA inducing widespread and potent RNAi effects in coleopteran species [16,17]. A dsRNA-binding protein, Staufen C, is essential for effective RNAi in the coleopteran *Leptinotarsa decemlineata*. This gene is known only in Coleoptera and is essential for the processing of dsRNA into siRNA in these insects [18]. RNAi effectiveness varies across insect orders. Hemiptera, Orthoptera, Diptera, Hymenoptera, and Lepidoptera show varying sensitivity to dsRNA [19–23]. RNAi effectiveness varies even within the same insect order [19,24,25].

RNAi efficacy also varies depending on the delivery routes of dsRNA. Factors influencing RNAi efficacy via oral delivery include the stability of dsRNA and its uptake by digestive systems. The midgut environment is alkaline in Lepidoptera, several Orthoptera, and Hymenoptera species, destabilizing dsRNA and accelerating its degradation [26–28]. dsRNA may be removed from the hemolymph in Lepidoptera and Orthoptera or from the saliva in Hemiptera with nucleases [19,22,29,30]. dsRNA can be trapped in endosomes of *Spodoptera frugiperda*, and in the cell lines and tissues of *Heliothis virescens*, hindering its cleavage into siRNAs and contributing to inefficient RNAi response in Lepidoptera insects [22,31]. Tissue dependence in the RNAi response may lead

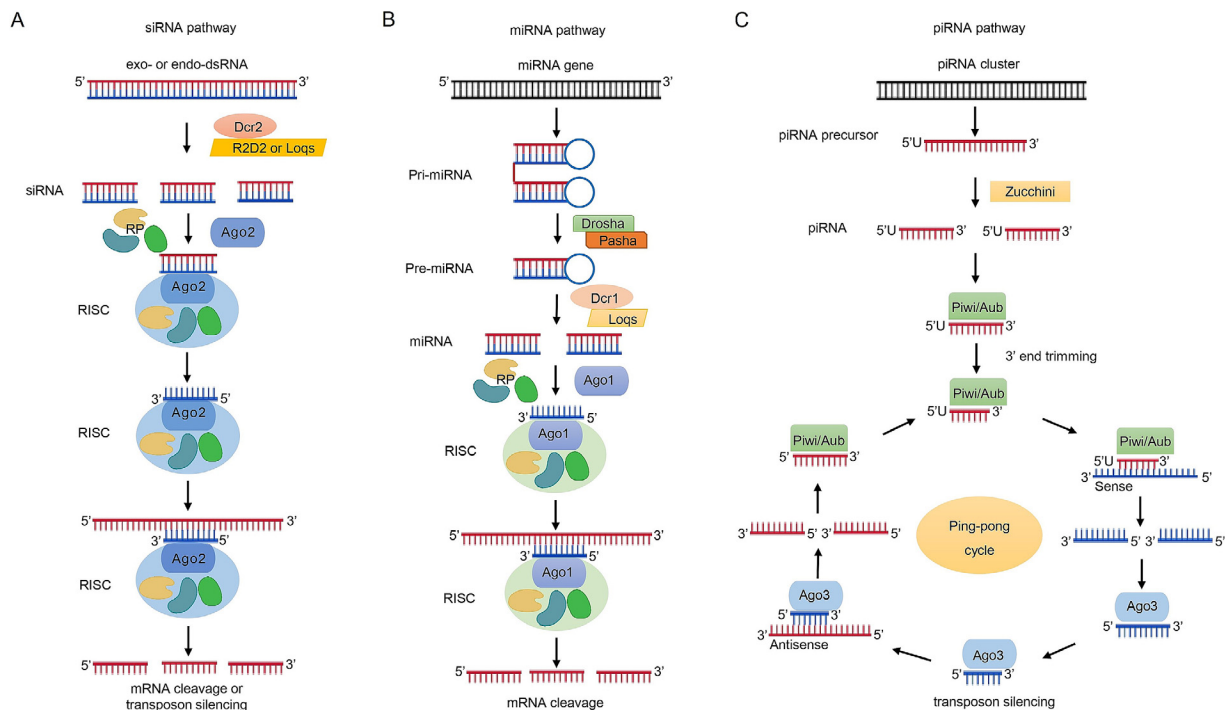


Fig. 1. Mechanisms of potential RNAi pathways in insects. (A) Schematic of the siRNA pathway. Dcr2 converts dsRNA into siRNAs in conjunction with Loqs or R2D2, which are then loaded onto a RISC that contains Ago2 and targets mRNA for degradation. (B) Schematic of the miRNA pathway. Primary miRNAs are transcribed from the genome, processed by Drosha and Pasha onto mature miRNAs. Dcr1 cleaves the pre-miRNA. After RISC has assembled, the active Ago1 enzyme within RISC cleaves complementary mRNA for endogenous gene regulation. (C) Schematic of the piRNA pathway. The piRNA precursor is cleaved by Zucchini to produce primary piRNA. After being loaded onto the Piwi/Aub complex, the sense transposon transcript is cleaved. It also produces a novel sense piRNA. This novel sense piRNA is employed by Ago3 to degrade the antisense transposon transcript.

to inconsistent outcomes. Although *D. melanogaster* is generally resistant to external dsRNA, hemocytes of *D. melanogaster* can absorb dsRNA from the extracellular environment, leading to a functional RNAi response [32]. Similarly, the *D. melanogaster* S2 cell line, derived from hemocytes, shows high sensitivity to dsRNA and is commonly used for *in vitro* RNAi studies [33]. The mechanisms underlying the tissue-specific RNAi responses may vary across species and even among tissues within the same species.

2.2. Cellular uptake of dsRNA and systemic RNAi

Two distinct pathways for dsRNA uptake in insect have been described: transmembrane channel-mediated and endocytosis-mediated uptake. The transmembrane protein system RNA interference defective 1 (*Sid1*), which is highly conserved and ubiquitously expressed (except in neurons), mediates the release of endosomal dsRNA into the cytoplasm during environmental RNAi, which is caused by dsRNA taken up by a cell from the environment, and systemic RNAi, which refers to the extension of the silencing signal from the cell in which the dsRNA is applied to other cells and tissues [34,35]. *Sid1* homologs have been identified in most insects, except for those of the superorder Antliophora such as Diptera, Mecoptera and Siphonaptera [36]. The insect *Sid1*-like protein shows high similarity with *C. elegans* Tag 130/ChUP1 protein [37], and is involved in dsRNA uptake in brown planthopper (*Nilaparvata lugens*) and the Colorado potato beetle (CPB, *L. decemlineata*) [38,39]. In contrast, the *Sid1*-like proteins in *Schistocerca gregaria* or *Plutella xylostella* appear to play no role in RNAi efficacy [40,41].

The clathrin-dependent endocytic pathway is the most well-documented mechanism of dsRNA uptake in insects. It was first characterized in *Drosophila* S2 cells, which lack *Sid*-like proteins [42]. Clathrin-dependent, receptor-mediated endocytosis can facilitate dsRNA uptake in several insect species [43,44]. Both *in vitro* and *in vivo* pharmacological suppression of this pathway in gut epithelial cells reduces dsRNA uptake, particularly in Coleoptera species such as the CPB. Phagocytosis, another major endocytosis pathway, is active primarily in specialized hemocytes, such as plasmatocytes [39]. Pattern recognition receptors (PRRs) and scavenger receptors are crucial for identifying dsRNA and facilitating its uptake via phagocytosis. In *D. melanogaster*, scavenger receptors such as SR-CI and Eater, accounted for more than 90% of the dsRNA uptake into S2 cells, indicating that receptor-mediated endocytosis functions in dsRNA uptake in this species [42]. However, inhibiting or blocking these receptors does not completely stop the absorption process, indicating that dsRNA internalization is a complex process [45,46].

Systemic RNAi is the process by which RNAi spreads between cells and can be transmitted to the offspring after dsRNA is introduced into a specific tissue or area [47]. In a dsRNA artificial diet feeding assay, the accumulation of Cyt3-labeled deoxycytidine triphosphate (dCTP) was initially found in the mouthparts of grain aphids. The labeled signal centralized in the midgut before spreading throughout the entire body when the compound was added with the synthesized dsRNA [48,49]. Combined with the fact that dsRNA-mediated gene silencing typically extends beyond the site of dsRNA delivery, the diffusion of fluorescence signal across body tissues suggests the presence of sustained systemic RNAi effects in aphid species [50]. Oral delivery of dsRNA targeting the *ecdysone receptor* (*Ecr*) and *ultraspiracle protein* (*USP*) reduced the survival rates of aphids, affecting both the treated generation and their progeny, indicating that the silencing effect was persistent and transgenerational [51]. In *C. elegans*, *Sid1* is required for dsRNA transport between cells to enable systemic RNAi [52]. Systemic RNAi in the desert locust *Schistocerca gregaria* exhibits tissue-specific efficiency and variable sensitivity [53]. In *D. melanogaster*, nanotube-like

structures facilitate systemic RNAi by transporting dsRNA and RNAi components between neighboring cells [54]. Exosome-like vesicles produced by immune cells (hemocytes) involved in immune priming transport siRNAs, mediating systemic RNAi in response to viruses [55].

2.3. Differences in RNAi machinery components in three RNAi pathways

Genes encoding core enzymes for three RNAi pathways have been identified across numerous species across different insect orders [56] (Table 1). These core elements or genes involved in RNAi pathways undergo diverse patterns of duplication and deletion across different insect species [36]. In the siRNA pathway, gene duplications have been reported for *Ago2*, *Dcr2*, and *R2D2*. The CPB has a duplicated *Ago2* gene, and both copies are essential for the RNAi response [57]. The increased RNAi efficiency observed in the red flour beetle (*Tribolium castaneum*) compared to *D. melanogaster* is due to the presence of extra copies of *Ago2* and *R2D2* in *T. castaneum*, while *D. melanogaster* possesses only a single copy of each [58,59]. The Asian citrus psyllid (*Diaphorina citri*), despite its high RNAi sensitivity, lacks the *R2D2* gene [60]. In the miRNA pathway, only a single gene copy of *Ago1* is present in mosquitoes *Anopheles gambiae* and *Culex pipiens*, but *Aedes aegypti* possesses two copies of the *Ago1* gene [61]. The pea aphid (*Acyrtosiphon pisum*) possesses two copies of *Ago1*, *Loqs*, and *Dcr1*, along with four copies of *Pasha* and one *Drosha* [62]. In the piRNA pathway, *A. pisum* and *Glossina morsitans* possess two to three copies of *Ago3* [19,36]. Two to three copies of *Aubergine* (*Aub*) genes, belonging to the Piwi family and crucial for silencing transposons and maintaining genome stability in animal germline cells, have been reported in the parasitoid wasp (*Nasonia vitripennis*) and the Indian jumping ant (*Harpegnathos saltator*) [63]. And 5–8 copies of *Piwi* genes have been identified in several insect species, including *A. pisum*, *A. aegypti*, *A. gambiae*, and the Russian wheat aphid *Diuraphis noxia* [36,58,63]. In systemic RNAi, the number of *Sid1* genes varies among insect orders. Most orders possess a single *Sid1* gene, while some coleopterans have two or three copies, and dipterans lack *Sid1* genes entirely [37,39,64]. Duplications of core RNAi elements or genes could result in subfunctionalization or neofunctionalization within RNAi pathways, potentially increasing RNAi efficacy in insects. In contrast, loss of these genes may reduce RNAi efficacy in some insect lineages [36,56].

The expression levels of RNAi pathway genes may also affect RNAi efficacy. Overexpression of *Dcr2* increased RNAi efficacy in transgenic *D. melanogaster* engineered to express hairpin RNAs [73]. Increased RNAi efficacy was observed in a Lepidoptera species, *Bombyx mori*, upon overexpression of the *Ago2* gene [74]. In the Western corn rootworm (WCR), the RNAi response is blocked when *Ago2*, *Ago1*, and *Aub* genes are silenced [57]. Core siRNA enzymes are basally expressed across life stages and tissues. In *L. decemlineata*, the expression of core siRNA enzymes is highest in young larvae, coinciding with higher RNAi efficacy at that developmental stage [57,75]. The expressions of core siRNA elements/genomes are influenced by various factors, such as repeated exposure to dsRNA, environmental conditions, and pathogens. These factors may account for variations in RNAi efficacy even within the same insect species [42].

2.4. Delivery methods of dsRNA

Major dsRNA delivery methods include microinjection, feeding, and soaking [76]. Microinjection involves precise injection of a small volume of dsRNA into the insect body or embryo [77]. The first successful application of dsRNA microinjection to silence the *frizzled* and *frizzled 2* genes was reported in *D. melanogaster* [78].

Table 1
Identified components involved in RNAi in insects.

RNAi pathway	Protein	Order	Species	Reference
siRNA	Argonaute2	Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. [57]
		Diptera	<i>Drosophila melanogaster</i>	Lewis et al. [58]
		Hemiptera	<i>Nilaparvata lugens</i>	Xu et al. [38]
		Lepidoptera	<i>Bombyx mori</i>	Wan et al. [65]
	Dicer2	Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. [57]
		Diptera	<i>Aedes aegypti</i>	Lewis et al. [58]
		Hemiptera	<i>Bemisia tabaci</i>	Upadhyay et al. [66]
			<i>Nilaparvata lugens</i>	Xu et al. [38]
	R2D2	Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. [53]
		Coleoptera	<i>Agilus planipennis</i>	Zhao et al. [67]
		Diptera	<i>Drosophila melanogaster</i>	Marques et al. [59]
		Hemiptera	<i>Diaphorina citri</i>	Taning et al. [60]
	Staufen Translin/Trax		<i>Bemisia tabaci</i>	Upadhyay et al. [66]
		Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. [18]
		Coleoptera	<i>Tribolium castaneum</i>	Garbutt et al. [68]
		Lepidoptera	<i>Manduca sexta</i>	Garbutt et al. [68]
miRNA	Argonaute1	Diptera	<i>Aedes aegypti</i>	Lewis et al. [58]
			<i>Culex pipiens</i>	Campbell et al. [61]
			<i>Anopheles gambiae</i>	Campbell et al. [61]
		Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. [62]
	Dicer1	Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. [69]
		Diptera	<i>Aedes aegypti</i>	Lewis et al. [58]
		Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. [62]
			<i>Nilaparvata lugens</i>	Xu et al. [38]
	Drosha	Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. [69]
		Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. [62]
			<i>Diaphorina citri</i>	Taning et al. [60]
		Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. [69]
	Loquacious	Diptera	<i>Drosophila melanogaster</i>	Marques et al. [59]
		Hemiptera	<i>Diaphorina citri</i>	Taning et al. [60]
			<i>Acyrtosiphon pisum</i>	Jaubert et al. [62]
			<i>Drosophila melanogaster</i>	Denli et al. [70]
piRNA	Argonaute3	Diptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. [62]
			<i>Aedes aegypti</i>	Lewis et al. [58]
			<i>Glossina morsitans</i>	Dowling et al. [36]
		Hemiptera	<i>Acyrtosiphon pisum</i>	Christiaens et al. [19]
	Aubergine		<i>Nilaparvata lugens</i>	Xu et al. [38]
		Lepidoptera	<i>Bombyx mori</i>	Wang et al. [65]
		Diptera	<i>Aedes aegypti</i>	Lewis et al. [58]
		Hemiptera	<i>Diaphorina citri</i>	Taning et al. [60]
	Piwi	Hymenoptera	<i>Nasonia vitripennis</i>	Mongelli et al. [63]
		Diptera	<i>Aedes aegypti</i>	Lewis et al. [58]
		Hemiptera	<i>Acyrtosiphon pisum</i>	Dowling et al. [36]
		Diptera	<i>Drosophila melanogaster</i>	Nishimasu et al. [71]
	Zucchini Sid 1	Diptera	<i>Aphis glycines</i>	Bansal et al. [72]
		Hemiptera	<i>Sitobion avenae</i>	Wang et al. [49]
			<i>Diabrotica virgifera virgifera</i>	Miyata et al. [64]
		Coleoptera	<i>Leptinotarsa decemlineata</i>	Cappelle et al. [39]

While microinjection is a reliable method for functional genomic studies, it is not suitable for controlling insect pests in the field. This approach is limited by high technical requirements and application to small or aquatic species [79,80]. Another basic delivery method is oral, in which insects are fed on a diet enriched with synthetic or microorganisms-expressed dsRNA [81]. This approach is less labor-intensive and more practical for pest control, and is particularly effective in coleopterans [82]. However, the efficacy of RNAi via oral delivery is lower in many insect species. In *Locusta migratoria*, RNAi is highly effective after injection but minimally effective with feeding. The main difference between the two dsRNA delivery methods is the degradation of dsRNA by double-stranded ribonucleases (dsRNases) in the insect gut [83]. The soaking delivery approach is commonly applied to insect cell lines by introducing dsRNA into the culture medium [33,84]. Topically applied dsRNA/siRNA formulations may breach the insect cuticle and cause mortality [85,86].

Recent technologies for improving delivery are cationic liposome-assisted, nanoparticle-enabled, and microbe-mediated delivery systems. Cationic liposomes serve as transfection agents,

enabling the effective transfer of dsRNA into animal cells through a process called lipofection [87]. Positively charged cationic lipids interact with negatively charged dsRNA to form lipoplexes through electrostatic forces, which is widely used for dsRNA delivery in insect cell cultures [88]. Combining RNAi with nanotechnology presents an alternative innovative methodology for pest control, as recently reviewed [89]. In a pioneering study of nanoparticle-mediated dsRNA delivery, chitosan nanoparticles were used to silence the *chitin synthase* gene in *A. gambiae*, resulting in increased RNAi efficacy [90]. Chitosan along with other dsRNA nanoparticles have been used to increase RNAi efficacy in insect species [91,92]. Microbe-based dsRNA delivery, including the symbiont-mediated and pathogen-mediated RNAi, has emerged as a promising strategy. Symbiont-mediated RNAi exploits symbiotic bacteria to continuously produce dsRNA, inducing RNAi in the insect hosts. RNase III-deficient bacterial strains derived from the symbionts of the kissing bug (*Rhodnius prolixus*) and the western flower thrips (WFT, *Frankliniella occidentalis*), permitted stable generation of dsRNA molecules that effectively suppress targeted insect genes [93]. Pathogen-mediated RNAi involves engineering ento-

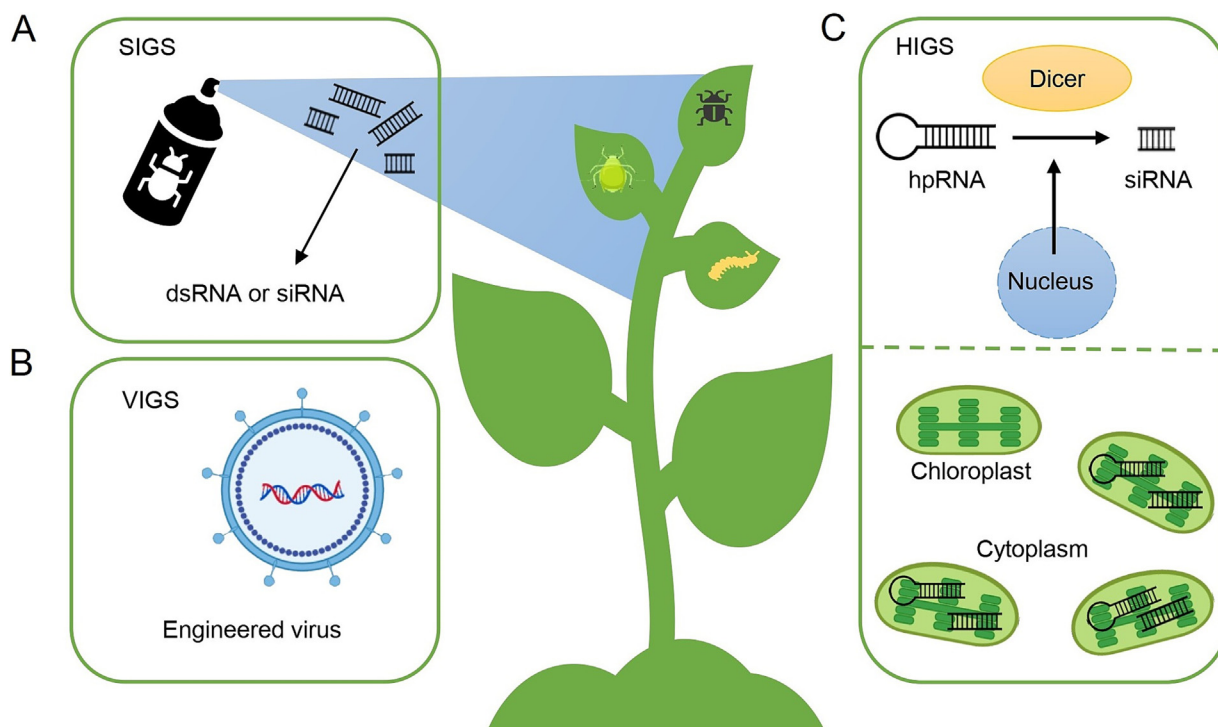


Fig. 2. Diverse methods for RNAi application. (A) Spray-induced gene silencing (SIGS), a spray-based non-transgenic method, dsRNA or siRNA that target pest genes are sprayed directly onto plants. (B) Virus-induced gene silencing (VIGS) is a delivery method that employs viral expression vectors to generate dsRNA in plants, specifically targeting insect genes upon ingestion. (C) Host-induced gene silencing (HIGS) refers to the development of transgenic plants expressing dsRNA in the nuclei or the chloroplasts, allowing insects to acquire dsRNA or siRNA through feeding. Long dsRNAs can be stably produced in the chloroplast, a cellular compartment that appears to lack Dicer enzymes.

mopathogenic fungi to express miRNAs targeting host immune genes, dramatically increasing fungal virulence against *Aedes aegypti* and *Galleria mellonella* [94].

3. Applications of RNAi for insect control in crop protection

The application of RNAi in agricultural pest control is divided into three main categories: host-induced gene silencing (HIGS), virus-induced gene silencing (VIGS), and spray-induced gene silencing (SIGS) (Fig. 2; Table 2). HIGS was first demonstrated by enabling transgenic crops to produce pathogen-targeting dsRNA, thereby silencing fungal virulence genes during infection [95]. The first proof of concept of HIGS for insect pest control was demonstrated in WCR. Transgenic maize plants expressing hairpin RNA (hpRNA) targeting the *snf7* gene in WCR protected the plants from larval feeding damage [96]. HIGS targeting *CYP6AE14*, a cytochrome P450 gene, was effective against the cotton bollworm *Helicoverpa armigera* in transgenic cotton [97]. Expression of dsRNA homologous to the *shp* gene in transgenic barley reduced the reproduction and survival rates of grain aphids (*Sitobion avenae*) [98]. Transgenic cotton expressing dsRNA targeting *FATTY ACYL-COA REDUCTASE* (*AsfAR*) showed reduced predation by cotton aphids (*Adelphocoris suturalis*), and diminished insect fertility [99]. Transgenic wheat expressing dsRNA targeting a zinc finger protein encoding gene *SaZFP* and the salivary protein gene *SmDSR33*, respectively, led to increased mortality and reduced fecundity of aphids [100,101]. dsRNA can be expressed at high levels in plant plastids that lack RNAi mechanisms. This transplastomic approach has been successful in protecting plants from chewing insects like the CPB, the WFT, and willow leaf beetle *Plagioderma versicolora* [102,103], as well as sap-sucking insects like the green peach aphid *Myzus persicae* [104]. Transplastomic poplar

that expressed dsRNAs targeting the β -Actin and *Srp54k* genes of *Plagioderma versicolora* showed higher larval mortality than nuclear transgenic plants [105].

VIGS is an RNA-based reverse genetics technology that has been developed to produce insect-specific dsRNAs in plants for pest management. Tobacco with *Tobacco rattle virus* (TRV) targeting the hornworm *Manduca sexta* Cytochrome P450 gene *CYP6B46* made *M. sexta* more susceptible to nicotine damage [132]. Similarly, recombinant *Tobacco mosaic virus* (TMV) targeting the citrus mealybug (*Planococcus citri*) in tobacco resulted in reduced fecundity and increased mortality of *P. citri* [149]. In addition to phyto-virus, VIGS can also directly use recombinant viruses to infect insects, inducing RNAi-mediated gene silencing in insect cells [157]. The recombinant *Citrus tristeza virus* (CTV)-RNAi vector, engineered with the *abnormal wing disc* (*Awd*) gene of Asian citrus psyllid (*D. citri*), resulted in malformed-wing phenotype in adults and increased mortality [148].

SIGS is a non-transgenic method in which dsRNAs or sRNAs targeting pest and pathogen genes are sprayed directly onto plants. These RNAs are then absorbed by the pest and pathogen, inducing the silence of target genes [158,159]. SIGS can also be applied via root absorption and trunk injection, enabling insects to ingest dsRNA via sucking or chewing [77]. The first study demonstrating SIGS targeted the *acetylcholine esterase* gene of the diamondback moth (*Plutella xylostella*). Second-instar larvae fed on treated leaves showed a mortality rate of 89% [160]. Another study demonstrated that foliar application of naked dsRNA targeting the *actin* gene was stable under greenhouse conditions for at least 28 days, resulting in effective insect control [85]. SIGS targeting the salivary effector *Sg2204* in the greenbug (*Schizaphis graminum*) led to induced plant defense responses, altered feeding behavior, and decreased aphid survival rate [141].

Table 2
Insect control in crops via RNAi using HIGS, VIGS, and SIGS methods.

Application method	Crop	Insect species	Target gene	Reference
HIGS	Barley (<i>Hordeum vulgare</i>)	<i>Sitobion avenae</i>	<i>Shp</i>	Abdellatef et al. [98]
	Cotton (<i>Gossypium hirsutum</i>)	<i>Adelphocoris suturalis</i>	<i>fatty acyl-coenzyme A reductase</i> ; <i>hormone receptor 3</i>	Luo et al. [99]; Han et al. [106]
	Maize (<i>Zea mays</i>)	<i>Helicoverpa armigera</i>	<i>CYP6AE14</i>	Mao et al. [97]
		<i>Apolygus lucorum</i>	<i>AlucV-ATPase-E</i>	Liu et al. [107]
		<i>Chilo partellus</i>	<i>Chitinase</i>	Adeyinka et al. [108]
		<i>Diabrotica virgifera virgifera</i>	<i>vitellogenin receptor, boule</i> ; <i>SSJ1</i> ; <i>Snf7</i> ; <i>wings up A</i>	Niu et al. [109]; Hu et al. [110]; Bolognesi et al. [96]; Fishilevich et al. [111]
	Potato (<i>Solanum tuberosum</i>)	<i>Diabrotica virgifera virgifera</i>	<i>ecdysone receptor</i>	Hussain et al. [112]
		<i>Halyomorpha halys</i> , <i>Phenacoccus madeirensis</i>	<i>v-ATPase</i>	Kaplanoglu et al. [113]
		<i>Henosepilachna vigintioctopunctata</i> , <i>Myzus persicae</i> , <i>Spodoptera litura</i>	<i>β-actin</i>	Ren et al. [114]
		<i>Leptinotarsa decemlineata</i>	<i>cuticular protein, glutathione synthetase</i> , <i>cytochrome P450 monooxygenases</i> ; <i>v-ATPase</i> ; <i>Ecdysone receptor</i>	Naqqash et al. [115]; Kaplanoglu et al. [113]; Hussain et al. [112]
	Rice (<i>Oryza sativa</i>)	<i>Myzus persicae</i>	<i>macrophage inhibitory factor</i>	Murtaza et al. [116]
		<i>Chilo suppressalis</i>	<i>Krüppel homologue 1</i> ; <i>heat shock protein 19</i> ; <i>fatty acyl-coenzyme A reductase</i> ; <i>disembodied</i> ; <i>Spook and Ecdysone receptor</i> ; <i>small heat shock protein gene</i>	Tang et al. [117]; Wu et al. [118]; Sun et al. [119]; Zheng et al. [120]; He et al. [121]; Mao et al. [122]
	Soybean (<i>Glycine max</i>)	<i>Nilaparvata lugens</i>	<i>glutathione S-transferase</i>	Yang et al. [123]
		<i>Apolygus lucorum</i>	<i>AlucV-ATPase-E</i>	Liu et al. [107]
		<i>Leguminivora glycinivorella</i>	<i>SpbP0</i>	Meng et al. [124]
		<i>Tetranychus urticae</i>	<i>coatomer subunit α, aquaporin 9</i>	Dubey et al. [125]
	Tobacco (<i>Nicotiana tabacum</i>)	<i>Bemisia tabaci</i>	<i>Acetylcholinesterase</i> ; <i>ecdysone receptor</i> , <i>aquaporin, α-glucosidase</i> ; <i>β-actin</i>	Malik et al. [126]; Raza et al. [127]; Dong et al. [128]
		<i>Chilo partellus</i> , <i>Maruca vitrata</i> , <i>Spodoptera litura</i>	<i>chitin synthase A</i>	Rana et al. [129]
		<i>Frankliniella occidentalis</i>	<i>β-actin, Tubulin v-TPase B, Snf7</i>	Wu et al. [103]
		<i>Helicoverpa armigera</i>	<i>Chitinase</i>	Mamta et al. [130]
VIGS			<i>acetylcholinesterase 1</i>	Saini et al. [131]
		<i>Leptinotarsa decemlineata</i>	<i>β-actin, Shrub</i>	Zhang et al. [102]
		<i>Manduca sexta</i>	<i>CYP6B46</i>	Kumar et al. [132]
		<i>Myzus persicae</i>	<i>v-ATPaseA</i>	Poreddy et al. [133]
			<i>CYP82E4v1</i> ; <i>cathepsin L</i> ; <i>Dhc64C</i>	Zhao et al. [134]; Rauf et al. [135]; Dong et al. [104]
	Tomato (<i>Solanum lycopersicum</i>)	<i>Bemisia tabaci</i>	<i>Phenolic glucoside malonyltransferase 1</i> ; <i>vacuolar ATP synthase catalytic subunit A</i>	Xia et al. [136]; Pizetta et al. [137]
		<i>Frankliniella occidentalis</i>	<i>coatomer protein subunit epsilon</i>	Venkatesh et al. [138]
		<i>Helicoverpa armigera</i>	<i>Chitinase</i> ; <i>ecdysone receptor</i>	Mamta et al. [130]; Yogindran et al. [139]
	Wheat (<i>Triticum aestivum</i>)	<i>Tetranychus evansi</i>	<i>β-Actin</i>	Wu et al. [140]
		<i>Schizaphis graminum</i>	<i>Sg2204</i>	Zhang et al. [141]
		<i>Sitobion avenae</i>	<i>lmf2-like gene</i> ; <i>Chitinase</i> ; <i>Gqα</i> ; <i>ZFP</i>	Xu et al. [142]; Zhao et al. [143]; Hou et al. [144]; Sun et al. [100]
		<i>Sitobion miscanthi</i>	<i>DSR33</i>	Zhang et al. [101]
		<i>Aphis gossypii</i>	<i>callose synthase 5</i>	Mbiza et al. [145]
	Cotton (<i>Gossypium hirsutum</i>)	<i>Helicoverpa armigera</i>	<i>peptidyl prolyl trans-isomerase 5</i>	Wang et al. [146]
	Maize (<i>Zea mays</i>)	<i>Ostrinia furnacalis</i>	<i>EXP-A20</i>	Batool et al. [147]
	Tobacco (<i>Nicotiana attenuate</i>)	<i>Manduca sexta</i>	<i>CYP6B46</i>	Kumar et al. [132]
	Tobacco (<i>Nicotiana benthamiana</i>)	<i>Diaphorina citri</i>	<i>phytoene desaturase, Awd</i>	Hajeri et al. [148]
		<i>Planococcus citri</i>	<i>Actin, chitin synthase 1, V-ATPase</i>	Khan et al. [149]
		<i>Tetranychus evansi</i>	<i>protein disulfide isomerase, APX3-1</i>	Cui et al. [150]
		<i>Phenacoccus solenopsis</i>	<i>Bursicon, V-ATPase</i>	Khan et al. [151]
	Tobacco (<i>Nicotiana tabacum</i>)			
	Wheat (<i>Triticum aestivum</i>)	<i>Sitobion miscanthi</i>	<i>chemosensory protein 4</i>	Zhang et al. [152]

Table 2 (continued)

Application method	Crop	Insect species	Target gene	Reference
SIGS	Barley (<i>Hordeum vulgare</i>)	<i>Sitobion avenae</i>	<i>Shp</i> ; migration inhibitory factors	Biedenkopf et al. [153]; Liu et al. [154]
	Potato (<i>Solanum tuberosum</i>)	<i>Leptinotarsa decemlineata</i>	<i>Actin</i>	San et al. [85]
	Soybean (<i>Glycine max</i>)	<i>Aphis glycines</i>	<i>TREH, ATPD, ATPE and CHS1</i>	Yan et al. [155]
	Tomato (<i>Solanum lycopersicon</i>)	<i>Myzus persicae</i> , <i>Tetranychus urticae</i> , <i>Trialeurodes vaporariorum</i> ,	helper component-proteinase	Gogoi et al. [156]
	Wheat (<i>Triticum aestivum</i>)	<i>Schizaphis graminum</i>	<i>Sg2204</i>	Zhang et al. [141]

HIGS, host-induced gene silencing; VIGS, virus-induced gene silencing; SIGS, spray-induced gene silencing.

4. Advantages and disadvantages of RNAi-based insect control

While the application of RNAi holds great promise in insect control, employing it in the field will require careful consideration of its advantages and disadvantages. HIGS shows potential for controlling species in the order Hemiptera. Many hemipteran insects, such as aphids, are agricultural pests, making HIGS a promising strategy for pest management [161]. The transplastomic approach can overcome the limitations of nuclear transformation in which most of the dsRNAs are processed into siRNAs by Dicer enzymes, which are absent in plastids. The transplastomic resistant crops do not transmit their genetic material via pollen, thereby benefiting ecological sustainability [162]. However, owing to the limitation of plastid transformation technology, only a few plant species can be subjected to this method. Genetically modified plants expressing dsRNAs may take many years to develop and require millions of dollars to commercialize due to the stringent regulations. The general lack of public acceptance toward genetically modified crops is a barrier, delaying the broader application of HIGS in agriculture [163]. However, owing to its effectiveness, HIGS has been approved for commercial use. In 2023, the transgenic corn variety SmartStax Pro was launched in the U.S. market. This variety expresses dsRNA targeting the *snf7* gene in the WCR and is combined with two *Bacillus thuringiensis* (*Bt*) Cry proteins (Cry3Bb1 and Cry34/35Ab) to delay the development of resistance to *Bt* proteins. In 2024, the U.S. Environmental Protection Agency (EPA) approved DP23211, an RNAi-based maize product that is both herbicide-tolerant and insect-resistant. This genetically modified maize contains *DvSSJ1* dsRNA and the IPD072Aa protein, which are designed to combat corn rootworm (*Diabrotica* spp.) infestations. Application of HIGS goes beyond pest control. Bayer's "Vistive Gold" high-oleic soybeans (Mon87705), targeting a gene in the fatty acid biosynthesis pathway, have been approved for food, feed, and cultivation in the USA, Canada, and Japan, respectively [164].

The advantage of VIGS lies in its high efficiency even in refractory cells, because viruses can naturally penetrate cells, bypassing the obstacles to dsRNA uptake in insects. The host specificity of viruses ensures that dsRNA functions in the intended target species, reducing off-target effects and minimizing potential ecological disruptions [165]. However, the use of recombinant viruses for dsRNA delivery may be constrained by virally encoded RNAi inhibitors, which can modulate the silencing effect. The ecological consequences of releasing recombinant viruses into the environment must be carefully evaluated to avoid harm to non-target organisms and ecosystems [157].

The application of SIGS minimizes the risk of toxicity to animals following ingestion or exposure. Topical application of dsRNA is unlikely to pose an environmental hazard, as dsRNAs degrade rapidly in soil and water [166]. But the instability of dsRNA is also

a challenge for SIGS application in agriculture, which reduces its effectiveness compared to genetically modified HIGS plants that produce dsRNA continuously. SIGS may require specialized formulations to increase dsRNA stability and RNAi efficacy in target insects. Foliar application may be more effective against insects that are particularly sensitive to dietary dsRNA intake [165]. Compared to chewing insects, delivering dsRNA through SIGS to piercing-sucking insects such as aphids remains challenging. These insects consume minimal plant tissue while feeding on phloem sap, making foliar application of dsRNA unlikely to elicit an effective RNAi response [11]. Microinjection is an effective method for delivering dsRNA into various insect orders, including Hemiptera [167]. However, it is impractical as a strategy for crop protection in the field. A promising SIGS application is the sprayable biopesticide Ledprona, which contains long dsRNAs that target the *psmb5* gene of CPB [168]. This product is approved for commercialization by EPA in 2023, marking a step toward the commercial use of SIGS in pest management.

5. Strategies for improving RNAi effectiveness in pest control

Numerous strategies have been devised to meet the challenges that hinder the effectiveness of RNAi and its application in insect pest control (Fig. 3). One approach is using chemically modified molecules to improve dsRNA delivery, increasing RNAi efficacy [169]. siRNAs modified with two 2'-methoxyl-nucleotides at both terminuses have shown effective silencing in the diamondback moth (*P. xylostella*) [160,170]. Chemical modifications can also reduce off-target effects of short siRNAs [171], suggesting this approach as a promising strategy for pest control. Combining RNAi with other pest management strategies, such as *Bt* toxins, biological agents and chemical treatments, is a more comprehensive approach for insect control. Transgenic cotton that integrates *Bt* toxins with RNAi targeting insect *JHMT* and *JHBP* genes has shown promise in delaying resistance development to *Bt* toxins in pests [172]. RNAi targeting the *IAP1* gene of *L. migratoria* combined with entomopathogenic fungi *Metharhizium acridum*, resulted in a lethal effect in *L. migratoria*, making it more susceptible to *M. acridum* infection [173]. The chemical ethylenediaminetetraacetic acid (EDTA), which can inhibit metalloenzymes and increase dsRNA stability, has been used as a co-formulant to increase RNAi effectiveness in the Neotropical stinkbug (*Euschistus heros*) [174].

Recently, several new delivery strategies of dsRNA in insects have been reported, offering promising alternatives to increase RNAi efficacy for fundamental research and pest control applications. Viral-like particles (VLPs) can be produced in microorganisms and can self-assemble *in vitro*, allowing the production of the dsRNA inside the particle [175]. VLPs are considered biologically safe owing to the lack of reproducing viral genetic material. VLPs were described in several insect viruses [176]. Novel VLPs

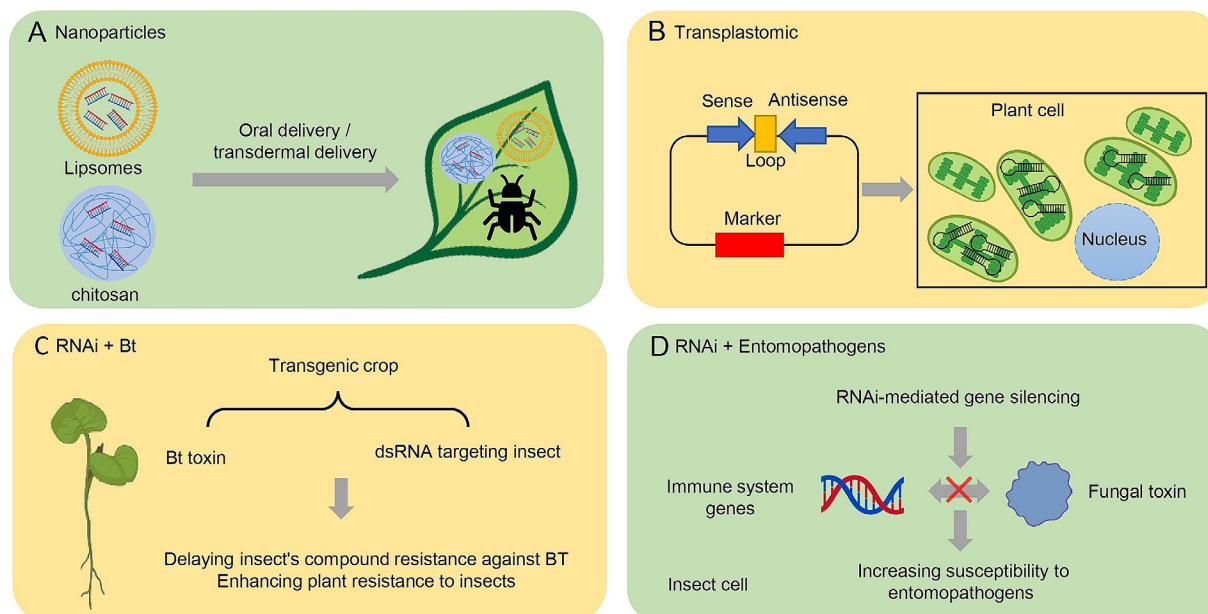


Fig. 3. Improved strategies using RNAi for insect control. (A) Synthetic nanoparticles can be employed to increase dsRNA delivery by insect feeding or spraying. (B) Transplastomic plants effectively increase the accumulation of long dsRNA in the encapsulated chloroplast. (C) Delaying insect's compound resistance against *Bacillus thuringiensis* (Bt) toxins. (D) Increasing plant resistance to insects with the combination of RNAi and entomopathogenic fungi.

derived from the *Drosophila* X virus (DXV) permit delivery of dsRNA both *in vitro* and *in vivo* in insects [177]. This finding suggests that insect virus-based VLPs can serve as nano-vehicles for dsRNA delivery. Combining the transplastomic RNAi technology with a bacteriophage MS2 VLP-based RNAi delivery system could increase plant resistance to cotton bollworm [178]. This approach effectively controlled lepidopteran pests through both bacteria- and plastid-mediated RNAi. Bacteria-expressed and nanocarrier-delivered RNA pesticides were used for pest control in a greenhouse trial [179]. The V-type proton ATPase subunit d (ATP-d) and ATP-G were selected as the RNAi target genes, and the hairpin RNAs (hpRNAs) targeting these two genes were fused together to be simultaneously expressed in *E. coli* BL21(DE3). Via spray application, the RNA pesticide achieved a promising control effect on *M. persicae* in the greenhouse. Encapsulating dsRNA within polymeric nanocarriers represents a promising strategy. Polymeric systems such as polycations, chitosan, guanylated polymers (GNPs), and star-polycation (SPC) have shown potential in protecting dsRNA from degradation, increasing cellular uptake, and ensuring controlled and precise release [180]. Examples include the BioClay nanocarrier platform, which uses the molecular complexation with layered double hydroxides (LDHs) to increase the environmental stability and biocidal efficacy of topically applied dsRNA on phylloplane surfaces [181]. Guanidinium-containing disulfide assembled sRNA nanoparticles (Gu^+ -sRNA NPs) facilitated the long-distance translocation of siRNAs through the vascular system of plants, which is essential for increasing whole-plant resistance to insect pests [182]. To increase dsRNA delivery and RNAi efficacy in lepidopterans, a fusion protein combining *Galanthus nivalis* agglutinin (GNA) and a dsRNA binding domain (dsRBD) was used to create a lectin-based dsRNA delivery system [183]. GNA:dsRBD increased dsRNA uptake and transfection efficiency in lepidopteran midgut cells, leading to RNAi increase.

6. Conclusions and perspectives

Some barriers need to be surmounted before the application of RNAi technology for pest control becomes a routine practice in the field. The mechanisms of dsRNA uptake and transport, sys-

temic RNAi, and differing RNAi responses in insects remain largely unknown. Developing strategies that can delay or prevent the occurrence of insect resistance would complement RNAi application. A combined approach involving gene pyramiding can be employed. Combining genes with distinct functions, such as Bt toxins and dsRNAs targeting RNAi pathways, could control lepidopteran, coleopteran, and hemipteran pest species simultaneously [184]. The synergistic effects of targeting multiple essential genes could increase the ability of RNAi to control aphids [185]. According to the behaviors of the pests, dsRNAs can be designed for developmental stage- or tissue-specific expression with specific promoters in RNAi plants. Aphid feeding behavior indicates that phloem-specific promoters are particularly useful as they drive high expression of exogenous genes in the phloem, which can strengthen the resistance of RNAi plants against phloem-feeding aphids by increasing defensive dsRNAs in the phloem sap while minimizing the exposure of non-target insects to these compounds [186]. At last, insect-specific RNAi-based biopesticides offer potential for wide applications in pest control in agricultural practice, avoiding societal and public concerns about chemical pesticides and the widespread reluctance toward GMOs. However, production of cost-effective dsRNA pesticides remains a major challenge. Current dsRNA synthesis methods include *in vitro* transcription (IVT), microbial expression in bacteria or fungi, and cell-free synthesis [187]. Each method has trade-offs scalability, cost, and complexity. While IVT kits allow small-scale dsRNA synthesis for initial RNAi screening, their high cost and low yield make them impractical for field use [188]. In contrast, microbial expression systems, such as the L4440-HT115 (DE3) strain, offer a sustainable and scalable alternative [189]. Recent advances in microbial expression system include optimized strains, genetic engineering, improved expression vectors, fermentation techniques, and dsRNA extraction methods [46,188,190–192]. Cell-free systems could eliminate the need for cell viability, simplifying the dsRNA production process [193,194]. Cell-free transcription has recently been used to produce dsRNA on a large scale, reducing production costs (<https://www.greenlightbiosciences.com/in-the-pipeline-colorado-potato-beetle/>).

CRediT authorship contribution statement

Xue Zhong: Writing – review & editing, Writing – original draft, Data curation. **Xiudao Yu:** Writing – review & editing. **Jiahui Zhang:** Writing – review & editing, Methodology, Formal analysis. **Jiajing Xu:** Writing – review & editing. **Mengchao Qin:** Writing – review & editing. **Mingxin Cao:** Writing – review & editing. **Frédéric Francis:** Writing – review & editing, Supervision, Funding acquisition. **Lanqin Xia:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We apologize to those whose work we were unable to cite due to space and reference limitations. Part of work mentioned in this review is funded by the National Natural Science Foundation of China (32188102 to Lanqin Xia) and Natural Science Foundation for Distinguished Young Scholars of Jiangxi province (20212ACB215001 to Xiudao Yu). Xue Zhong was supported by the China Scholarship Council (202303250062) and the GSCAAS-ULg Joint PhD Program.

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