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RNAi in Plant Protection: A Targeted Approach for Insect Pest Suppression

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ABSTRACT

The biggest challenge faced by humanity in the 21st is how to increase crop yields in a profitable, efficient, and sustainable way. There are several issues constraining agricultural productivity, such as damage by insect pests, diseases, and weeds. Currently, chemical pesticides remain the major approach used for suppressing insect pests owing to their well-controlled effect. Unfortunately, the excessive application of chemical pesticides has caused some serious problems threatening the environment and human health. RNAi is a post-transcriptional gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into a cell. This knockdown mechanism of gene by dsRNA is known as RNA interference (RNAi) in animals and post-transcriptional gene silencing in plants. The basic dsRNA delivery methods include microinjection, feeding, and soaking. To improve dsRNA delivery, various new technologies, including cationic liposome–assisted, nanoparticle-enabled, symbiont-mediated, and plant-mediated deliveries, have been developed. Chemically synthesized and modified siRNA corresponding to P. xylostellaAChE genes cause significant mortality of the insect both under laboratory and field conditions, which provides a novel strategy to control P. xylostella and to develop bio-pesticides based on RNA interference technology. However, the widespread adoption of RNAi for insect pest management faces several key challenges, including the high cost of dsRNA synthesis, the need for efficient delivery to the target site, concerns over off-target and non-target effects, and the potential development of resistance. Furthermore, innovative approaches such as cell-free RNAi production and nanotechnology-mediated RNAi transfer offer promising solutions to challenges like high synthesis costs and efficient dsRNA delivery, paving the way for the practical application of RNAi in sustainable insect pest management.

Keywords: *RNAi, post-transcriptional, insect pest, knockdown, pest suppression, siRNA, dsRNA and gene silencing.*

INTRODUCTION

Currently, chemical pesticides remain the major approach for suppressing insect pests owing to their well-controlled effect. Unfortunately, the excessive application of chemical pesticides has caused some serious problems threatening the environment and human health (27). The ability of herbivorous insects to adapt to insecticide use in agricultural systems presents an on-going challenge for pest management (3). By the end of 2020 nearly 16,920 cases of insecticide resistance were reported (33). Therefore, a great demand for novel and effective alternative approaches has developed in recent years owing to growing consumer awareness and pressure for safer and healthier food. A pest management strategy should be economically, environmentally and farmer friendly (37). In recent years, further advancements in several trends such as design, synthesis, and delivery of dsRNA led to the development of RNAi-based applications for plant protection (43). In RNA interference (RNAi), a key gene responsible for a vital biological process in the insect is identified and silenced. Since this gene is essential for the insect's survival, its suppression prevents the insect from carrying out critical functions, ultimately leading to its death. If an insect does not have a proper systemic RNAi system, the dsRNA treatment may not work effectively.

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This means that the gene silencing will only happen in specific areas, like the insect's midgut (where the dsRNA is absorbed), instead of spreading throughout the body. As a result, the insect may not die or may only experience mild effects, making the treatment less effective. Since genes are highly specific, RNAi only affects the targeted pest species and does not harm non-target organisms. This makes it a safe and sustainable method for pest control, reducing the need for chemical pesticides while minimizing environmental impact.

What is RNAi?

RNAi, first described in *Caenorhabditis elegans* and is known as post-transcriptional gene silencing because exogenous RNAs can induce sequence-specific mRNA degradation. Among the mature biotechnological tools, RNAi has not only provided a novel and powerful reverse genetics tool for identifying gene functions, but also showed great potential in pest management. One promising area is the development of transgenic crops expressing dsRNA against key genes of insect pests, which are now realized by some commercial products.

It is a post-transcriptional gene silencing mechanism that is initiated by the introduction of double-stranded RNA (dsRNA) into a cell. This knockdown mechanism of gene by dsRNA is known as RNA interference (RNAi) in animals and post-transcriptional gene silencing in plants (16, 2). RNA interference has now emerged as an important tool for the study of gene functions by sequence specific gene silencing via mRNA degradation (20).

Mechanism of RNAi

In the last few years, important insights have been gained in elucidating the mechanism of RNAi. A combination of results obtained from several in vivo and in vitro experiments have gelled into a two-step mechanistic model for RNAi/PTGS (Post-transcriptional Gene silencing).

The mechanism of RNAi is divided into two steps (20)

1. Initiation Step
2. Effector step

- **Initiation Step**

Introduction of long exogenous dsRNA, presence of endogenous dsRNA or infection by RNA viruses triggers the RNAi machinery of cells. This results in the recruitment of RNase III protein known as Dicer (14, 6) Presence of ATP Dicer cleaves the dsRNA and processes it into 21-23 base pairs long RNA fragments known as siRNAs

- **Effector step**

These siRNAs are recognized by a complex of protein called RISC (RNA-Induced Silencing Complex) which distinguishes the two siRNA strands as the sense or antisense strand. RISC complex unwinds the siRNA duplexes. Sense strands are degraded and the antisense strands are incorporated into the RISC. The antisense strand acts as a guide strand to target mRNA. Antisense strand and RISC complex degrades the target mRNA (1). The activated RISC can repeatedly participate in mRNA degradation, inhibiting protein synthesis. Thus, in this way, the degradation of mRNA takes place in sequence specific manner that bind the complementary target mRNA.

- **Pathways for RNA interference**

1. siRNA-mediated pathways are mostly used for defense against viral infections and plant protection. (7, 25, 34)
2. Regulations of gene expression via the miRNA-mediated pathway (38, 40, 32).
3. Suppression of germline transposon expression via the piRNA-mediated pathway (26, 35).

Commercial Applications and Regulatory Approvals for RNAi-Based Products

Over the last two decades, the screening and functional analysis of potential target genes and the design of RNAi-based strategies for crop protection and crop improvement, have led to the first commercial RNAi-based products entering the market. The first product of this kind was approved by US regulators in 2017 and recently also by Chinese regulators in 2021. On 15 June 2017, the United States Environmental Protection Agency (EPA) approved the world's first insect-resistant transgenic corn MON87411 expressing double-stranded RNA (dsRNA) targeting the DvSnf7 gene for control of rootworms, opening the biggest revolution in the history of pesticides (10, 11).

The Bayer "SmartStax Pro" maize (Mon87411) combines the expression of the *Bacillus thuringiensis* (Bt) Cry3Bt1 toxin with glyphosate resistance and the expression of a dsRNA targeting the Snf7 gene of the western corn rootworm (*Diabrotica virgifera virgifera*). RNA interference-mediated silencing of this gene involved in the transport of transmembrane proteins causes lethality in *D. v. virgifera*, ultimately leading to reduced root damage.

This product is available to farmers in the US in 2022 and from 2023 in Canada. In Europe, the "SmartStax Pro" maize has been authorized for the market for all uses except cultivation (11). The Bayer "Vistive gold" high-oleic soybeans (Mon87705), in which a gene in the fatty acid biosynthesis pathway is targeted, has been approved for food, feed, and cultivation in the USA, Canada, and Japan, and for feed and food use in the EU market (19). In 2023, the Australian scientific agency, CSIRO (Commonwealth Scientific and Industrial Research Organization) and the Australian clean technology business, GO Resources, are expected to release "Super-High Oleic" (SHO) safflower in Australia (11). Recently, Syngenta and Greenlight Biosciences have successfully concluded independent field trials showing a better performance of dsRNA treated plants to control the Colorado potato beetle (8, 28). Field tests under "BioDirect" a SIGS-based platform developed by Bayer; have confirmed the potential of RNAi technology to control Varroa destructor mites in honeybees, by reducing mite levels and increasing colony survival rates (23).

Production of ds RNA:

The production of double-stranded RNA (dsRNA) is a crucial process in RNA interference (RNAi). Chemical Synthesis, In Vitro Transcription, Fermentation, and Green Light Cell-Free are the different methods used for the production of ds RNA. It highlights key parameters such as the source of nucleotides, DNA template, and polymerase, along with scalability, cost of goods, and capital intensity. Chemical synthesis and in vitro transcription rely on purified or synthetic materials, making them expensive and less scalable. Fermentation and Green Light Cell-Free, on the other hand, utilize biomass, making them more cost-effective and scalable. However, fermentation has some product length limitations, while Green Light Cell-Free does not, giving it an advantage in producing longer RNA molecules. Green Light Cell-Free stands out as the most cost-effective and scalable platform, with a cost of goods below $0.50 per gram, significantly lower than other methods. It also requires less capital investment, making it more accessible for large-scale applications. The platform is described as flexible, proprietary, and patent-pending, offering a single manufacturing process capable of producing multiple RNA products. This efficiency allows dsRNA products to be produced at a commercially viable cost, making RNAi technology more practical for large-scale agricultural use.

Table 1. Different methods for production of dsRNA

Parameter	Chemical Synthesis	In Vitro Transcription	Fermentation	Green Light Cell-Free
Source of Nucleotides	Chemically synth.	Purified	Biomass	Biomass
Source of DNA template	NA	Purified	Biomass	Biomass
Source of Polymerase	NA	Purified	Biomass	Biomass
Product Length Limitations	Yes (<100 base pairs)	No	Some	No
Cost of Goods ($/g)	$100,000/g	$1,000/g	$1.00/g	<$0.50/g
Scalability	mg-mg	mg-g	kg	kg-MT
Capital Intensity	High	Med	High	Low

In vitro transcription kits utilizing purified RNA polymerases and nucleotides have been widely used in laboratory experiments, but the high cost (~$700 per mg dsRNA) limits the large-scale application in the field. However, a large-scale cell-free production platform that uses endogenous cellular RNA to synthesize the desired dsRNA has been developed by Green Light Biosciences. Compared to fermentations that cost $1 to produce 1 g of dsRNA, this cell-free platform can produce 1 g of dsRNA for as little as $0.50, making it highly competitive in the market endogenous RNA was depolymerized into nucleoside monophosphates (NMPs) with nucleases and then phosphorylated with kinases to form nucleotide triphosphates (NTPs).These NTPs were then polymerized into the target dsRNA using the corresponding DNA template and RNA polymerases.

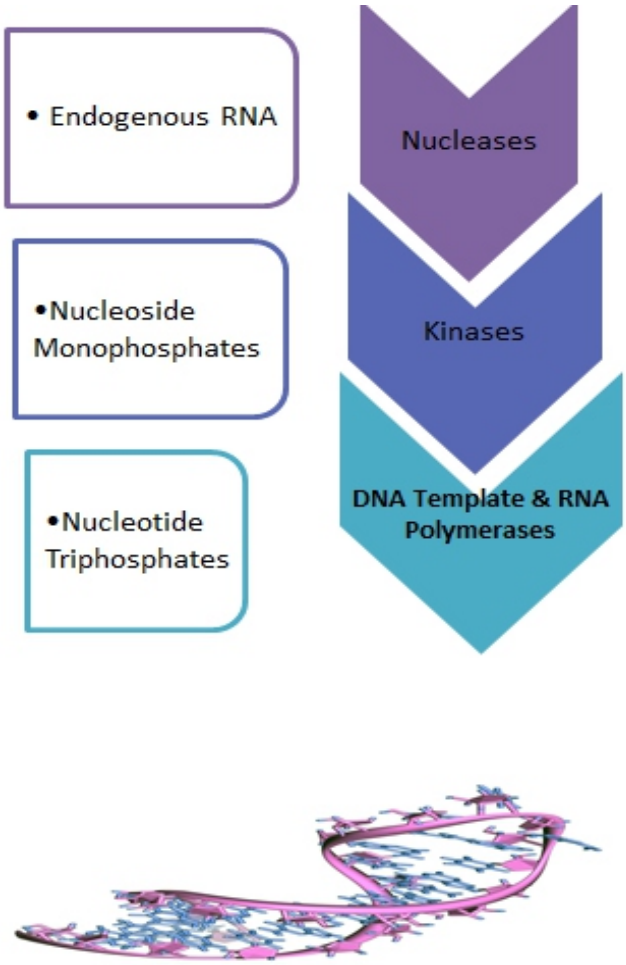

Figure 1: Cell-free production technique of dsRNA.

Novel Delivery methods

The overall success of RNAi-based pest control depends largely on the mode of dsRNA delivery, making it a key factor in designing effective insect management strategies. There are two main approaches: non-transformative delivery and transformative delivery.

1. Non-transformative delivery methods

Non-transformative delivery methods include foliar spray, irrigation, trunk injection, bacterial dsRNA, and symbiont-based delivery. These methods offer several advantages, such as being GMO-free, ensuring higher consumer acceptance, and having a low application cost. Additionally, they are feasible and affordable, making them a practical choice for large-scale agricultural use.

However, the main limitation is the continuous need for dsRNA application, as the effects are temporary and do not provide long-term pest control.

2. Transformative delivery methods

Transformative delivery methods, on the other hand, involve genetic modification, including conventional transgenic crops and transplastomic crops. These methods provide efficient and long-lasting pest control since the plants continuously produce dsRNA against target pests. However, they face significant challenges such as extensive regulatory approval processes and low consumer acceptance, particularly in countries with strict regulations on genetically modified organisms (GMOs). In a country like India, where the use of GMO crops is highly restricted, non-transformative methods remain the most viable option for pest management. These methods allow for the implementation of RNAi-based pest control without facing regulatory hurdles while ensuring acceptance among farmers and consumers. Thus we need to focus majorly on non-transformative delivery methods.

A. Non-transformative delivery
a. Foliar spray
b. Irrigation
c. Trunk injection
d. Bacterial dsRNA

Foliar spray

The dsRNA formulation can be directly sprayed on insect pests, which may penetrate the cuticle to induce lethal effects, and also be sprayed on the crops to feed pests. Study was conducted by Gong *et al*., 2013 using six siRNAs targeting acetylcholine esterase genes of *Plutella xylostella* revealed that the best insecticidal activity with 89% mortality rate was observed when second instar *P. xylostella* were fed with *Brassica* spp. Leaves sprayed with siRNAs. Spraying the dsRNA might be useful to control some pest population in the field, but not all. For example, Li *et al* reported that the spraying method would not affect the piercing-sucking pests feeding on phloem sap, or stem borer pests feeding in the plant stems (25). Penetration ability of dsRNA into the cuticle is different among insects, and the topical application is not fit for all insect pests.

Irrigation

The delivery of dsRNA via crop roots can trigger RNAi in insect pests, and the irrigation of RNAi-based products seems to be an alternative for suppressing pests feeding/growing in stems and fruits (37). One study exploring the applications of dsRNA via crop roots was conducted against the brown planthopper and the Asian corn borer (25). When *Nilaparvata lugens* fed on rice that had been irrigated with carboxyl esterase dsRNAs, the mortality rate reached nearly 50% at 5 days post-treatment. Irrigation is a simple yet practical method to deliver dsRNA; however, dsRNA may be degraded within approximately 2 days after the application to soil, regardless of texture, pH, clay content and other soil differences (13). Thus, the success of this delivery strategy relies on the advances of formulations to protect dsRNA from degradation.

Trunk injection

The efficiencies of foliar spray and irrigation are relatively low, and sometimes this method is impractical for trees.

Trunk injection is a promising method to deliver agrochemicals in many tree species while reducing the environmental impacts, risk for users and consumer exposure (5, 36). Phloem is considered a preferential channel for the transport of dsRNA/siRNA where it can remain stable for long periods, owing to the Rnase-free environment in phloem sap (12, 24). Citrus trees (2.5-m height) and grapevines were treated with dsRNA via root drench and trunk injection, and the dsRNA was taken up into the whole plant system over 3 months to suppress insect pests (18). The control of some insect pests has been difficult, especially for underground root-feeding pests, and the trunk injection may solve this problem. This strategy may be more effective for sap-sucking pests than for chewing pests feeding largely on leaves (21).

Bacterial dsRNA

The delivery of dsRNA using bacteria has many advantages when compared with plant-mediated dsRNA delivery or in vitro synthesized dsRNA delivery (Transgenic crop). The application of bacteria expressed dsRNA is less costly when compared with in vitro synthesized dsRNA. The bacteria-expressed dsRNA pesticides can be sprayed on crops at any time because of the ease of producing large amounts of bacteria expressing dsRNAs. Colorado potato beetle, which was fed on different *E. coli* transformations targeting five different mRNAs (42). An RNAase III - deficient *E. coli* was used for dsRNA production; after beetles had ingested the bacteria, significant mortality and loss of body weight were observed.

Improved delivery efficiency of dsRNA by nanoparticles:

Nanoparticles are defined as any particle between 1 and 100 nm. In addition to shielding and protecting the dsRNA from environmental nuclease degradation, nanoparticles promote the translocation and penetration of dsRNA across the peritrophic membrane, cell membrane, and insect cuticle (31, 41). In most cases, the nanoparticle combines with dsRNA into the nanoparticle/dsRNA complex through the electrostatic interactions between the cationic groups in the nanoparticle and the phosphate groups in the dsRNA (39, 17). The complex usually retains a net positive charge that facilitates the interaction with the negatively charged cell membrane surface (29). When the complex is bound to the cell membrane, it can penetrate the cell membrane into the cytoplasm through endocytosis (9, 4). Complexation of the nanoparticle and dsRNA can avoid the degradation within the endocytic vesicles and this process is known as the sponge effect (30).

Challenges and Future Perspective in the Application of RNAi-Based Products

There is a risk of RNAi resistance in insect pests. Resistance can develop through mutations in target genes or core RNAi machinery genes, enhanced dsRNA degradation, or reduced dsRNA uptake. Sequence polymorphism in target genes can also create mismatches between dsRNA and mRNA, leading to reduced efficacy and the evolution of resistance. The application of bacteria expressed dsRNA is less costly when compared with in vitro synthesized dsRNA. The bacteria expressed dsRNA pesticides can be sprayed on crops at any time because of the ease of producing large amounts of bacteria expressing dsRNAs. Genetically modified (GM) crops require significant investment, take a long time to develop, and may contribute to unintended ecological consequences, such as foreign gene escape. Additionally, public and NGO opposition remains a barrier to widespread acceptance of transgenic solutions. Another concern is the potential risk of nanoparticle-mediated dsRNA delivery. While nanoparticles offer a promising way to improve dsRNA stability and uptake, they may pose risks to human and environmental health, including contamination of water sources and residues on food products. However, research on the environmental impact of nanoparticle-based dsRNA formulations is still limited, highlighting the need for further studies before widespread implementation.

Advancements in the systemic properties of dsRNA and improvements in delivery methods are ongoing. In the coming years, these developments will lead to innovative breakthrough applications for pest management, offering a unique mode of action in the field. One promising approach is the use of transgenic plants that express dsRNA to selectively suppress insect pests. However, due to strict regulatory processes, alternative non-transformative strategies with similar efficiency can be explored. For transplastomic crops, scientists need to develop chloroplast transformation protocols for major crops to expand the range of chloroplast-transformed plants. Additionally, when dsRNA stability within insect bodies is a concern, nanoparticles and bacteria-based delivery methods could be effective solutions. However, before nanoparticles can be widely used in agriculture, their environmental impact must be thoroughly evaluated. Among various production methods, bacteria-based expression of dsRNA is considered the most cost-effective approach. Many biotech companies are investing in this technique to produce affordable dsRNA, making RNAi-based pest control solutions accessible to both small and large-scale farms.

CONCLUSION

RNAi technology has been an effective tool in functional genomics studies and its application toward pest management is already close to a reality. Because of the high specificity of its sequence specific gene-silencing mode of action. RNAi will be safer than any pesticide currently available in the market. The high specificity reduces the negative impact produced by broad-spectrum insecticides, however impact on natural enemies and beneficial fauna should be evaluated carefully. Delivery of dsRNA using nanoparticles, viruses or bacteria, increase efficacy in attaining a potent RNAi response. Expression of dsRNA through transplastomic plants is a preferable strategy to achieve improved results. Increase in the effectiveness of RNAi strategies achieved by utilizing multiple targets.

Future Scope of Study

RNAi is just the beginning of new era for plant protection. However there is a great future scope focusing on overcoming existing challenges such as dsRNA stability, cost-effective production, and delivery efficiency. Additionally, further exploration of gene targets and RNAi pathways will help improve specificity and minimize off-target effects. Regulatory approvals and consumer acceptance remain critical for widespread adoption, requiring continued engagement with policymakers and the public. Ultimately, integrating RNAi-based pest management with sustainable agricultural practices can revolutionize crop protection while reducing environmental impact and pesticide dependency.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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