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Chloroplast engineering and RNA interference: A dual-technology approach for insect pest control

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Abstract

The integration of chloroplast engineering and RNA interference (RNAi) technologies represents a groundbreaking approach for sustainable insect pest management in agriculture. This review highlights the combined potential of these technologies to address the escalating challenges posed by insect pests, particularly in the context of increasing resistance to conventional insecticides. Chloroplast engineering provides a robust platform for the high-level expression of double-stranded RNA (dsRNA), essential for effective RNAi-mediated gene silencing in pests. The unique features of chloroplasts, such as their high copy number, transgene stability, and maternal inheritance, significantly reduce the risk of gene flow and non-target effects, making them an ideal system for dsRNA production. Recent advancements in transplastomic plants have demonstrated the efficacy of this approach in controlling economically significant pests, including aphids, whiteflies, and cotton bollworms. The review discusses the mechanisms underlying plant-mediated RNAi and explores innovative dsRNA delivery methods, such as nanoparticle-based systems, which have the potential to enhance RNAi efficacy. Additionally, the review addresses the environmental and biosafety considerations of RNAi-based pest control, emphasizing the need for eco-friendly alternatives to chemical pesticides. By leveraging the strengths of chloroplast engineering and RNAi, this integrated approach not only provides a sustainable and effective strategy for pest management but also paves the way for future innovations in crop protection and agricultural sustainability. This review underscores the importance of continued research and development in this field to address the growing challenges of global food security and environmental protection. The integration of these technologies offers a promising solution to reduce the reliance on chemical pesticides, mitigate the impact of insect pests on crop yields, and promote a more sustainable agricultural future.

Keywords: Chloroplast engineering, dsRNA delivery, Gene silence, Pest resistance, Sustainable agriculture, Transplastomic plants

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Introduction

The question of how to eradicate global hunger is one of the Sustainable Development Goals, and feeding the future world population is a major global societal challenge (Iqbal, 2018). Between 2040 and 2050, there is estimated to be a 35% to 56% growth in the overall global food demand, whereas individuals at risk of starvation are predicted to increase by -9% to +8% throughout that time (van Dijk et al., 2021; Falcon et al., 2022; Lee et al., 2024). While a variety of factors, including farming techniques, climatic events (Omokhafe et al., 2024), and pathogen outbreaks (Rubab et al., 2020; Hameed et al., 2022; Ullah et al., 2023), have an impact, arthropod pests are responsible for about 20% of the world's yearly crop losses (Ali et al., 2018; Ahmad & Ahmad, 2018; Mateos Fernández et al., 2022; Qasim et al., 2023). Since the introduction of Bt crops in the 1990s, numerous insect pests have developed practical resistance, including control failure, to the insecticidal proteins expressed by these crops. In response, insect molecular biologists have developed the next generation of insecticides, which are eco-friendly and unconventional. RNA interference (RNAi) technology is a promising strategy in this regard (Lu et al., 2024).

Insect control via plant-mediated RNA interference (RNAi) saw its initial proof of concept published (Baum et al., 2007). Since its 1st discovery in

nematode *Caenorhabditis elegans* (Fire et al., 1998). These powerful reverse genetics tools are being created as a potentially useful technology for insect management (Zhu & Palli, 2020). There are various mechanisms of RNAi to control harmful insects of crops, like foliar spray formulations coated with clay or nanoparticles to stabilize dsRNA, mechanical, infiltration, trunk injection, or the best strategy to get more expression by transgenic expression of dsRNA in the plastid (Jin et al., 2015). Ni chloroplast offers several advantages gene containment (not disturbing the non-target beneficial insects), no position effects, and transgene stability. This review aims to highlight the recent advancements in RNA interference (RNAi) technology for managing harmful agricultural pests. We will specifically explore strategies for controlling cotton pests, including pink bollworms and whiteflies, as well as other sap-sucking insects like aphids. Despite significant progress, current research still lacks comprehensive solutions for controlling *Pectinophora gossypiela*. This review will also delve into potential antisense technology strategies that could be employed for more effective management of pink bollworms. Additionally, we will emphasize the importance of risk assessment in the application of RNAi technologies to ensure their safety and efficacy in sustainable pest management.

Revolutionizing the plastid genome engineering

The plastome has an extraordinarily high copy number, which may reach roughly l0,000 copies per mesophyll cell, one of its interesting characteristics. In addition to being essential for plastome stability by preventing damaging mutations by homologous recombination, a huge to create and maintain the photosynthetic equipment throughout plant growth, the copy number is required. To experimentally convert the plastome, the present protocol begins with constructing a transforming plasmid with two cassettes: an expression cassette with the relevant genes or genes that must be expressed, and the other for antibiotic resistance that works in chloroplasts. There are two plastome segments next to these cassettes, which are often referred to as flanking areas or targeted regions, with sizes generally ranging from l to 2 kb can aid in the integration of foreign genes via homologous recombination at plastome locations (Nawkarkar et al., 2020). The homologous recombination machinery of the Chloroplast promotes targeting DNA into specific genome areas. The expression of multiple GOIs from a single operon-like structure is made possible by polycistronic gene expression, which also makes the construction of a transformation vector simpler and allows for the integration of multiple transgenes in a single transformation step (Fig. 1). Recombinant protein expression levels, which are typically significantly higher for chloroplasts than nuclear transgene, are further increased because of copy correction, which causes the expression cassette to be duplicated to the homologous site on opposite inverted repeats A and B.

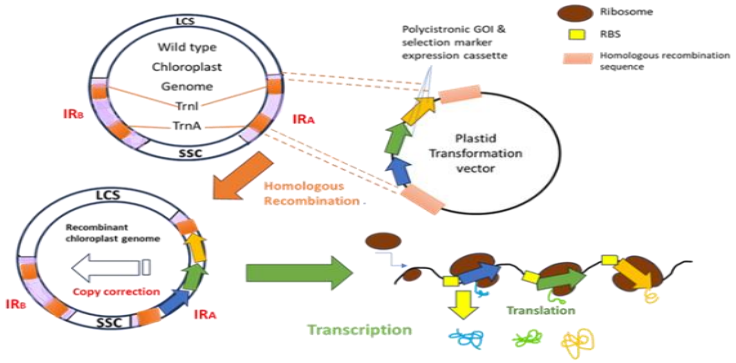

Fig. 1 Schematic structure and prokaryotic functional features of the plastid transformation vector (Meyers et al., 2010)

By coating DNA on 0.4-l.0 µm gold or tungsten microparticles, Plastids receive these structures through a particle delivery mechanism mediated by a gene gun approach. The method used to deliver particle bombardment is a biolistic delivery device, sometimes referred to as a "gene gun," which was created in 1997. With the aid of externally applied high pressure, exogenous biomolecules linked to gold particles cross the second wall of the plant and enter the nucleus and other organelles. These biomolecules include proteins, peptides, DNA, and RNA (Zhi et al., 2022; Smeekens et al., 2022). A maternally inherited restriction methylase in the chloroplast would defend against a nuclear-encoded, plastid-targeted restriction endonuclease. Because of the maternally inherited plastid genome, gene flow will not occur to the other crops, and superweeds will not be formed in others, meaning no genetic pollution happens (Tonti-Filippini et al., 20l7). Hence, Plastome genome engineering is regarded as an excellent technique for maintaining the containment of transgenes and enhancing the biosafety of genetically modified plants (Mathur, 2018; Yang et al., 2022). Plastids in Nicotiana tabacum are exclusively inherited from the mother plant to her progeny. Other techniques, such as polyethylene glycol-mediated transformation and microinjection, can transfer foreign DNA into plastids; however, their efficiency is lower than that of biolistic DNA delivery (Singh et al., 2022; Kumar and Ling, 2021). The antibiotic resistance gene selects the transgene that has been stably inserted into the plastid genome (Douchi et al., 2021; Ghandour et al., 2023). Selectable markers that are most frequently used express the bacterial aadA gene, which is produced by elements regulating plastid expression. Adenylyl transferase, also known as 3-aminoglycoside, is encoded by eliminating the

activity of spectinomycin and streptomycin. This positive dominant selection marker is known for its high transformation efficiency, resulting from selective amplification of transformed plastid DNA copies (Yu et al., 2020), also the reporter gene, like GFP, as a selective agent for the transformed plastome. Upon the introduction of transgene into plastids, a limited number of plastome copies transform, leading to the establishment of a heteroplasmic state. The attainment of homoplasmy, whereby all instances of the plastome include the transgene, is accomplished via the process of subculturing a bombarded explant in vitro while applying a selective agent. The marker gene will be eliminated to create a plant without markers once homoplasmy has been established. Flanking direct insertion is used in several techniques, such as intrinsic homologous recombination, integrase (Int) phage recombinase, and Cre/loxP (Yau & Stewart, 2013; Qamar et al., 2024). Chloroplast engineering holds significant potential in the field of biotechnology, particularly for its environmentally sustainable applications (Fig. 2).

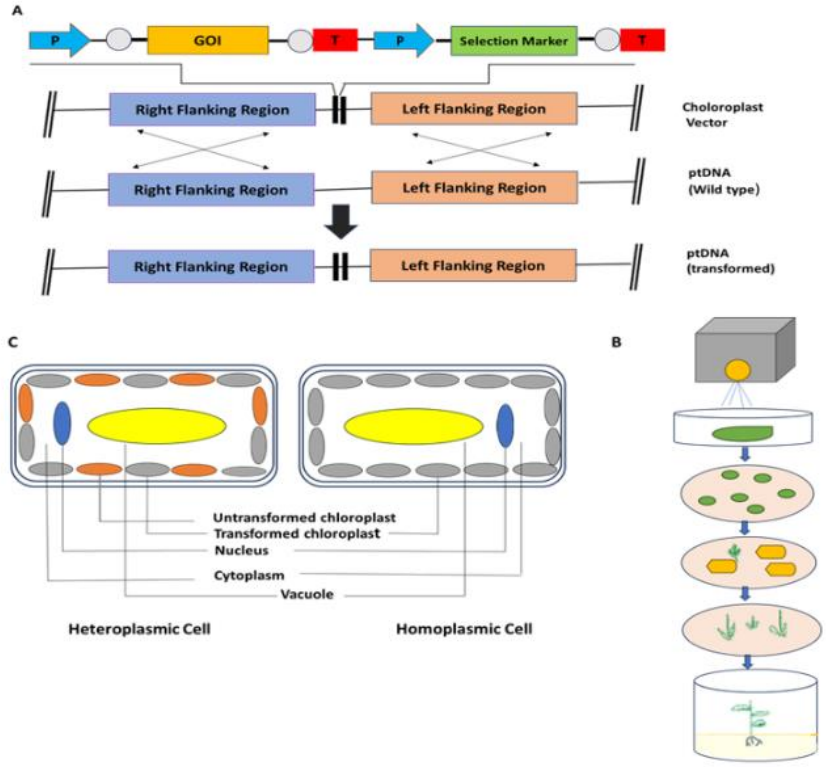

Fig. 2 Diagrammatic depiction of the plastid genome transformation process (Ahmad et al., 2016)
(A) Typical vector's basic design for plastid genome transformation. The two plastid areas are separated by cassettes for expression and selection. These flanking portions are provided by a particular species of plant whose plastome is to be modified; they are then taken out of its wild-type plastid genomes. This makes it possible for a crossover event to take place and merge their DNA sequences. In the chloroplast transcription vector, green arrows indicate the transcription direction, while red rectangles are used to indicate terminators (T). White circles indicate the areas that are not translated. The homologous recombination is indicated by the arrows on the thin, marked lines. (B) Transformative plasmids are introduced into the chloroplasts of leaf cells by a piezoelectric delivery technique.

Plasmid DNA is introduced onto the 4- to 6-week-old sterile leaves' abaxial layer by injecting it into the leaves using a gene cannon. After being blasted, the leaves are sliced into small discs, incubated in the dark for 48 hours, and then placed on regeneration media that has been treated with the necessary hormones and antibiotics. Primary shoots often emerge in two to three months. (C) The procedure for obtaining a transplastomic plant line with homoplasmy. Since there are initially very few transformed copies of the plastome, the heteroplasmy is the state in which an explant possesses both transformed and untransformed copies. Plasmid DNA is introduced onto the abaxial layer of sterile leaves that are 4-6 weeks old by injecting it into microparticles that plasmid DNA using a gene cannon. After being blasted, the leaves are sliced into small discs, incubated in the dark for 48 hours, and then placed on regeneration media that has been treated with the necessary hormones and antibiotics. Primary shoots often emerge in two to three months. (C) The procedure for obtaining a transplastomic plant line with homoplasmy. Since there are initially very few transformed copies of the plastome, heteroplasmy is the term for the explant's mixture of transformed and untransformed copies. Following two or three cycles of regeneration under consideration, to achieve homoplasmy, an environment in which all plastome copies are altered, the wild-type copies represented by ovals with light colors are gradually separated.

Superior role of chloroplast engineering over the nuclear genome

Maternal Inheritance Dominates

Transgenes included in annual crops may inadvertently be transferred into the genomes of other crops (Mehmood et al., 2020; Mehmood et al., 2021; Zia et al., 2023). There is concern that these transgenes may endure in the environment and result in adverse ecological effects. The exchange of material with their relatives through pollen-mediated introgression (Maqbool et al., 2025). Are certain crops or transgenic characteristics more concerning than others? Are the natural genetic barriers to reducing gene escape? Can the process of genetic transformation be used to generate novel mechanisms that impede the transfer of genes? (Kogan & Heinrichs, 2020) For instance, such genes have been transferred from nuclear sources and encode resistance against herbicides like Roundup, Glyphosate (Fig. 3), and Liberty, and Pursue genome engineering of crops to weeds- superweeds like super insects (Bain et al., 2017; García et al., 2019).

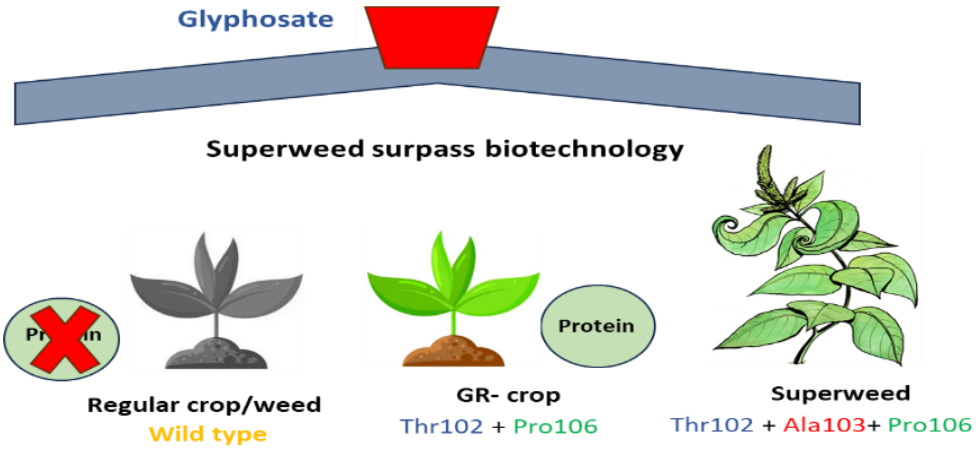

Fig. 3 Superweed surpasses biotechnology (García et al., 2019).

A genetic engineer methylated the tobacco chloroplast genome. The suggested plant transgene containment method must tolerate plastid genome methylation. A maternally inherited restriction methylase in the chloroplast would defend against a nuclear-encoded, plastid-targeted restriction endonuclease. Because of the maternally inherited plastid genome, gene flow will not occur to the other crops, and superweeds would not be formed in others, meaning no genetic pollution happens. (Kumar and Ling, 2021; Singhal et al., 2023; Jansen and Ruhlman, 2012; Greiner et al., 2015; Tonti-Filippini et al., 2017). Hence, plastome genome engineering is regarded as an excellent technique for maintaining the containment of transgenes and enhancing the biosafety of genetically modified plants (Mathur, 2018; Yang et al., 2022). Plastids in *Nicotiana tabacum* are exclusively inherited from the mother plant to her progeny. Nevertheless, there have been documented cases of paternal plastid transmission occurring at low frequencies in crosses between parents with different cytoplasmic origins. The insertion of transgenic chloroplasts into pollen up to a range of .00024 - .008% in tobacco (Svab and Maliga, 2007).

Potential use for 10,000 plastome copies

There could be many copies (l0000) of plastome present per cell, which results in the gene expression being high as compared to the nuclear genome, in which the number of chromosomes varies depending on the species; however, each chromosome present in each cell has two copies, resulting in few copies of transgene per cell (Kusnetsov, 2018). Less than 0.0l % of the total amount of soluble proteins (TSP) for the tobacco nuclear genome expressed the β-subunit of enterotoxigenic E. coli (LT-B). When compared to LTB expressed through nuclear genome integration, the 2.5% LTB protein of the total soluble protein that was seen by chloroplast transformation was expressed around 250 times higher. So that same protein is expressed more in the plastid genome as compared to the nuclear genome (Kim and Kang, 2019). The toxicity of foreign protein is detected in the nuclear genome up to a total soluble protein of 0.3% in tobacco cytosol, resulting in the plant growth being stunted (Rascón-Cruz et al., 2021; Mbongue et al., 2023). So, we need to focus on superior alternative sources like the plastid genome. It will protect the plants from the toxic effects of protein. There are some examples in which foreign proteins affect the chloroplast functionality. Pleiotropic effects seen in transplastomic plants often include a decrease in the synthesis efficiency of recombinant proteins and pigment reduction in foliage, stunted growth, and male sterility (Scotti & Cardi, 2014).

Potential of plastome engineering to overcome gene silencing

Site-specific integration occurs in the plastid genome, which eliminates gene silencing and unwanted mutation because of random integration in the case of nuclear transgene integration. The gene silencing in a nuclear genome is because of its conserved portion (heterochromatin). Agrobacterium mediated transformation often leads to random integration of the transgene. Plastid triggers the dsRNA expression, which is

silenced in nuclear-encoded genes (Bélanger et al., 2023). Foreign genes may interact with native nuclear genes, perhaps suppressing their activity or vice versa. Such barriers have not been observed in the case of plastid genome engineering (Dorogova & Sidorchuk, 2023).

Engineering the chloroplast for insect pest control

Half of the approximately l million insect pest species discovered to date are herbivores and represent a persistent threat to agricultural production. Despite widespread efforts, including the use of billions of pounds of chemical pesticides, major infestations persist. With a changing climate and cultivation of vast agricultural monocultures, the abundance of various pests shows an increase, and their activity is shifting, expanding, and intensifying (Jampílek & Kráľová, 2022). Global climate changes have significant impacts on agriculture and agricultural insect pests. Crops and their corresponding pests are directly and indirectly affected by climate change. Insect physiology is very sensitive to changes in temperature, and their metabolic rate tends to approximately double with an increase of l0 °C. Elevated concentrations of atmospheric CO_2 can affect the distribution, abundance, and performance of herbivorous insects. Such increases can affect consumption rates, growth rates, fecundity, and population of insect pests. The ranges of insect pests are expected to shift to higher altitudes by 2055, with an increase in the number of generations in central Europe (Skendžić et al., 2021; Azenzem & Kassout, 2023). The greatest worry for farmers is the decline in productivity due to illnesses and pests. Insects, weed pests, and plant pathogens destroy more than 40% of all potential food production every year. Insecticides are indispensable for modern agriculture to ensure crop protection and optimal yields. However, their excessive use raises concerns regarding their adverse effects on agriculture and the environment.

To ensure food security, we must create new, effective, and eco-friendly crop-security measures. New crop protection methods target chloroplasts, providing effective plant protection from insects by transplastomic recycling. Although based on the superiority of plastid genetic engineering, genetic engineers prefer chloroplast engineering over the nuclear genome. Because in chloroplast genome engineering, gene expression is high, no gene flow, and gene silencing as compared to the nuclear genome (Malhi et al., 2021; Tanwar et al., 2023). Genetic engineers need to take advantage of high transgene expression. The first superiority of the transplastomic approach for pest control was the Bt gene. Here we report the incorporation of a hybrid cry gene (*SN-l9*, comprised of the domains I and III of c*rylBa* and domain II of c*rylIa*) into the chloroplast genome. *SN-l9* can ensure a durable resistance against insect species belonging to the orders Coleoptera and Lepidoptera, as established by earlier researchers; the mortality rate was l00% (Salehian et al., 2021). By expressing double-stranded RNA (dsRNA) in potato plastids targeting the β-Actin (ACT) gene of the Colorado potato beetle (CPB). The *BaciIIus thuringiensis* (Bt) *cry3Bb* gene was successfully introduced into the poplar plastid genome, leading to transplastomic poplar with high mortality to *Plagiodera versicolora* (Xu et al., 2020). The expression of crylC from the plastid genome of poplar leads to high mortality of leaf-eating caterpillars (Wu et al., 2019).

Plastid-mediated RNA interference against insect pests

We introduced Plastid-mediated RNA interference in tobacco against four genes of western flower thrips (WFT; *Frankliniella occidentalis*). This damaging pest and viral vector target many outdoor crops, greenhouse vegetables, and flowering crops. The feeding study revealed that, unlike nuclear transgenic plants, producing high insect mortality and effective targeted gene deletion by chloroplast engineering (Wu et al., 2022). The management against *Henosepilachna vigintioctopunctata* by expressing long double-stranded RNAs in potato plastids (Xu et al., 2023). Double-stranded RNA expression in plastids presents a great deal of potential for the effective control of chewing insects by attacking the MpDhc64C gene, a recently discovered effective RNAi target gene, whose silencing renders the Myzus persicae green peach aphid deadly (Dong et al., 2022). RNA Interference in the Tobacco Hornworm, Manduca sexta (Burke et al., 2019). Transplastomic tomatoes that express dsRNA against the conserved β-Actin mRNA region of spider mites (Table 1). Transplastomic tomatoes that express dsRNA against the conserved β-Actin mRNA region of spider mites with a higher mortality rate (Fig. 4). Our study demonstrates the potential of PM-RNAi as an efficient pest control measure for spider mites and extends the application range of the technology to non-insect pests (Wu et al., 2023). In this study, we aimed to test whether silencing dsRNase could increase the RNAi effect in CPB via PM-RNAi. Various researchers have attempted to create transplastomic-resistant plants against insect pests by chloroplast engineering. Where in the propensity of chloroplast genomes to express genes at high amounts of protein is taken advantage of by other transplastomic-resistant plants that fend off pests and dangerous insects, are included in Table 2.

Delivery methods dsRNA

A trustworthy delivery method of dsRNA is necessary for testing and confirming gene silencing's effectiveness (Fig. 5). Many dsRNA delivery methods have been developed, depending on the species and goals; these can be broadly classified into three categories: topical administration, oral ingestion, and microinjection. When dsRNA and nanoparticles are mixed, RNA interference (RNAi) can be improved because the latter stops the degradation of molecules of dsRNA/siRNA and promotes the preferential uptake of whole molecules.

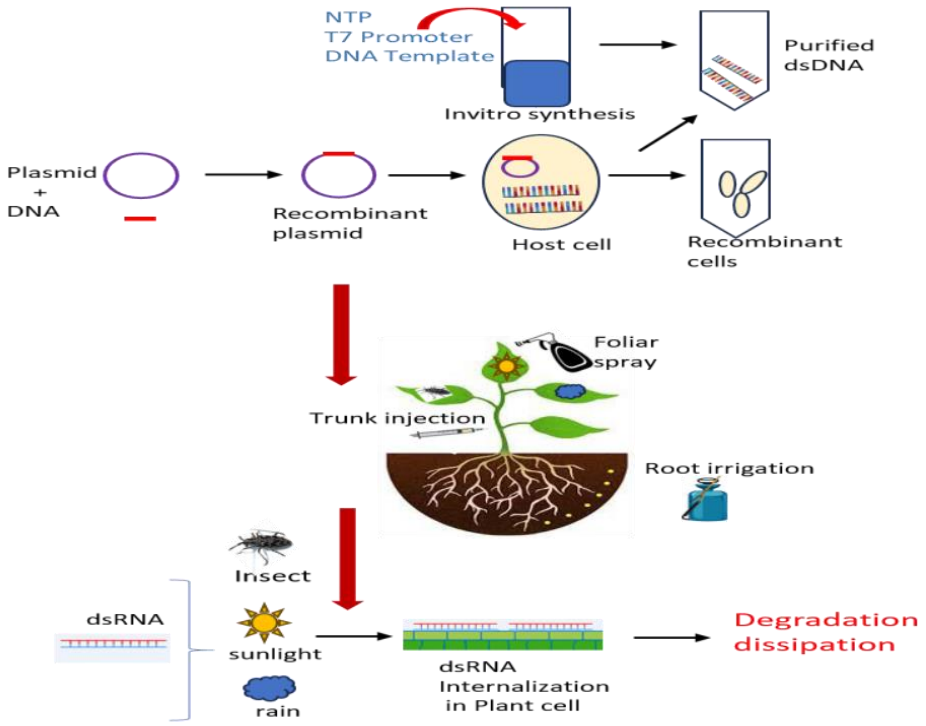

Fig. 4 Process of RNA interference biotechnology

Table 1 PM-RNAi as insect pest management

Taxonomy	Species	Target genes	Target crops	Reference
Coleoptera	Henosepilachna vigintioctopunctata, Leptinotarsa decemlineata,	(β-Actin, SRP54, and SNAP), v-ATPaseA	Potato, Tomato	(Xu et al., 2023; Kaplanoglu et al., 2022)
Hemiptera	White fly, Halyomorpha halys, Myzus persicae, Phenacoccus madeirensis	*BtACTB* gene, v-ATPaseA,	Tobacco, Tomato	(Dong et al., 2020; Kaplanoglu et al., 2022; Dong et al., 2022)
Lepidoptera	Cotton Bollworm, Helicoverpa zea,	acetylcholinesterase gene of the cotton bollworm, genes for chitin synthase, V-ATPase, and cytochrome p450 monooxygenase.	*Nicotiana benthamiana.*	(Bally et al., 2016; Jin et al., 2015; Adeel et al., 2021; Fu et al., 2022)
Acar	*Tetranychus evansi*, *Tetranychus truncatus*, and *Tetranychus cinnabarinus*	Spider mite *β-Actin* mRNA.	Tomato plants	(Wu et al., 2023)

Table 2 Various plastome-resistant plants for pest control

Gene Name	Role	Target species	Approach	Host	References
T. palmi UHRFlBPl and *PFAS* in GBNV infection,	To manage thrips and restrict the spread of tospovirus.	(*Thysanoptera: Thripidae*)	Silencing	Thrips palmi	(Priti et al., 2022)
Western flower thrip Actin (ACT), Western flower thrip Tubulin (TUB), Western flower thrip Endosomal sorting complex for transport III subunit SNF7, Western flower thrip Catalytic subunit B of vacuolar ATPase (VAT).	Efficient weapons to control thrips and other sucking plant pests (WFT).	*Frankliniella occidentalis*	Silencing	Nicotiana tabacum	(Wu et al., 2022)
Phenacoccussolenopsis v-ATPaseA	While lacerate-and-flush feeding insects and leaf-chewing insects can be suppressed by plasmid-produced dsRNA, sap-sucking insects may not be affected.	(*Halyomorphahalys*), (*Phenacoccus madeirensis*), (*Leptinotarsa decemlineata*)	Silencing	Solanum lycopersicum	(Kaplanoglu et al., 2022)
MpDhc64C gene, NDUFVII	Effective control of sap-sucking insect pests	*Acyrthosiphonpisum, Adelphocorissuturalis, Rhopalosiphumpadi, Myzuspersicae, and Nilaparvata lugens*	Silencing	Tobacco	(Dong et al., 2022)
Sl l02 gene	(l) a strongly reduced level of *Sl l02* transcripts, (2) enhanced biopesticide activity	*Spodoptera littoralis* larvae	Silencing s	Tobacco plants	(Caccia et al., 2020; Khalil et al., 2023; Gouda et al., 2024)
phenolic glucoside malonyltransferase gene BtPMaTl	By genetically transforming tomato plants to produce small interfering RNAs that silence BtPMaTl	(*Bemiciatabaci*)	Silencing	Tomato	(Xia et al., 2021)
crylC	Effective toxic Hyphantria cunea	*H. cunea* and L. dispar	Overexpressed	Hybrid Popular	(Wu et al., 2019)

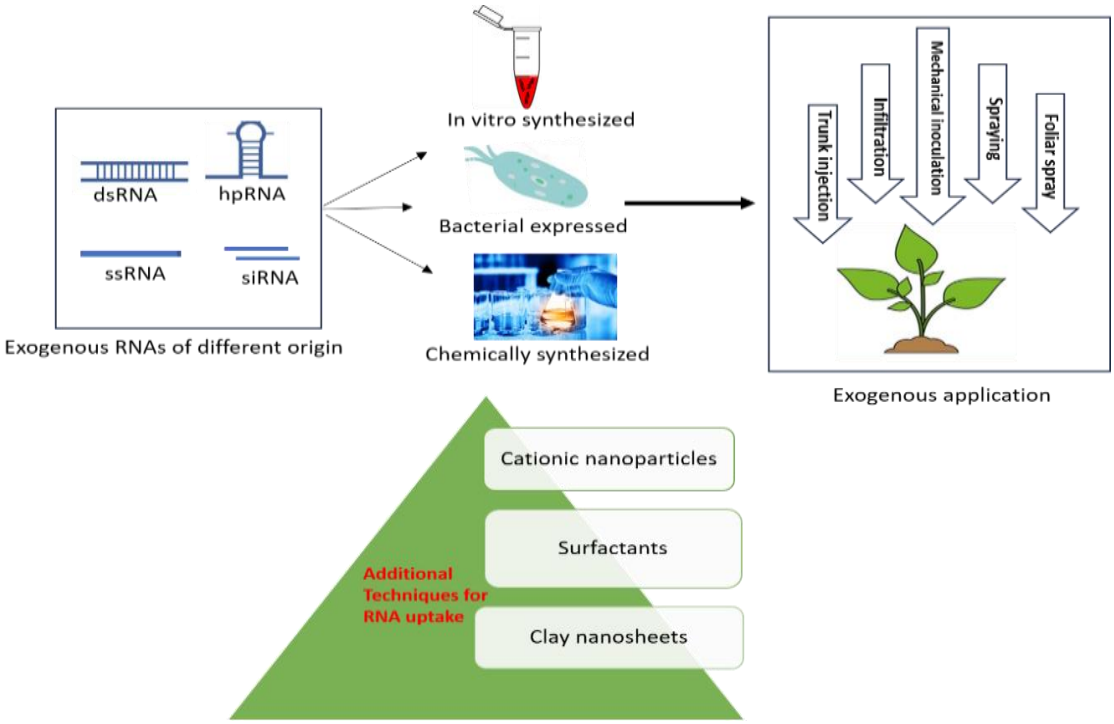

Fig. 5 Schematic diagram of dsRNA delivery methods

Applications of RNA interference antisense technology for control of chewing and piercing-sucking insect pests

By using antisense technology, RNA interference pathways, and gene knockdown, *Virgifera*, also known as the southwestern corn rootworm, is probably going to play a significant role in future technologies designed to control this significant corn pest (Camargo et al., 2018; Ali et al., 2024). By oral feeding of dsRNA to check the mortality rate at different larval instars, in almost all stages, the mortality rate of the potato lady beetle *Henosepilachna vigintioctopunctata*, was very high. Consequently, it might serve as an appropriate molecular target for biopesticides based on RNA interference to manage H. *vigintioctopunctata* (Liu et al., 2024). According to genome-wide research, RNA interference (RNAi) (Fig. 6) may be a more effective means of controlling the target genes (905, 92) of the *Tribolium castaneum* red flour beetle. The *ds*RNA, also used to control *furnacalis* larvae, was diluted (5 µg/µL) with diethylpyrocarbonate (DEPC) water. Three 4th instar *O. furnacalis* larvae groups were sterilized with 75% alcohol and injected with 20 µg of *ds*ERK, 20 µg of *ds*GF (Li et al., 2024). To induce apoptosis, the related genes of *H. armigera* were silenced via RNAi, using the merging tool to control this cotton pest. We investigated the effects of HDAC3 loss-of-function affecting the expansion and maturation of H. armigera by treating it with HDAC3siRNA and RGFP966, a particular inhibitor. A recent study demonstrated that HDAC3 knockdown dysregulated genes associated with apoptosis and juvenile hormone (JH) in H. armigera (Chang et al., 2022). Plant-mediated RNA interference (RNAi) has been a successful method for silencing aphid gene expression, which has been used to research the gene function of aphids (Fig. 7). The target genes' level of expression, ApCht7 and ApChtl0, by RNAi, the mortality rate ranged from 3% to 26% (Li et al., 2024). In Host-induced gene silencing, silencing the insecticides detoxifies genes directly in the host. In virus-induced gene silencing, take dsRNA from the virus and knock off the genes of White fly, pink bollworm, American bollworm, etc. By spray-induced gene silencing, make a formulation of siRNA or dsRNA and spray it on the crops to eradicate the target insects.

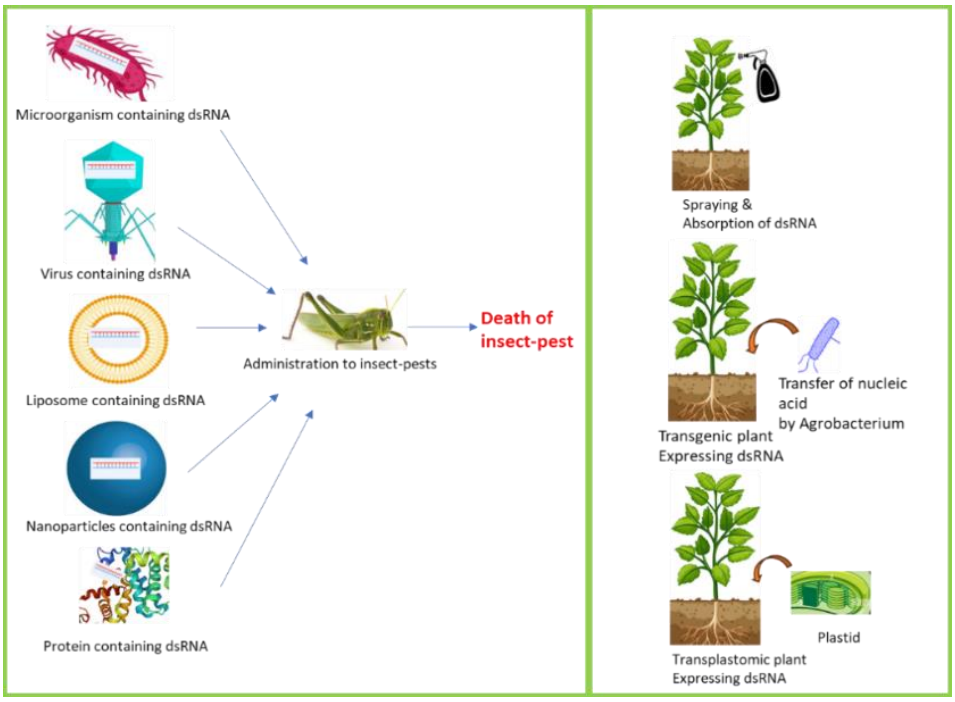

Fig. 6 Different ways of using RNAI biotechnology for the management of insects

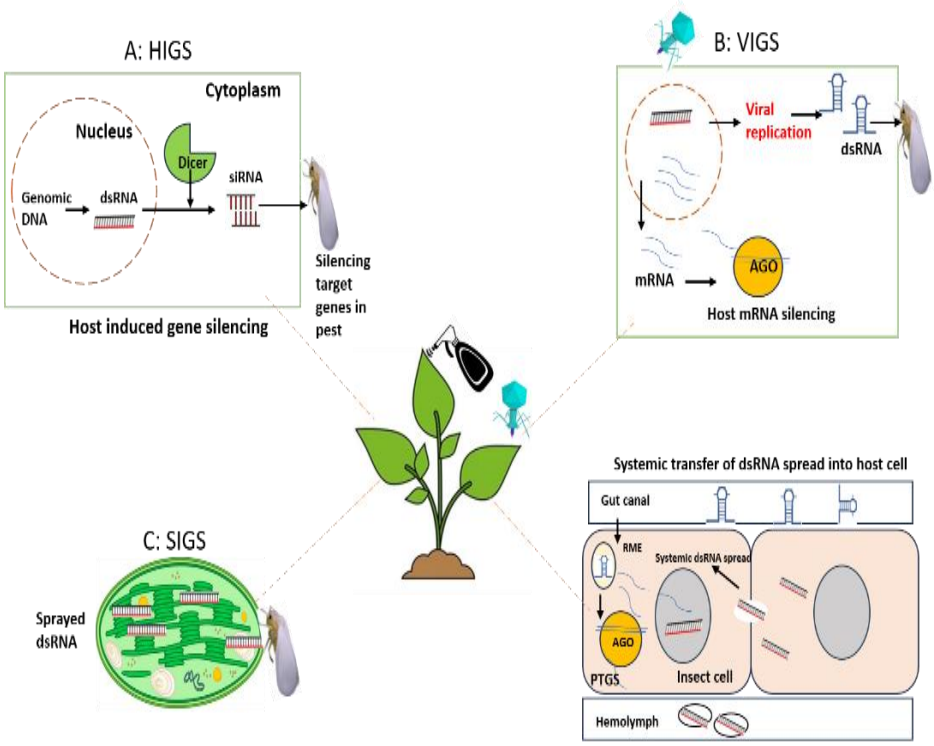

Fig. 7 Ways to control the harmful insect species

Eradicating whitefly and pink bollworm with RNAi technology

The silverleaf whitefly, *Bemisia tabaci*, has emerged as a major problem among sucking insects in recent years. It is thought to be one of the most significant plant viral vectors in the world since it is global and polyphagous, feeding on more than 600 host plants (Grover et al., 2019). The extensive use of chemical pesticides resulted in several issues with *B. tabaci* control efforts, including the development of resilience to the bulk of the pesticides on the market, insecticide-induced resurgence, and disturbance of parasitoids' and predators' populations. These problems have emphasized the necessity for more research on ecologically beneficial, targeted, sustainable, and alternative management strategies for *B. tabaci.* Since RNA interference (RNAi) may induce post-transcriptional gene silencing, it has shown promise as a pest management strategy (Fig. 8) against *B. tabaci*. While RNA interference (RNAi) can be used to create scalable, highly effective pest management methods, genomic information, and other resources (Luo et al., 2017; Shelby et al., 2020;

Hunter & Wintermantel, 2021; Suhag et al., 2021; Jain et al., 2022). We employed the exciting new strategy of plant-mediated artificial miRNA (amiRNA) expression to target three crucial whitefly genes. Acetylcholinesterase (AChE), orcokinin (Orc), sex lethal (Sxl) protein, and the Arabidopsis thaliana miRl59 precursor were the three essential genes of the whitefly that were targeted by artificial miRNAs (amiRNAs) by modification and engineering. The creation of resistance against whiteflies in cotton plants may be made possible by the amiRNA-mediated resistance against whiteflies in transgenic plants (Zubair et al., 2020; Gong et al., 2023; Karthik et al., 2023; Goswami et al., 2024). Using recyclable Chitosan Nanoparticles (CNPs), a hydrogel containing EcR dsRNA with morbidity over 80%, the current study examined RNAi-mediated silencing of the Ecdysone Receptors (EcR) gene in *B. tabaci* Asia-I. This effectively down-regulated the expression of the EcR gene, as confirmed by qRT-PCR analysis (Keppanan et al., 2024). In another study, we identified that the white fly gene TPS genes might be the primary cause of serious harm and might be useful targets for managing whiteflies. Subsequently, in adult whiteflies, RNA interference (RNAi) of BtTPSl and BtTPS2 caused a notable death rate and altered the expression of related metabolism-related genes of energy and chitin production, with 90% mortality in tobacco plants (Gong et al., 2022). The house fly could be controlled genetically (Khalid et al., 2025).

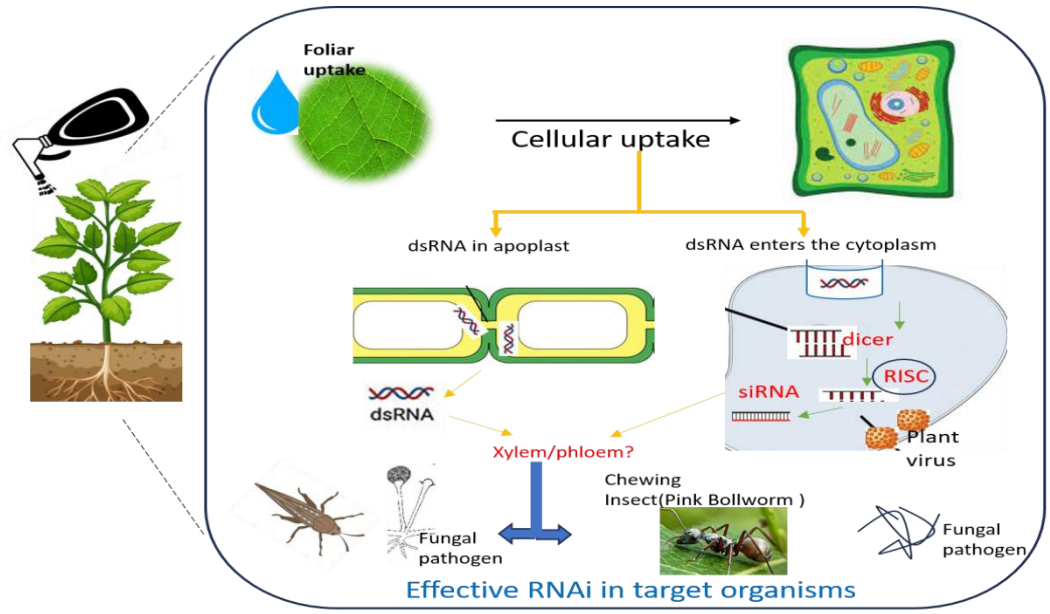

Fig. 8. Mechanisms of Effective RNA Interference for Pest Management (Hoang et al., 2022)

Temperature

One of the main factors influencing the eco-geographical dispersion and population growth of invasive insects is temperature. Our earlier research has demonstrated that by removing ROS, CAT helps whiteflies adapt to high temperatures (Fig. 9). In this work, we examined the function of CAT at three distinct temperatures: 25 °C, 20 °C, and 4 °C. When BtCATs were silenced, *B. tabaci* MED became much more sensitive to low temperatures. The findings demonstrated that silencing BtCYP 4Cl and BtCar3 greatly reduced the heat tolerance of invading whiteflies. Furthermore, after BtCYP 4Cl was silenced, whiteflies showed a greater ability to withstand cold. These findings suggest that two crucial regulators in *B. tabaci*'s adaptability are BtCYP 4Cl and BtCar3 to temperature. Furthermore, they might have a significant impact on how *B. tabaci* spreads throughout China's landscape and becomes an invasive species (Shen et al., 2021). The impact of high temperatures on the insecticide thiamethoxam-resistant BTQ was examined in a different investigation. The elevated temperature affected P450 activity, which in turn affected BTQ tolerance. The adults' mRNA levels of the target gene were dramatically reduced when fed double-stranded RNA (dsRNA) of CYP6CM1. Additionally, their tolerance to thiamethoxam, which was produced at a temperature of 3°C for six hours, was dramatically reduced (Guo et al., 2018; Nyamukondiwa et al., 2022; Barman et al., 2023).

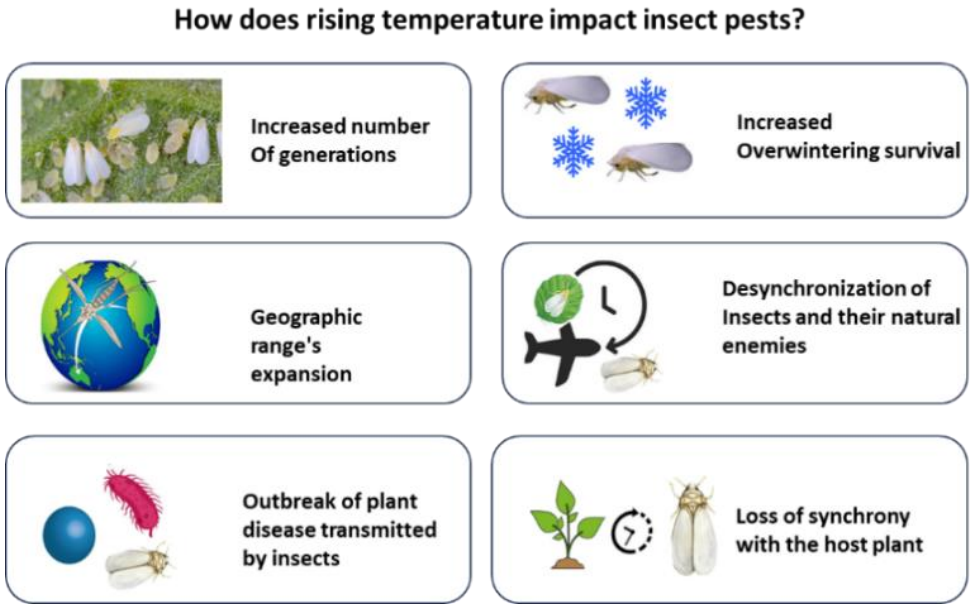

Fig. 9 Impacts of climate change on insect pests and agricultural systems

Atmospheric carbon dioxide

Increasing atmospheric concentrations of carbon dioxide (CO_2) and warmer temperatures are two of the greatest obvious consequences of climate change on a worldwide scale this century (Fig. 10). The effects of CO_2 alone enrichment on the biology and physiology of herbivorous insects are well-studied (Li et al., 2017). In the figure effect of Carbon dioxide that how it increased the genes of the whitefly, which could be lethal for crop species. Under elevated temperature, CO_2 concentration enhancement resulted in a 167.5% increase of the activity of GST, a l03.6% increase in AchE activity, and a 31.6% reduction of CAT activity (Li et al., 2016). In the uncontrolled greenhouse with changing temperature conditions (FTC) ranging from springtime and summer, 0°C to 60°C, we saw an elevated rate of survival of *B. tabaci* MED. According to our research, *B. tabaci* MED's survival rate gradually rose after receiving heat shock treatment at 50 °C for 0.5 hours, even if it was raised under FTC for 0 weeks from April to June (Yao et al., 2019; Jhan & Lee, 2022).

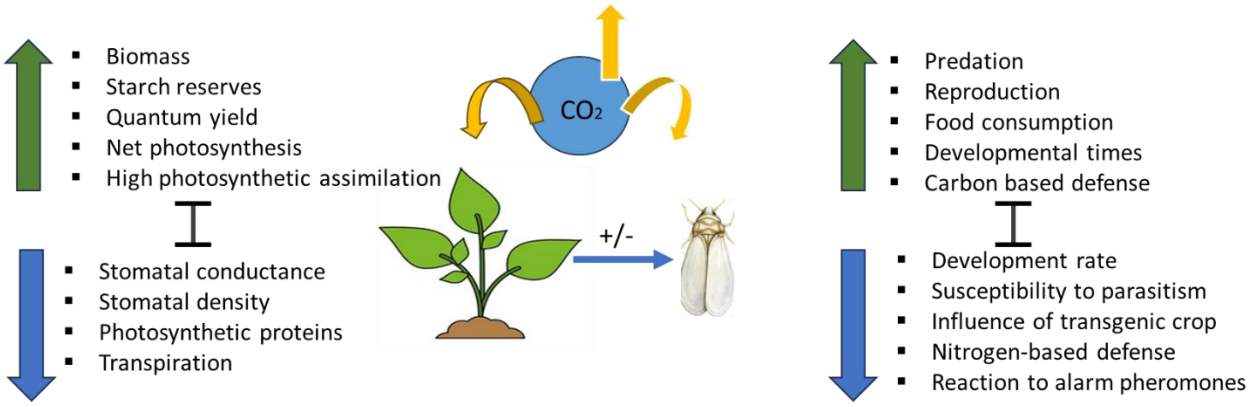

Fig. 10 The effect of increased atmospheric carbon dioxide on insect pests

Sustaining growing hormone titters in insects is mostly dependent on juvenile hormone esterase, which also regulates the metamorphosis and reproduction of insects. Advanced techniques like RNAi are used to knock down the juvenile gene of the whitefly. Decreased gene expression was seen in adult whiteflies following dsRNA feeding at 2.5 and 1.0 µg/µl, according to qRT-PCR studies. The gene knockdown negatively impacts whitefly survival and reproduction in a dose-dependent way (Grover et al., 2019; Hunter & Wintermantel, 2021). One of the deadliest polyphagous insects in the world, *Bemisia tabaci* (Gennadius) damages a variety of crops by sucking sap both as an adult and during the nymphal stage. The increase in pesticide resistance makes the chemical treatment of whiteflies challenging (Fig. 11). Silencing of ETHr by RNAi, along with higher adult mortality (68.88%) and higher mortality (81.35%) at the nymphal stage as compared to the control. Therefore, it may be a possible target for the creation of insecticides based on dsRNA to control whiteflies (Devi et al., 2024). The current study focuses on nine potential genes from whiteflies that have been linked to several essential physiological processes and the transmission of viruses, Apoptosis inhibitor and non-fermentable sugar substitute (SNF7) (IAP).

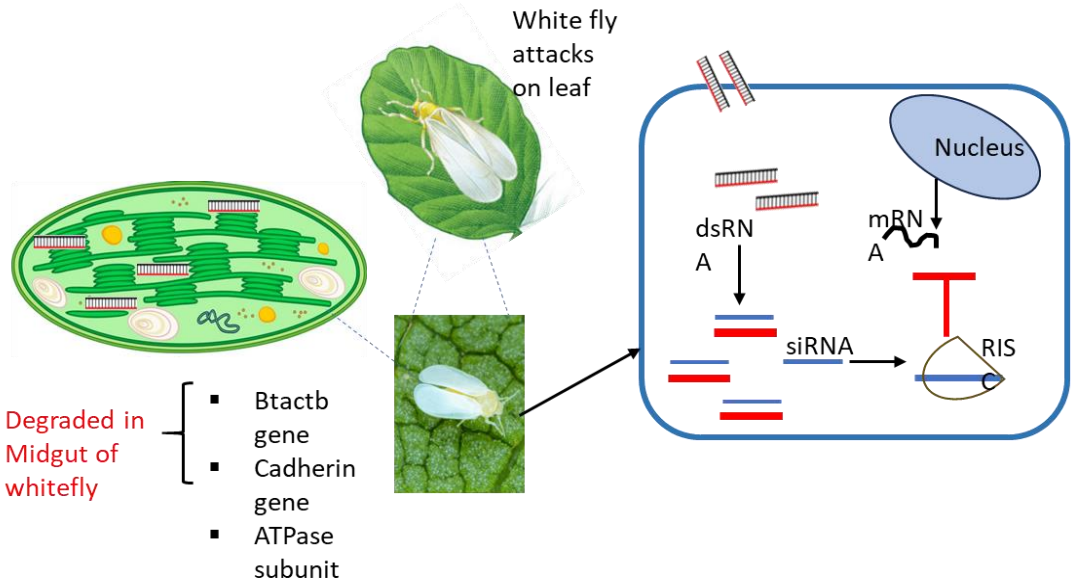

Fig. 11 Limitations of nuclear genome transformation: chloroplast engineering as a viable alternative

Pectinophora gossypiella (Saunders), often known as the pink bollworm, is a highly damaging insect pest that affects cotton production worldwide. The Indo-Pakistan region is most likely where the pink bollworm originated (Fand et al., 2021). Pink bollworm populations were successfully controlled by commercial Bt cotton planting. Moreover, some pink bollworm field populations have grown practically resistant to Bt cotton that expresses CrylAc or even produces CrylAc and Cry2Ab, which poses a significant risk to the long-term viability of transgenic Bt cotton. Pink bollworms could be a more serious threat to the survival of Cotton. Foliar pesticides like chlorpyrifos, fenvalerate, lambda-cyhalothrin, and bifenthrin are used as pesticides on pink bollworms. However, due to their excessive use, this pest is resistant to chemical insecticides. This chewing-type pest contains insecticide detoxifying enzymes, so insect molecular biologists have decided to knock off these genes with new emerging technology like RNA interference in the form of transgenic plants, to make the formulations as a foliar spray or inside the host insects. The creation of innovative methods for managing insect pests was prompted by RNAi's demonstration of effective gene silencing in insects, which involved removing the genes generating vacuolar ATPase (V-ATPase) components a and c from the midsection of pink bollworms. When both genes were silenced by 200 ng of dsRNA, mortality ranged from 8.9 to 26.7%.

Reasons for not controlling the Pink Bollworm

Mutations in Cadherin genes

Mutation in the Cadherin gene is a key factor for Pink Bollworm resistance to Bt Cotton in China. Another study reported that Transposon insertion causes cadherin mis-splicing and confers resistance to Bt cotton in pink bollworms from China (Wang et al., 2019). Previous research with pink bollworms selected in the lab and the field revealed that variations in the arrangement of amino acids of a midgut cadherin protein (PgCadl) that binds CrylAc in vulnerable larvae are the cause of resistance to CrylAc. Previous research with pink bollworms selected in the lab and the field revealed that variations in the arrangement of amino acids of a midgut cadherin protein (PgCadl) that binds CrylAc in vulnerable larvae are the cause of resistance to CrylAc. The ability of pink bollworm and other major insect pests presents challenges for keeping an eye on and controlling Bt crop resistance (Fabrick *et al.*, 2020). There could be the 5 cadherin repeated mutations associated with Bt resistance in a field-derived strain of pink bollworm (Gao et al., 2018; Wang et al., 2020). In pink bollworms. According to Wang et al. (2019) and Fabrick et al. (2023) transgenic cotton produces the novel aIIele (rl6) of the adhesive gene (PgCadl), which is associated with resistance to the Bt toxin CrylAc. In the given (Table 4) gene mutations in the pink bollworm.

One of the most destructive plant pests is the aphid, which uses its stylets to pierce plant tissue and ingest copious volumes of phloem sap, depriving the plant of photoassimilates and other nutrients feeding-related (Fig. 12). Genes to counteract plant defenses (Nalam et al., 2019; Yates & Michel, 2018). This network contains important genes in locating the host (encoding receptors for smell and signaling components), defeating the host's defenses (producing salivary proteins), and metabolizing foods (encoding digestive and detoxifying enzymes). Such genes are very efficient targets for RNAi-based aphid control because they cause death, decrease the quantity of progeny, and interfere with growth and development (Ye et al., 2019). Research carried out on citrus (An et al., 2024). Plant-mediated RNA interference (RNAi) (Table 5) has been a successful strategy for researching the gene function of aphids. It targets the genes ApCht7 and ApChtl0, which are involved in chitin metabolism, and has a death rate ranging from 3.3% to 26% (Li et al., 2024).

Table 4 Gene Mutations in Pink Bollworm

Mutated Gene	Resistant against genes	References
PgCadl, ATP-binding cassette transporter protein PgABCA2	CrylAc, Cry2Ab	(Fabrick et al., 2023; Tabashnik & Carrière, 2019; Wang et al., 2020)
Cadherin aIIeles (*rl9* and *r20*)	CrylAc	(Wang et al., 2020)
rl5A and *rl5B*	CrylAc	(Wang et al., 2019)
rl4 allele of the pink bollworm cadherin gene (*PgCadl*)	CrylAc	(Wang et al., 2020)
rl3PgCadl	CrylAc	(Wang et al., 2018)
PgABCA2	Cry2Ab	(Fabrick et al., 2021; Mathew et al., 2018)
PgCadl alleles (*rl-r20*), PgABCC2	CrylAc, *PgABCC2* confers low-level resistance to CrylAc	(Wang et al., 2018)

Table 5 RNA Interference gene silencing against Aphids

Target Gene	Silencing mechanism	Mortality rate	Dose	Insect Stage	Cite
CHS gene	dsRNA	44.7% mortality rate	600 ng through injection, l200 ng·μL^{-l} through plant mediated	fourth-instar nymphal stage	(Ye et al., 2019)
ApCht7 and *ApChtl0*	dsRNA	ds*ApCht7*-treated aphids were about 3.3%, ds*ApChtl0*-treated aphids were 3.8%	l.0 mL of dsRNA (l200 ng/μL)	Nymphal stage	(Li et al., 2024)
CHSl	pBAC–RNAi-*CHSl*	50.29% and 45.32%	-	second-instar larvae	(Zhao et al., 2018)
Aphunchback gene	dsRNA	74%	600 ng	-	(Ye et al., 2019)
VGSCs gene	siRNA, double-stranded RNA	6l%	(20 ng/L)	nymph stage	(Liu et al., 2024)
MpPar6	dsRNAs	70%,	l0 ng μL^{-l}	Different stages	(Zhang et al., 2024)
*β*2 divergent nicotinic acetylcholine receptor (nAChR) subunit	dsRNA	l9% and 80%	400 ng	Adult stage	(Ligonniere et al., 2024)
AgCLK-l	dsRNA	35 %.	l μg/μL	3rd instar	(Liu et al., 2024)

Factors that affect the fate of RNAi-based pesticides

Non-target insect species

The major concern of dsRNA-based pesticide effects on non-target insect species like pollinators and the beneficial natural enemies of insect pests. Pollinators (primarily honeybees, *Apis mellifera* (Hymenoptera: Apidae), but including hoverflies, bumble bees, and other insects) increased oilseed rape seed weight by l8% and market value by 20% per plant. Using spray-based and nuclear-encoded insecticides highly affects the natural ecosystem (Chen et al., 2015; Zioga et al., 2023; Nugnes et al., 2023).

Trigger the Epigenetic modifications

Especially in plants nucleus mRNA could be degraded, and the concern is that when dsRNA or siRNA is delivered to plants, the epigenetic modifications will be boosted in the form of DNA methylates and histone protein alterations (Law & Jacobsen, 2010; Gutbrod & Martienssen, 2020) . Here, we show that plants may experience de novo methylation of regulatory sequences using high-pressure dsRNA spraying. The insertion Adding a methyl group to the 5th carbon of the six-ring amino acid, called a residue, is known as methylation of eukaryotic DNA, and is a significant epigenetic change that controls a wide range of developmental features. Aptly

named RNA-directed DNA methylation (RdDM) occurs when RNA molecules mediate de novo DNA methylation in plants. RNA interference (RNAi) and RdDM are closely related; RNAi is its epiphenomenon (Dalakouras & Ganopoulos, 2021). However, the applied dsRNAs cause unintentional epigenetic changes that lead to epigenetically changed plants, a problem that hasn't been adequately addressed and needs more thought (Dalakouras & Papadopoulou, 2020). If the molecular biologist used large dsRNA about 24nt, then clearly RNA-directed DNA methylation (RdDM) will occur. It has been seen that if 2lnt, then the above case may not occur. Thus, searching for the small matches of dsRNA to exclude the concern of epigenetics by dsRNA given to crop applications (Dalakouras et al., 2016). Currently, this issue is rising with European GMO legislation (Faltus, 2023). Moreover, one of the superior functions of dsRNA is to affect insect epigenome modifications besides plants (Glastad et al., 2019). It has been seen that insect divers could degrade the excessive amounts of dsRNA, neglecting the endogenous dsRNA and saturating with exogenous dsRNA and negatively affecting the development and antiviral defense of non-target organisms, even seen in mice, causing lethality, also seen in the case of dsGFP in *Telenomus podisi* (Castellanos et al., 2022).

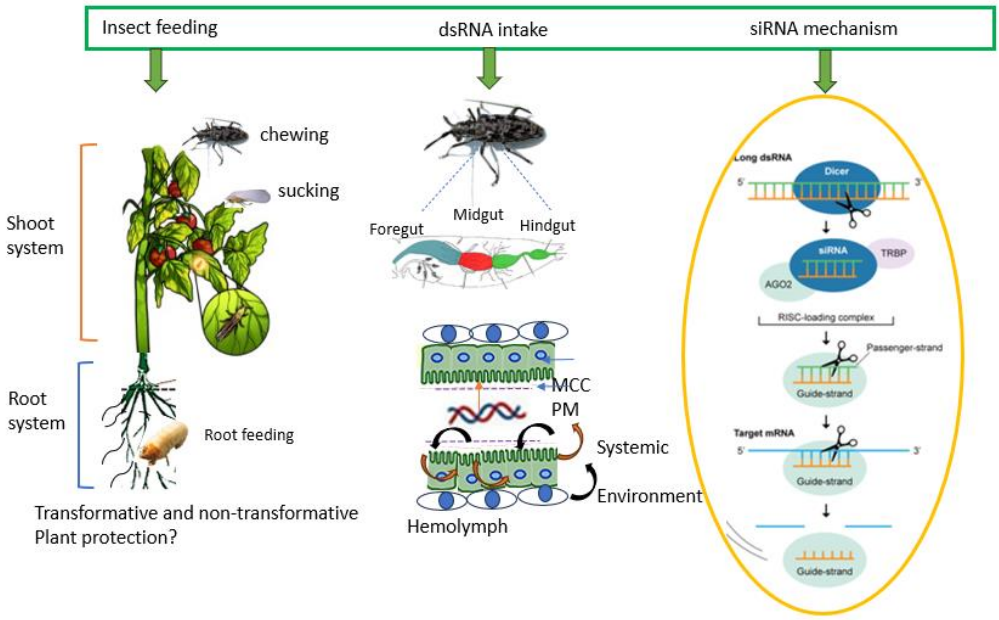

Fig. 12 Mechanisms of plant protection via RNA interference (RNAi) pathway utilized by Hemiptera insects

Factors that affect DSRNA stability

Microorganisms could degrade the dsRNA, $UV_{254,}$ affecting its stability and washing off by rain or dew drops (Qiao et al., 2018; Parker et al., 2019; Bachman et al., 2020; Das & Sherif, 2020). In another study, Light intensity affects RNA silencing of a transgene in *Nicotiana benthamiana* plants (Kotakis et al., 2010). The mechanism of exogenously applied DSRNA entry, transport, and processing in plants is still poorly understood. The price of dsRNA and the legal environment will be important factors in determining whether this technique is used (Mitter et al., 2017). When dsRNA was applied on the leaf surface in controlled conditions provided 5 days of protection against viruses before degradation was confirmed at the molecular level. Moreover, Cy3-labeled dsRNA applied on the leaf surface was rinsed after 24 h, mimicking rain, and washed away (Mitter et al., 2017). The efficacy of dsRNA on potato leaves was up to 28 days. once it dried on the surface, it could not be degraded, but incorporated with gel and exposed to UV, it was seen to be inactive (San Miguel and Scott, 2016; Dietz-Pfeilstetter et al., 2021; He et al., 2022).

RNAi (The next generation insecticides) target the genes of harmful insect pests at different stages

Degradation of the Energy Metabolism process of Insects Pest of Cotton bollworm

The midgut-resident cytochrome P450 monooxygenase gene of insects is essential for the energy metabolism of bollworms. *V-ATPase* gene resource of the plasma membrane for alkaline and amino acid absorption in insects, and Key enzymes for the formation of the trachea, cuticles, and midgut are called pest chitin synthases (CHS). It is well known that the primary structural element of fungi and arthropods is chitin, a material lacking in plants and vertebrates. Therefore, chitin synthases might serve as a perfect RNAi technology target for controlling insect growth (Jin et al., 2015). The enzyme acetylcholinesterase is crucial to *Helicoverpa armigera*'s central nervous system and is the target of numerous organophosphorus and carbamate insecticides (Bally et al., 2016). The same case has been seen in *Diaphorina citri* to target gene is disturbed to disrupt its energy metabolism of that gene (Guo et al., 2023).

Arginine kinase (*NfArgK) is* a cellular energy reserve regulatory gene present in different insects, like the tawny crazy ant (*Nylanderia fulva*) (Meng et al., 2020)**,** PGRPl, Toll, Domeless SPNI, and Lysozyme in leaf beetle (68.7% gene silencing effect by RNAi (Tu et al., 2023)The clones of male-specific lethal 3 (MSL3), glycerol 3-phosphate dehydrogenase (GPDH), and NADH dehydrogenase (ND) were used to produce double-stranded RNAs (dsRNAs) when fed bacteria that knocked down the transcript levels of those genes, with a higher mortality rate at the newly hatched larval stage (Jin et al., 2021).

Actin, Tubulin, and other complexes of insects are ideal targets for RNAi

l µL of dsRNA solution into the drastic reduction of the critical α-tubulin gene in the lower abdomen of *B. germanica* suggests that this method could be a viable strategy for controlling insect pests. It also targets tubules other than the midgut, like the head, antennae, fat body, and Malpighian tubules, to arrest translation (Lin et al., 2017). Sec23 was found to be a particularly deadly RNA interference target. Coatomer protein, which is encoded by Sec23, mediates ER-Golgi transport and is a component of the coat protein (COPII) complex. In a different investigation, we looked at the lethal and sublethal effects of orally given double-stranded (ds)RNAs that target the endoplasmic reticulum-Golgi transport and organelle acidification processes of *P. chrysocephala* orthologs of Sec23 and vacuolar adenosine triphosphatase subunit G (VatpG), causing mortalities of 76% and 56%.

Conclusion and Future Perspectives

Chloroplast engineering combined with RNA interference (RNAi) technology holds significant promises for sustainable insect pest management. This integrated strategy leverages high transgene expression, stability, and reduced non-target effects of chloroplasts to enhance the efficacy of RNAi. However, several challenges and limitations must be addressed to fully realize its potential. The technical complexity of chloroplast transformation and the stringent regulatory frameworks for GMOs pose significant barriers to the widespread adoption of these technologies. Additionally, public perception and acceptance of GMOs remain critical factors influencing their implementation. Despite these challenges, the eco-friendly nature of RNAi and chloroplast engineering offers a sustainable alternative to traditional chemical pesticides, which are increasingly facing resistance issues and environmental concerns.

To further develop this strategy, future research should focus on broadening the range of insect pests that can be targeted. This includes investigating the creation of transplastomic plants that express dsRNA against a wider variety of economically significant pests, such as aphids, whiteflies, leafhoppers, and other sap-feeding insects. Additionally, exploring the potential of RNAi against chewing insects like borers, beetles, and caterpillars could lead to more comprehensive pest control measures. Research into the synergistic effects of combining multiple RNAi targets within the chloroplast genome could enhance the effectiveness and longevity of this strategy against evolving insect populations. As our understanding of insect molecular biology and RNAi processes advances, designing more effective and targeted dsRNA constructs will be crucial for gaining better control over diverse insect pests. Moreover, integrating chloroplast-mediated RNAi with other sustainable practices, such as biological control agents and integrated pest management (IPM) techniques, could provide a more holistic approach to crop protection. This combination would leverage the strengths of each method, offering a comprehensive and eco-friendly framework for managing insect pests. While RNAi and chloroplast engineering offer a sustainable alternative to chemical pesticides, it is important to recognize that chemical pesticides may still be necessary in certain scenarios where immediate control is required. Additionally, cultural practices like crop rotation and intercropping can complement RNAi to reduce pest populations effectively. In conclusion, the combination of RNAi and chloroplast engineering represents a transformative technique for sustainable insect pest management. By addressing the current limitations and exploring new research directions, this technology has the potential to revolutionize crop protection, leading to a more secure and sustainable agricultural future.

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