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Review

Spray-Applied RNA Interference Biopesticides: Mechanisms, Technological Advances, and Challenges Toward Sustainable Pest Management

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Abstract

Spray-induced gene silencing (SIGS) represents a transformative paradigm in sustainable pest management, utilizing the exogenous application of double-stranded RNA (dsRNA) to achieve sequence-specific silencing of essential genes in arthropod pests. Unlike transgenic approaches, sprayable RNA interference (RNAi) biopesticides offer superior versatility across crop systems, flexible application timing, and a more favorable regulatory and public acceptance profile. The 2023 U.S. EPA registration of Ledprona, the first sprayable dsRNA biopesticide targeting *Leptinotarsa decemlineata*, marks a significant milestone toward the commercialization of non-transformative RNAi technologies. Despite the milestone, large-scale field deployment faces critical bottlenecks, primarily environmental instability, enzymatic degradation by nucleases, and variable cellular uptake across pest taxa. This review critically analyzes the mechanistic basis of spray-applied RNAi and synthesizes the recent technological breakthroughs designed to overcome physiological and environmental barriers. We highlight advanced delivery strategies, including nuclease inhibitor co-application, liposome encapsulation, and nanomaterial-based formulations that enhance persistence on plant foliage and uptake efficiency. Furthermore, we discuss how innovations in microbial fermentation have drastically reduced synthesis costs, rendering industrial-scale production economically viable. Finally, we outline the roadmap for broad adoption, addressing essential factors such as biosafety assessment, environmental fate, resistance management protocols, and the path toward cost-effective manufacturing.

Keywords: RNAi; biopesticides; dsRNA; environmental stability; commercialization

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1. Introduction

Modern agricultural systems are increasingly constrained by the escalating complexity of arthropod pest management. The long-term sustainability of conventional control methods is increasingly compromised by the rapid evolution of insecticide resistance, the regulatory phase-out of key active ingredients, strict residue limits, and the critical need to protect beneficial fauna [1]. Compounding these challenges, global climate change and expanding international trade are driving the range expansion and resurgence of invasive

and secondary pests, rendering outbreaks more volatile and unpredictable. These converging crises necessitate the development of innovative, mechanism-driven, and ecologically sound strategies to augment or supersede traditional chemical interventions [2].

In this landscape, RNA interference (RNAi) has emerged as a promising avenue for next-generation pest control [3,4]. As an evolutionarily conserved gene-silencing mechanism, RNAi is triggered by double-stranded RNA (dsRNA), which orchestrates the sequence-specific degradation of homologous messenger RNA (mRNA) via complementary base pairing. By operating at the post-transcriptional level, RNAi facilitates the precise suppression of target genes, resulting in robust loss-of-function phenotypes with high molecular specificity [5,6]. Biopesticides based on this mechanism utilize polynucleotides to silence essential genes in pathogens or pests, inducing physiological failure or mortality while preserving crop health [7–9]. Distinguished by their high target specificity, rapid mode of action, and minimal impact on non-target organisms, RNAi-based biopesticides align with the principles of sustainable agriculture. Consequently, they are widely regarded as a transformative innovation, frequently cited as the third revolutionary milestone in the history of pesticide development [10].

Driven by the immense potential of this technology, academic and commercial entities are investing heavily in its advancement [11]. Since Monsanto (now Bayer) introduced the concept of RNAi biopesticides in 2007, the agrochemical industry has pivoted toward leveraging RNAi for pest and disease management, resulting in a surge of patent filings for innovative solutions [12]. Current applications generally fall into two categories: plant-incorporated protectants (transgenic plants expressing dsRNA) and sprayable dsRNA formulations, which are applied analogously to conventional agrochemicals [13]. Both strategies exploit the RNAi pathway to selectively silence key genes in pests such as Coleoptera and Lepidoptera, providing a precise and sustainable alternative to traditional pest management methods [14].

The commercialization of the genetically modified corn event MON87411, the first insect-resistant crop expressing dsRNA, received cultivation approval from the U.S. EPA in 2017. Subsequent transgenic events, such as DP23211 and VT4PRO, have also been authorized [15]. However, the transgenic approach is restricted by significant technical and social barriers. Currently, fewer than 40 crop species are amenable to the genetic modification required for dsRNA expression, and plastid transformation—often critical for high RNAi efficacy—remains technically challenging for many crops [16]. Furthermore, genetically modified organisms (GMOs) encounter substantial market opposition and require rigorous, time-consuming environmental risk assessments, limiting their global adoption.

In contrast to transgenic crops, spray-induced RNAi is gaining regulatory traction. A milestone was reached on December 28, 2023, when the U.S. EPA registered Ledprona, the world's first sprayable dsRNA biopesticide targeting Colorado potato beetle *L. decemlineata*. This approval marks a significant step toward commercializing non-transformative RNAi technologies. Unlike GMOs, externally applied dsRNA formulations generally face fewer regulatory hurdles and are viewed more favorably by the public [17,18]. Nevertheless, large-scale field efficacy is currently limited by the environmental instability of dsRNA, which is susceptible to rapid degradation by nucleases, ultraviolet light, and extreme temperatures, as well as by difficulties in ensuring efficient cellular uptake by the target pest [19]. These technical hurdles must be overcome to achieve widespread commercialization [20].

This review comprehensively synthesizes the principles and application potential of spray-applied RNAi biopesticides. We critically examine the barriers impeding their efficacy, particularly environmental instability and delivery inefficiencies, and discuss emerging strategies to enhance their performance. Finally, we highlight essential con-

siderations for large-scale deployment to provide a roadmap for future research and commercial applications.

2. Discovery and Mechanisms of RNAi

RNA interference (RNAi) is a ubiquitous and evolutionarily conserved regulatory mechanism in eukaryotes mediated by RNA molecules, which facilitates the silencing or suppression of specific gene functions. Functionally, RNAi operates by recognizing and binding to target gene transcripts through complementary base pairing, thereby inhibiting expression at either the transcriptional or post-transcriptional level [21]. The phenomenon was first observed in plants during investigations into the *chalcone synthase* (*CHS*) gene in *Petunia hybrida*. In an attempt to deepen flower pigmentation by overexpressing *CHS*, researchers paradoxically observed the suppression of anthocyanin biosynthesis, resulting in white or variegated flowers. This effect, initially termed "co-suppression," was later identified as a manifestation of gene silencing [22]. Similarly, observations were made in the fungus *Neurospora crassa*, where Romano and Macino described a similar silencing phenomenon they termed "quelling" [23]. foundational work began in 1995 when Guo and Kemphues injected both sense and antisense RNA strands of the *par-1* gene into *Caenorhabditis elegans*. They discovered that both strands unexpectedly triggered the specific degradation of *par-1* mRNA [24]. However, the definitive mechanism was elucidated in 1998 by Fire et al., who demonstrated that double-stranded RNA (dsRNA) was the potent initiating agent. Their experiments in *C. elegans* revealed that dsRNA induced gene silencing with an efficiency 10- to 100-fold greater than single-stranded RNA, a post-transcriptional process they coined "RNA interference" [25].

Subsequent research has firmly established dsRNA-induced gene silencing as a conserved pathway across diverse lineages, including insects, fungi, plants, and vertebrates. Typically, the pathway is triggered by long dsRNA or single-stranded RNA (ssRNA) that folds into a hairpin structure (hairpin RNA or hpRNA) [26]. A defining characteristic of RNAi is the processing of these precursor molecules into small RNAs that possess sequence-specific regulatory capabilities. Intracellular Ribonuclease III enzymes, such as Dicer and Drosha, cleave the precursor dsRNA into small interfering RNAs (siRNAs) approximately 22 base pairs (bp) in length. These siRNAs are subsequently incorporated into the RNA-induced silencing complex (RISC), a multi-protein assembly containing Argonaute protein 2 (AGO2) and associated enzymes, including endonucleases and exonucleases [27]. Within the RISC assembly, helicase activity unwinds the siRNA duplex; the passenger (sense) strand is degraded, while the guide (antisense) strand is retained. In an ATP-dependent process, the activated RISC utilizes the retained antisense strand to recognize and bind to complementary target mRNA sequences [28]. Once bound, the nucleolytic activity of RISC cleaves the target mRNA with high precision, preventing protein translation and effectively mediating post-transcriptional gene silencing [29].

3. Application Potential of RNAi Technology in Pest Control

RNA interference (RNAi) offers a versatile platform for pest management by specifically suppressing the expression of genes essential for growth, development, and survival. The inhibition of these critical targets can induce mortality or significantly reduce pest population densities. This strategy has demonstrated efficacy across a diverse range of insect orders, including Lepidoptera, Diptera, Hemiptera, and Isoptera [30]. For instance, Turner et al. validated the systemic nature of RNAi in *Epiphyas postvittana* by silencing an antennal pheromone-binding protein gene through the ingestion of in vitro synthesized dsRNA, confirming that oral delivery can effectively disrupt physiological functions in distal tissues [31]. Similarly, targeting the *aquaporin* gene in *Acyrthosiphon pisum* via an

artificial diet resulted in significant gene downregulation and a subsequent increase in hemolymph osmotic pressure, underscoring the potential of RNAi to disrupt fundamental physiological homeostasis [32].

Beyond direct mortality, RNAi serves as a potent tool for managing insecticide resistance. Bautista et al. targeted *CYP6BG1*, a cytochrome P450 gene implicated in cypermethrin resistance in *Plutella xylostella* [33]. Oral delivery of specific dsRNA successfully downregulated this gene, thereby restoring susceptibility to cypermethrin in the larvae. In another application involving the termite *Reticulitermes flavipes*, silencing genes encoding cellulase and storage proteins compromised the population's environmental adaptability. When this RNAi strategy was combined with juvenile hormone treatment, it induced fatal defects during the molting process [34]. These studies underscore the feasibility and effectiveness of RNAi as a targeted, gene-specific pest control method.

Promising RNAi targets generally encode proteins involved in critical physiological processes such as chitin biosynthesis, energy metabolism, molting, and immune responses. Key candidate genes identified to date include *chitin synthase*, *trehalose*, *chitinase*, *ecdysone receptor*, *juvenile hormone receptor*, *allatostatin*, *allatotropin*, *HSP90*, *Snf7*, *aminopeptidase*, *tryptophan oxygenase*, *arginine kinase*, *tyrosine hydroxylase*, *V-ATPase*, *vitellogenin*, *serine protease*, *acetylcholinesterase*, *P450 enzyme*, *glutathione-S-transferase*, *catalase*, *hydroxy-3-methylglutaryl coenzyme A reductase*, etc. [35]. These target genes can be broadly categorized into three categories based on their function and application profile:

- Insect-specific genes: Genes such as chitin synthase are unique to arthropods, offering a high safety profile. The selection of precise target sequences within these genes maximizes control efficacy while ensuring biosafety by minimizing off-target effects on non-pest species.
- Housekeeping genes: Genes like *V-ATPase* are fundamental to essential cellular processes across many life forms. While silencing these targets is often highly lethal, they require rigorous bioinformatic screening to ensure sequence specificity to the pest, thereby preventing unintended harm to beneficial organisms.
- Resistance-associated genes: Genes involved in detoxification, such as *P450 monooxygenases*, play key roles in metabolic resistance to chemical pesticides. Targeting these genes via RNAi can re-sensitize resistant pest populations by impairing insecticide detoxification, potentially enabling effective control with lower application rates and/or fewer spray events, and thus reducing overall reliance on conventional chemical pesticides (Table 1).

Table 1. RNAi target genes (proteins) with promising applications.

Target Gene	Species	Mode of Action	Action Effect	Reference
Category 1. Insect-specific target genes				
CHSA1	*Nilaparvata lugens*	Injection	High mortality rate under low-dosage treatment	[36]
TPS	*Nilaparvata lugens*	Feeding	The survival rate decreased by about 30%	[29]
CHSA	*Sitobion avanae*	Genetically modified plants	The number of aphids on host plants decreased by half	[37]
Cht9	*Musca domestica*	Injection	The expression level of target genes significantly decreased, resulting in a mortality rate of over half and accompanied by wing deformities	[38]
Cht2	*Musca domestica*	Injection	Abnormal emergence accompanied by increased mortality rate	[39]
Chts	*Leptinotarsa decemlineata*	Feeding	The metamorphosis and development of larvae were significantly inhibited, accompanied by an increase in mortality rate	[40]

Table 1. *Cont.*

Target Gene	Species	Mode of Action	Action Effect	Reference
EcR	*Helicoverpa armigera*	Genetically modified plants	The harm to the host was significantly reduced and about 40% of developmental abnormalities occured	[41]
CHSA	*Helicoverpa armigera*	Genetically modified plants	The pupation rate decreased to 46.7%	[42]
Cht5/7/10	*Plutella xylostella*	Injection	The emergence rate significantly decreased	[43]
Cht5/6/8	*Chilo suppressalis*	Injection	The emergence rate significantly decreased	[44]
TPS	*Spodoptera exigua*	Injection	The survival rate on the 2nd day after treatment was only 49.1%	[45]
CHSB	*Spodoptera exigua*	Feeding	The expression level of target genes was significantly reduced, leading to developmental disorders and increased mortality rates	[46]
Chts	*Agrotis ipsilon*	Injection	Obstructive molting and increased mortality rate	[47]
Category 2. Efficient housekeeping genes				
Snf7	*Diabrotica virgifera virgifera*	Feeding	Significant lethality at low doses	[48]
Snf7	*Diabrotica virgifera virgifera*	Feeding	The target gene level was significantly reduced and produced a systematic RNAi effect, which not only achieved a high mortality rate but also had a lethal effect on non-target pest *Diabrotica undecimpunctata howardi*	[49]
V-ATPase A	*Holotrichia parallela*	Feeding	Damage to the cuticle and midgut structures, hindered development, and increased mortality rate	[50]
V-ATP-E	*Diaphorina citri*	Feeding	Significant increases in mortality, weight loss, and midgut cell apoptosis	[51]
V-ATP	*Helicoverpa armigera*	Genetically modified plants	The vitality and pupation rate of larvae were significantly reduced	[42]
V-ATPase A	*Chilo suppressalis*	Genetically modified plants	Interacting with Bt toxins (Cry1Ca and Cry2Aa)	[52]
V-ATP	*Phenacoccus solenopsis*	Genetically modified plants	After consuming genetically modified tobacco, the corrected mortality rate reached 30%	[53]
V-ATPase A	*Amphitetranychus viennensis*	Feeding	The mortality rate has increased to around 90%, and the reproductive capacity has decreased by over 90%	[54]
Category 3. Resistance-associated genes				
CYP6ER1	*Nilaparvata lugens*	Injection	The mortality rate of imidacloprid increased by 34.42% compared to the control group	[55]
CYP6EM1	*Bemisia tabaci*	Feeding	Enhancing the sensitivity of *Bemisia tabaci* to dinotefuran, resulting in a significant increase in mortality rate	[56]
CYP6CY22	*Aphis gossypii*	Feeding	The mortality rate of *Aphis gossypii* exposed to cyantraniliprole increased by 2.08 times	[57]
CYP9A3	*Locusta migratoria*	Injection	The mortality rate of nymphs was greatly increased by deltamethyrin and permethrin	[58]
CYP9AQ1	*Locusta migratoria*	Injection	The lethality of tau-fluvalinate to nymphs increased by 29.8–53.0%	[58]
CYP6AE14	*Helicoverpa armigera*	Genetically modified plants	Reducing tolerance to gossypol, leading to an increased mortality rate	[59]
CYP321A1	*Helicoverpa armigera*	Injection	Significantly reducing the tolerance to flavonoids	[60]
CYP321B1	*Spodoptera litura*	Injection	The mortality rates of chlorpyrifos and deltamethrin increased by 25.6% and 38.9%, respectively	[61]
GSTs1	*Plutella xylostella*	Feeding	Reducing the tolerance to four insecticides	[62]

Identifying a target gene with high preventive and control potential is the first step; however, elucidating its mechanism of action is essential to guarantee safety. Achieving an optimal balance between potent pest control and the preservation of non-target species and ecosystem health remains a critical priority in the development of RNAi biopesticides.

4. Development and Applications of RNAi-Based Biopesticides

In 2007, researchers from the Chinese Academy of Sciences and Monsanto published two influential papers in Nature Biotechnology demonstrating the significant potential of RNA interference (RNAi) technology for pest control [48,59]. These pivotal studies

provided the first empirical validation of RNAi efficacy in an agricultural context, marking a watershed moment for molecular pest management. In one study, Mao and colleagues engineered transgenic cotton to express dsRNA targeting *CYP6AE14*, a cytochrome *P450 monooxygenase* gene in *Helicoverpa armigera*. This gene is essential for detoxifying gossypol, a natural defense compound produced by cotton. When *H. armigera* larvae fed on this transgenic cotton, *CYP6AE14* expression was significantly suppressed, compromising the larvae's ability to tolerate gossypol and resulting in severe growth retardation [59].

Concurrently, Baum et al. developed transgenic corn expressing dsRNA targeting the *V-ATPase A* gene of the western corn rootworm, *Diabrotica virgifera virgifera*, aiming to disrupt its energy metabolism and ion balance, ultimately impairing growth and development. Ingestion of the dsRNA-expressing tissue led to significant larval mortality and a marked reduction in root damage [48]. These pioneering studies have established RNAi technology as a promising strategy for pest control, which has since become a major focus of research [30].

In recent years, attention has increasingly shifted toward spray-induced gene silencing (SIGS) as an alternative to transgenic approaches. This transition is driven by the limitations of genetically modified (GM) crops, including their restricted host range, variable efficacy, and the significant regulatory and public acceptance hurdles they face. Sprayable dsRNA formulations offer a versatile and non-transformative solution. The feasibility of this approach was first demonstrated by the research team led by Miao, who applied dsRNA (50 ng/μL) targeting the *DS10* (*chymotrypsin-like serine protease*) and *DS28* (an uncharacterized protein) genes onto the integument of newly hatched *Ostrinia furnacalis* larvae. This topical application resulted in 40–50% mortality. Using fluorescent labeling, the study confirmed that dsRNA could penetrate the larval body wall and enter the hemolymph to induce systemic gene silencing [63]. Similarly, Miguel and Scott successfully utilized spray-induced RNAi against the *L. decemlineata*, by targeting the housekeeping gene *actin*. Their research notably demonstrated that dsRNA sprayed on foliage remained stable and biologically active for at least four weeks under greenhouse conditions [64].

These findings underscored the potential of foliar spraying as a practical pest control strategy. Since then, the scope of spray-induced RNAi has expanded successfully to a broader spectrum of pests, including Hemipteran species such as *Diaphorina citri* and *Myzus persicae*, as well as the arachnid mite *Tetranychus urticae*. Furthermore, research has indicated that sprayed dsRNA can be absorbed by plant tissues and translocated systemically to untreated leaves, thereby extending the protective coverage of the biopesticide [65,66].

5. Application of Spray-Induced RNAi-Based Biopesticide in Pest Control

Spray-induced RNAi-based biopesticides are predominantly formulated as aqueous solutions of double-stranded RNA (dsRNA) designed for direct application to crop foliage. Upon application, the dsRNA exerts its control effect through two primary pathways: direct penetration of the insect cuticle or ingestion following systemic absorption and vascular transport within the plant tissues [7]. This spray-based methodology offers a versatile, rapid, and straightforward alternative to transgenic approaches (Host-Induced Gene Silencing). It allows for the precise targeting of specific pests and developmental stages and facilitates the simultaneous silencing of multiple genes through the application of dsRNA cocktails. This "stacking" capability significantly enhances interference efficiency and increases pest mortality rates. Furthermore, spray-induced RNAi resolves many of the technical constraints associated with plant-mediated RNAi and effectively addresses public concerns regarding GMOs. In contrast to conventional chemical pesticides, sprayable dsRNA formulations exhibit a predictable and relatively short environmental half-life, char-

acterizing them as environmentally benign biocontrol agents and sustainable alternatives to synthetic chemicals.

The commercial landscape for spray-induced RNAi biopesticides has expanded rapidly in recent years. This approach circumvents the complex breeding and regulatory pipelines required for cultivating genetically modified plants, thereby avoiding the significant time and financial investments associated with GMO development. In 2019, Bayer submitted the world's first spray-mediated dsRNA product, BioDirect, to the U.S. EPA. This product demonstrated significant efficacy against Varroa destructor, a critical pest in the apicultural industry, while posing no adverse effects on honeybees [67]. In 2021, Bayer licensed the underlying patent to GreenLight Biosciences to facilitate production, with market entry anticipated in 2024. That same year, Syngenta validated the field efficacy of directly spraying dsRNA to control the *L. decemlineata* [68]. In 2022, Greenlight Biosciences' product, Ledprona, which protects against *L. decemlineata* through foliar spray application, received approval from the International Organization for Standardization (ISO). Following this, Greenlight Biosciences submitted a registration request for this product to the EPA. On 28 December 2023, the EPA granted approval for the registration of Ledprona, marking it as the world's first sprayable RNAi-based biopesticide.

6. Challenges in the Application of RNAi Technology

RNAi technology is widely utilized to inhibit gene expression, and its high efficiency and specificity make it a foundational technology for third-generation pesticides. Despite the successful registration of pioneering products like Ledprona and the presence of others in the regulatory pipeline, the widespread deployment of RNAi strategies faces significant hurdles. A primary limitation is the pronounced variability in RNAi efficiency across different insect orders. For instance, Coleopteran species such as *Tribolium castaneum* and Orthopterans like *Locusta migratoria* typically exhibit a robust RNAi response [69,70], whereas dipteran species like *Drosophila melanogaster* and many Lepidopteran species show comparatively low RNAi efficiency [71,72].

Furthermore, efficacy can vary drastically even within a single species depending on the method of dsRNA delivery. In *L. migratoria*, for example, injection of dsRNA induces potent gene silencing, whereas oral delivery (feeding) yields minimal to no effect [70,73]. Similar inconsistencies in RNAi outcomes can arise from factors such as the specific target gene selected, the developmental stage of the insect at the time of application, and the dosage of dsRNA administered [72].

Despite the promising potential of sprayable dsRNA, its practical efficacy is compromised by several biological and environmental factors. A critical physiological barrier is the presence of endogenous nucleases in the insect midgut, which can rapidly degrade dsRNA upon ingestion, thereby limiting its uptake and subsequent silencing activity [74,75]. Additionally, environmental degradation of dsRNA poses a significant barrier to the effective induction of RNAi, particularly in field applications [30,76–78]. To optimize the use of sprayable dsRNA, ongoing research must prioritize overcoming these stability issues, enhancing cellular delivery, conducting comprehensive environmental risk assessments, and developing proactive strategies to mitigate the evolution of resistance in target pests.

7. Nuclease That Degrades DsRNA

The hemolymph or intestinal fluids of many insects contain ribonucleases capable of degrading dsRNA. This enzymatic degradation prevents dsRNA from reaching target cells, thereby acting as a primary barrier to RNAi efficacy. Consequently, the stability of dsRNA within the insect host is a pivotal determinant of success. Extensive research has characterized various insect nucleases responsible for this activity, primarily categorized

into double-stranded ribonuclease (dsRNase) and RNAi efficiency-related nuclease (REase). Among these, dsRNase is the most intensively studied, as it can directly compromise RNAi efficacy by degrading dsRNA before uptake and processing. dsRNases are typically described as non-specific nucleases exhibiting activity against both DNA and RNA, and were originally identified in *Serratia marcescens* [79,80]. In insects, dsRNase was first isolated and characterized from the intestinal fluids of *Bombyx mori*, demonstrating its high efficiency in degrading dsRNA and other molecules [81].

The physiological role of dsRNases in *L. migratoria* has been systematically investigated [82,83]. As noted earlier, this species exhibits a robust RNAi response to injected dsRNA, but is refractory to oral delivery. To investigate this discrepancy, researchers identified four specific dsRNases. LmdsRNase1 and LmdsRNase4 were found predominantly in the hemolymph, whereas LmdsRNase2 and LmdsRNase3 were highly expressed in the midgut. Functional analysis revealed that silencing *LmdsRNase2* prior to feeding dsRNA targeting the molting genes *LmCht10* or *LmCHS1* resulted in significant gene suppression and distinct molting defects. In vitro assays confirmed that LmdsRNase2 rapidly degrades dsRNA across a broad pH range (6–10), whereas LmdsRNase3 showed minimal activity. This suggests that the high expression of LmdsRNase2 in the gut is the primary barrier to oral RNAi efficacy [73]. Conversely, although the hemolymph-derived nuclease LmdsRNase1 exhibits strong dsRNA-degrading activity in vitro, its catalytic function is strictly pH-dependent, with maximal activity under acidic conditions (optimal pH~5). Given that the hemolymph of *L. migratoria* is maintained near neutrality (approximately pH 7), LmdsRNase1 is expected to display minimal activity in vivo. As a result, injected dsRNA can persist in the hemocoel with enhanced stability, thereby facilitating efficient systemic RNAi induction [84]. Similar mechanisms have been observed in other species. Prentice et al. identified three dsRNases in the sweet potato weevil, *Cylas puncticollis*. By silencing the gut-expressed *CpdsRNase3* via injection, they successfully restored the efficacy of subsequent oral RNAi treatments [75]. Additionally, in various insect species, including Lepidoptera, *dsRNases* that influence the efficacy of RNAi have been identified.

Beyond dsRNases, a distinct class of enzymes termed RNAi efficiency-related nucleases (REases) has been identified. Guan et al. characterized a REase in *O. furnacalis*, capable of degrading a broad spectrum of nucleic acids, including dsRNA, dsDNA, ssRNA, and ssDNA. In vivo inhibition of this REase significantly enhanced the efficiency of RNAi targeting specific genes. Furthermore, ectopic expression of this REase in *D. melanogaster* attenuated the RNAi response, confirming its role in modulating RNAi efficiency through dsRNA degradation [74].

8. Endocytosis

A decisive factor governing the success of RNAi is the ability of insect cells to effectively internalize exogenous dsRNA. Two primary mechanisms for dsRNA absorption have been elucidated: transmembrane channel-mediated transport and endocytosis-mediated uptake. While certain insects possess transmembrane proteins homologous to the *C. elegans* SID-1 protein, the majority of insect species predominantly rely on endocytic pathways for dsRNA uptake [85–87]. These pathways are diverse and include clathrin-mediated endocytosis, folate-mediated endocytosis, macropinocytosis, and phagocytosis. The diversity of these endocytic routes is likely attributed to the various carriers involved [88–91].

Experimental evidence highlights significant interspecific variation in these uptake mechanisms. In *T. castaneum*, inhibitor-based studies have demonstrated that dsRNA uptake occurs primarily via a clathrin-dependent endocytic pathway [92]. Conversely, in the cotton boll weevil, *Anthonomus grandis*, macropinocytosis has been identified as the principal route for dsRNA internalization [93]. These findings underscore the complexity

of cellular absorption in insects, as different species utilize distinct mechanisms, and a single species may employ multiple, complementary endocytic pathways to ensure efficient uptake.

Upon entering the cell via endocytosis, dsRNA is enclosed within a vesicle, which then detaches from the plasma membrane and is internalized into an endosome. Late endosomes subsequently fuse with lysosomes for degradation. For dsRNA to remain functional and engage in RNA interference, it must successfully escape the endosome before being degraded in the lysosome. Therefore, the ability of dsRNA to traffic from the plasma membrane to the endosome and subsequently escape the endosome is a crucial factor influencing the efficiency of RNAi.

9. Methods to Improve the Efficiency of RNAi-Based Biopesticides

9.1. Dosage Optimization and Strategic Application

Enhancing the efficacy of RNAi-based biopesticides requires a multi-faceted approach involving the identification of potent target genes and the timing of applications to coincide with the pest's most vulnerable developmental stages. Additionally, increasing the application dosage represents a significant advantage of sprayable formulations over transgenic plants. Research indicates that, within a specific range, RNAi efficiency correlates positively with dsRNA dosage [94]. This is particularly important for insects like Lepidoptera, which are generally less responsive to RNAi, as certain genes may require higher doses to achieve effective interference [95]. Furthermore, studies in *L. migratoria* have demonstrated that genes highly expressed in the testes, such as *Piwi*, *Ago3*, and *Aubergine*, can only be effectively silenced through repeated injections during each instar stage [96].

9.2. Improve dsRNA Stability

The environmental and physiological stability of dsRNA is arguably the most critical determinant of RNAi success. Rapid degradation by nucleases in the insect gut or hemolymph often prevents cellular uptake, thereby nullifying the RNAi effect. Consequently, strategies to protect dsRNA are essential. These primarily involve: (a) inhibiting nuclease activity, and (b) encapsulating dsRNA within protective delivery vectors.

Since nuclease-mediated degradation represents a major constraint on RNAi efficacy, co-delivery of dsRNA with nuclease inhibitors has been proposed as a practical strategy to enhance dsRNA persistence. Effective implementation of this approach depends on identifying the key degradative nucleases—for example, the gut-expressed nuclease Lmd-sRNase2 in *L. migratoria* and mitigating their activity. Notably, many of these enzymes are metal-ion dependent; accordingly, co-application of chelating agents (e.g., EDTA) can suppress nuclease function and thereby improve dsRNA stability, helping to preserve the integrity and bioavailability of the delivered dsRNA [97].

Nanomaterials have emerged as superior delivery vehicles, offering enhanced transfection efficiency, protection, and stability. A milestone in this field was achieved in 2010 by Zhu and colleagues, who utilized chitosan nanoparticles to encapsulate dsRNA. Administration to *Anopheles gambiae* larvae resulted in significant gene silencing, demonstrating for the first time that nanomaterials could facilitate RNAi by improving dsRNA stability [98]. Building on this, Avila et al. engineered amphiphilic peptide-based nano-capsules loaded with dsRNA targeting a heat shock protein binding gene. Bioassays confirmed that these nano-capsules significantly accelerated mortality in *Aphis pisum* and increased mortality rates in *T. castaneum* compared to naked dsRNA [99].

Nanomaterials function by adsorbing dsRNA onto their surfaces or by encapsulating it, utilizing their nanoscale dimensions to create a physical barrier against nucleases. For example, Ma et al. developed a star polycation nanocarrier that formed a complex with

dsRNA, rendering it resistant to degradation by RNaseA and insect hemolymph [100]. Similarly, Christiaens et al. demonstrated that guanylate polymers effectively protected exogenous dsRNA from degradation in the highly alkaline midgut of *Spodoptera exigua* [101]. These findings confirm that nanocarriers can effectively navigate physiological barriers such as the peritrophic membrane and insect cuticle, ultimately improving pest control outcomes (Table 2). In addition to solid nanoparticles, liposome encapsulation has also been proven to enhance stability and interference efficiency [97].

Table 2. RNAi-based insecticide delivery systems based on nanomaterials.

Nanocarrier Material	Test dsRNA	Target Insect	Action Effect	Reference
Lipofectamine	ds*Tub*	*Blattella germanica*	Effectively preventing the degradation of ds*Tub* in the midgut, resulting in an RNAi efficiency of 60% and increased lethal effect	[102]
Lipofectamine 2000	ds*act-2* and ds*vATPaseA*	*Euschistus heros*	Increasing lethality and extending the effective period of dsRNA	[97]
Nanoliposome	ds*Met*	*Spodoptera frugiperda*	Reducing the use of dsRNA and improving the effectiveness of RNAi	[103]
Chitosan	ds*CHS1*	*Anopheles gambiae*	Improving the efficiency of RNAi	[98]
	ds*Vg*	*Aedes aegypti*	Improving RNAi efficiency, leading to higher mortality and teratogenicity rates	[104]
	ds*JHAMT*, ds*ACHE*, ds*HaLipn001*, ds*CHS1*	*Helicoverpa armigera*	Improving the silencing efficiency and efficacy of dsRNAs; Preventing the degradation of dsRNA under the action of intestinal nucleases	[105,106]
CS-TPP nanoparticles	ds*IAP*	*Aedes aegypti*	Forming a complex loaded with dsRNA with a particle size less than 200 nm and doubled the lethality rate	[107]
ROPE@C	ds*CHSA*	*Nilaparvata lugens*	Reducing the relative expression of CHSA by 54.3% and causing a 65.8% mortality rate of BPH	[108]
Fluorescent nanoparticle	ds*CHT10*	*Ostrinia furnacalis*	Causing significant weight loss, molting defects, and death of larvae by feeding mothed	[109]
Star polycation	ds*vATPaseD* ds*CHS1*	*Aphis glycines*	The silencing efficiency was 58.87%~86.86% and the mortality rate of *Aphis glycines* reached 78.5% by directly spraying on soybean seedlings	[110]
PAG87L	ds*ChSB*	*Spodoptera exigua*	The mortality rate was as high as 53.3%, significantly higher than the control group (16.7%)	[101]
Branched amphiphilic peptide capsule	ds*BiP* and ds*Armet*	*Tribolium castaneum*, *Acyrthosiphon pisum*	Significantly inhibiting the expression of target genes and greatly enhancing the lethal effect	[99]
Cell-membrane penetrating peptide	ds*ChSII*	*Anthonomus grandis*	Reducing the transcription level of target genes by 80%, significantly higher than naked dsRNA treatment (30%)	[111]
Layered double hydroxide	ds*Suc*, ds*Duox*, ds*Syx*	*Bemisia tabaci*	Effective controling of *Bemisia tabaci* at all stages through foliar spraying	[112]

10. Issues That Must Be Considered for the Large-Scale Application of RNAi-Based Biopesticides

RNAi-based biopesticides represent a paradigm shift in pest management. Significant progress has been made in demonstrating their feasibility, identifying high-value target genes, and optimizing delivery systems [17]. However, the majority of these validations remain confined to laboratory environments or limited small-scale field trials. As this technology transitions from nascent research to practical application, several critical barriers must be overcome to enable widespread field deployment.

The foremost consideration for any new crop protection agent is field efficacy. Despite the optimization strategies discussed previously, RNAi biopesticides currently may not

match the acute knockdown speed or broad-spectrum efficiency of conventional chemical insecticides. Consequently, the most viable immediate path for adoption involves integrating RNAi into Integrated Pest Management (IPM) frameworks—for instance, by targeting genes that confer resistance to chemical pesticides—thereby reducing reliance on synthetic chemistry while preserving control efficacy.

Beyond efficacy, commercial scalability hinges on the development of cost-effective, high-volume manufacturing processes. Furthermore, the long-term sustainability of RNAi biopesticides depends heavily on the proactive management of biological resistance [113]. To achieve successful commercialization and large-scale adoption, a comprehensive evaluation of biosafety, environmental fate, economic viability, and resistance risks is imperative. The following sections address these critical challenges, synthesizing insights from diverse fields to propose solutions for the successful market introduction of RNAi-based biopesticides [18].

10.1. Biosafety and Environmental Impact

While humans possess a fully functional RNAi system that relies on precise sequence matching, the risk posed by agricultural dsRNA is mitigated by the rigorous design of target sequences. Potential biosafety challenges can be effectively addressed during the screening phase by ensuring no homology exists with the human genome. Furthermore, the human immune system is robustly capable of identifying and rapidly eliminating exogenous dsRNAs that do not align with the genomic blueprint [114,115].

This sequence specificity is also the cornerstone of environmental safety regarding non-target organisms. When designing dsRNA for spray application or transgenic expression, sequences can be tailored to target specific pest genes while strictly excluding homologous sequences in beneficial organisms, such as pollinators, predators, and parasitoids. This precision makes it feasible to achieve high pest mortality while significantly mitigating biosafety risks to the broader ecosystem. However, vigilance is required; studies have suggested that processed small interfering RNAs (siRNAs) could theoretically exhibit off-target effects in non-target species [116]. This underscores the necessity for comprehensive and rigorous biosafety evaluations prior to the large-scale commercialization of any RNAi-based biopesticide.

The environmental stability of dsRNA is a critical factor that influences both field efficacy and ecological safety. As a biological macromolecule, dsRNA is inherently susceptible to degradation via photodegradation, rainfall wash-off, and microbial activity in soil and on plant surfaces. From a safety perspective, this rapid degradation is advantageous; research indicates that dsRNA typically degrades to undetectable levels in soil within 48 h and in aquatic environments within 7 days [117,118]. These findings suggest that the risk of long-term environmental accumulation or persistence is minimal.

However, this transience presents a significant challenge for pest control efficacy. The primary technical hurdle lies in enhancing the stability of dsRNA to ensure sustained bioactivity without compromising its safety profile. Recent innovations have shown promise in addressing this trade-off. For example, Australian researchers developed "BioClay," a formulation using clay nanosheets to stabilize dsRNA. This technology facilitates adhesion to plant surfaces and sustained release, protecting crops from viral diseases for 20–30 days [119,120]. Similarly, protecting dsRNA from photolysis and enzymatic degradation is crucial. Emerging solutions include the use of bacterial minicells (e.g., AgroSpheres), which encapsulate dsRNA within cross-linked biological membranes. This approach shields the payload from environmental ribonucleases and significantly extends the effective lifespan of the biopesticide in field conditions [121].

10.2. Low-Cost Mass Production of DsRNA

The commercial viability of spray-induced RNAi biopesticides is inextricably linked to the capacity for cost-effective, industrial-scale manufacturing. To support large-scale agricultural adoption, dsRNA production capacities must reach ton-scale levels—an ambition that initially seemed unattainable. Historically, production costs were a prohibitive barrier; in 2008, dsRNA synthesized via traditional chemical methods cost approximately $12,500 per gram, rendering agricultural application economically untenable. However, rapid technological innovations have dramatically altered this landscape. A transformative breakthrough occurred with the utilization of Escherichia coli strain HT115, which is deficient in dsRNA-degrading RNase III enzymes. This discovery enabled the efficient, fermentation-based production of dsRNA [122].

Driven by these biological advancements, production costs have plummeted. By 2016, the price of dsRNA had fallen to approximately $100 per gram. In 2017, the introduction of Apse RNA Containers (ARCs)—which utilize encapsulated bacteria to express and protect dsRNA—further reduced projected costs to roughly $2 per gram. Companies such as RNAgri have since pioneered the large-scale production and purification of ton-level quantities. Through strategic bacterial engineering and the optimization of downstream processing, RNAgri has reportedly achieved synthesis costs below $1 per gram. This dramatic reduction establishes a robust economic foundation for the widespread adoption of RNAi technology.

Current estimates suggest that effective pest control requires application rates ranging from 2 to 10 g of dsRNA per hectare. At current production costs, this makes RNAi-based biopesticides economically competitive with conventional chemical alternatives for many crops and regions. Furthermore, as market adoption accelerates, economies of scale are expected to drive prices down further, enhancing accessibility and cementing the role of RNAi in global pest management strategies [123–125].

10.3. Resistance of Pests to RNAi-Based Biopesticides

Although there are currently no reported cases of insect resistance to RNAi-based biopesticides in the field, the potential for resistance evolution remains an inevitable challenge for any pest control technology. Understanding the mechanisms that could drive this resistance is crucial for delaying its onset and ensuring product longevity. Resistance may arise primarily through genetic variability within pest populations; specifically, single nucleotide polymorphisms (SNPs) within the target gene sequence can disrupt the perfect complementarity required for effective dsRNA binding, thereby compromising silencing efficiency [126,127]. Alternatively, pests may evolve physiological barriers that prevent the uptake or processing of dsRNA.

To address the challenge of potential resistance, Monsanto established a laboratory-bred population of *D. v. virgifera* that exhibited resistance to the dsRNA targeting the Snf7 gene through rigorous multi-generational screenings. Further investigation revealed that the dsRNA ingested by these resistant insects failed to be efficiently absorbed by their intestinal tracts. Additionally, this population exhibited resistance to dsRNA targeting other genes as well, suggesting a broader mechanism of resistance. Genetic analysis identified mutations in a recessive gene located at the LG4 locus on autosomes, which contributed to the observed resistance. Remarkably, these resistant strains regained sensitivity to RNAi after being crossed with sensitive strains [128].

These findings provide valuable insights into the genetic basis of resistance to RNAi-based biopesticides. To mitigate resistance development, integrated pest management strategies could be implemented. These strategies may include the use of insect refuges, rotation of target genes, and the combination of RNAi with other pest control measures,

which would work synergistically to prolong the effectiveness and sustainability of RNAi-based biopesticides.

11. Prospects

As an emerging frontier in modern crop protection, RNAi-based biopesticides stand at the forefront of a post-chemical agricultural era. These bio-rational tools offer distinct advantages over traditional chemical pesticides, including exceptional target specificity, minimal toxicity to non-target organisms, and superior environmental compatibility. The convergence of advanced bioinformatics with nanotechnology has streamlined the design of dsRNA sequences, enabling the precise selection of targets that maximize pest mortality while minimizing biosafety risks [129]. However, the transition from laboratory promise to field reality requires navigating complex hurdles, specifically environmental variability, production scalability, and the transient stability of naked dsRNA.

To realize the full potential of sprayable RNAi, future research must prioritize interdisciplinary collaboration. Key milestones for widespread adoption include the establishment of standardized environmental risk assessment frameworks, the implementation of proactive resistance management protocols, and the refinement of regulatory pathways for product approval [130]. The commercial landscape is already shifting, with the first generation of products securing regulatory approval, signaling a maturing market. Looking ahead, continuous innovation in formulation stability and cost-effective mass production will be pivotal. As these technologies mature, commercial spray-induced RNAi biopesticides are positioned to revolutionize plant protection, providing a sustainable, precise, and socially acceptable alternative to synthetic chemicals within IPM systems [131,132].

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