

Generation of aphid-resistant wheat through plant-mediated RNA interference

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Original assay for graduation as a doctor in agricultural sciences and
biological engineering

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Abstract

Aphids (Aphididae) are major agricultural pests that cause substantial economic losses through direct feeding damages and efficient transmission of plant viruses. Current control strategies heavily rely on chemical insecticides, which pose environmental risks and can lead to resistance, underscoring the urgent need for sustainable and targeted alternatives. In this study, we explored plant-mediated RNA interference (RNAi) as a sustainable strategy for aphid control by targeting two distinct genetic elements: the endosymbiotic bacterial gene *Smrrl* from *Buchnera aphidicola*, which is essential for aphid survival, nutrient provision, and stress tolerance, and *SmDSR32*, a novel aphid gene encoding a salivary peptide that is crucial for successful feeding.

Initial *in vitro* double-stranded RNA (dsRNA) feeding assays confirmed that silencing these two genes significantly increased aphid mortality, demonstrating their importance as RNAi targets. Transgenic wheat lines expressing dsRNA targeting either *Smrrl* or *SmDSR32* were generated. Aphids feeding on these transgenic plants exhibited significantly reduced expression of the target genes, confirming successful uptake and RNAi silencing. This gene suppression led to severe physiological impairments, manifesting as significantly increased mortality, reduced fecundity, and overall decline in population growth over time. Detailed electrical penetration graph (EPG) recordings further revealed profound alterations in feeding behavior, including prolonged periods of stylet pathway activity and significantly shortened sustained ingestion phases. Notably, the silencing effects were not limited to the exposed generation. Offspring of aphids reared on RNAi plants also exhibited sustained fitness deficits, demonstrating that the RNAi-mediated suppression was both persistent and transgenerational. This prolonged impact highlights the robustness of this approach and its potential to provide sustainable control in agricultural settings.

In conclusion, our findings identify two promising RNAi targets, and provide a comprehensive proof-of-concept for using host-induced gene silencing (HIGS) to disrupt critical biological functions in aphids. This strategy offers a novel, effective, and environmentally sustainable approach for managing *Sitobion miscanthi* in wheat.

Keywords: Wheat (*Triticum aestivum* L), Grain aphid (*Sitobion miscanthi*), RNA interference (RNAi), *Buchnera aphidicola*, salivary peptide, aphid control

Résumé

Les pucerons (Aphididae) sont des ravageurs agricoles majeurs qui causent des pertes économiques substantielles through des dégâts alimentaires directs et la transmission efficace de virus végétaux. Les stratégies de lutte actuelles reposent largement sur les insecticides chimiques, qui posent des risques environnementaux et peuvent entraîner une résistance, soulignant le besoin urgent d'alternatives durables et ciblées. Dans cette étude, nous avons exploré l'interférence par ARN (RNAi) médiée par les plantes comme stratégie durable pour la lutte contre les pucerons en ciblant deux éléments génétiques distincts : le gène bactérien endosymbiotique *Smrrl* de *Buchnera aphidicola*, essentiel pour la survie, la nutrition et la tolérance au stress des pucerons, et *SmDSR32*, un nouveau gène de puceron codant pour une protéine salivaire cruciale pour une alimentation réussie.

Des tests préliminaires d'alimentation *in vitro* avec de l'ARN double brin (dsRNA) ont confirmé que le « silencing » de ces deux gènes augmentait significativement la mortalité des pucerons, démontrant leur importance en tant que cibles de ARNi. Des lignées de blé transgénique exprimant du dsARN ciblant *Smrrl* ou *SmDSR32* ont été générées. Les pucerons se nourrissant sur ces plantes transgéniques ont montré une expression réduite des gènes cibles, confirmant l'absorption et l'efficacité des ARNi. Cette suppression génétique a entraîné de graves impairments physiologiques, se manifestant par une mortalité accrue, une fécondité réduite et un déclin global de la croissance de la population au fil du temps. Des enregistrements détaillés par électropénétrographie (EPG) ont en outre révélé des altérations profondes du comportement alimentaire, incluant des périodes prolongées d'activité du stylet et des phases d'ingestion soutenue considérablement raccourcies. Notamment, les effets de « silencing » ne se sont pas limités à la génération exposée. La descendance des pucerons élevés sur les plantes ARNi a également présenté des déficits de fitness durablement, démontrant que la suppression médiée par ARN était à la fois persistante et transgénérationnelle. Cet impact prolongé souligne la robustesse de cette approche et son potentiel pour fournir un contrôle durable en milieu agricole.

En conclusion, nos résultats identifient deux cibles de ARNi prometteuses et fournissent une preuve de concept complète pour l'utilisation du « silencing » génique induite par l'hôte (HIGS) pour perturber les fonctions biologiques critiques des pucerons. Cette stratégie offre une approche novatrice, efficace et écologiquement durable pour la gestion de *Sitobion miscanthi* dans le blé.

Mots-clés: Blé (*Triticum aestivum* L), Puceron des céréales (*Sitobion miscanthi*), Interférence par ARN (RNAi), *Buchnera aphidicola*, peptide salivaire, lutte contre les pucerons

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List of acronyms

Ago	Argonaute
APOP	Adult preoviposition period
BLAST	Basic Local Alignment Search Tool
Bt	Bacillus thuringiensis
cDNA	Complementary DNA
CMV	Cucumber mosaic virus
CPB	Colorado potato beetle
CTAB	Cetyltrimethylammonium bromide
CTV	Citrus tristeza virus
DAF	Days after feeding
Dcr	Dicer
dCTP	Cy3-labeled deoxycytidine triphosphate
DIG	Digoxigenin
dsRBD	DsRNA binding domain
dsRNA	Double-strand RNA
dsRNases	Double-stranded ribonucleases
DXV	Drosophila X virus
EcR	Ecdysone receptor
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EPG	Electrical penetration graph
FAO	Food and Agriculture Organization
GFP	Green fluorescent protein
GLD	Glucose dehydrogenase
GMOs	Genetically modified organisms
GNA	Galanthus nivalis agglutinin
GNPs	Guanylated polymers
HIGS	Host-induced gene silencing
hpRNA	Hairpin RNA
IPM	Integrated pest management
IVT	<i>In vitro</i> transcription

LDHs	Layered double hydroxides
miRNAs	MicroRNAs
mRNA	Messenger RNA
MW	Molecular weight
NCBI	National Center for Biotechnology Information
pI	Isoelectric point
piRNAs	Piwi-interacting RNAs
PRRs	Pattern recognition receptors
qRT-PCR	Quantitative real-time PCR
RISC	RNA-induced silencing complex
RNAi	RNA interference
rRNA	Ribosomal RNA
SEM	Standard error of the mean
Sid1	System RNA interference defective 1
SIGS	Spray-induced gene silencing
siRNAs	Small interfering RNAs
SPc	Star-polycation
sRNA	Small RNA
TMV	Tobacco mosaic virus
TPOP	Total preoviposition period
TRV	Tobacco rattle virus
Ubi	Ubiquitin
USP	Ultraspiracle protein
VIGS	Virus-induced gene silencing
VLPs	Viral-like particles
WCR	Western corn rootworm
WFT	Western flower thrips

Chapter I

Review on RNAi technologies for insect control in crop protection

Abstract

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

Keywords: Crop protection; Double-stranded RNA (dsRNA); Insect control; RNA interference (RNAi)

1 Introduction

Insect pests could cause significant crops yield losses worldwide each year, competing with humans for food and fiber resources while spreading various plant viruses (Sarwar, 2020). Currently, undue applications of diverse pesticides for insect management are exacerbating environmental pollution and ecological deterioration (Beketov et al., 2013; Tang et al., 2021). Furthermore, the intensified applications of pesticides not only promotes the rapid evolution of pest resistance but also lead to the decrease of the population of non-target beneficial pest predators (Bass and Nauen, 2023). At the same time, the frequent occurrences of extreme weather aggravate the negative effects of chemical pollution caused by the intensive usage of pesticides. To ensure global food and ecosystem security, it is essential to have resilient crop production and minimize environmental pollution by using the cutting-edge technologies. RNA interference (RNAi)-based technology has emerged as a promising technology in pest control, offering a potentially species-specific, low-risk, effective, and environmentally friendly strategy for crop protection (Palli, 2023).

RNAi is an RNA-mediated regulatory mechanism present in most eukaryotic organisms (Agrawal et al., 2003). It was first discovered in the nematode *Caenorhabditis elegans* in 1998, demonstrating potent and sequence-specific interference that specifically knock down the expression of targeted gene (Fire et al., 1998). The potential RNAi mechanisms are highly conserved in eukaryotic cells, utilizing various small RNA (sRNA) groups to regulate messenger RNA (mRNA) degradation/translation or heterochromatin establishment in a sequence-specific mode (Couzin, 2002). In general, the endogenously synthesized or exogenously supplied dsRNAs are processed into sRNA duplexes typically ranging from 21 to 24 nucleotides (nt) in length by ribonuclease III enzymes known as Dicer (Dcr), which was initially identified from the *Drosophila melanogaster* (Jaskiewicz and Filipowicz, 2008). The sRNA duplexes are then loaded into specific members of the Argonaute (Ago) protein family, either in the cytoplasm or within the nucleus, to form an RNA-induced silencing complex (RISC) (Bologna et al., 2018). The guide strand of sRNA typically mediates the binding of RISC to complementary mRNA in the cytoplasm, thereby inducing RNAi. Besides, it can direct the RISC complex to a DNA target after RISC has entered the nucleus, leading to transcriptional gene silencing through DNA methylation (Liu et al., 2020). In insects and other animals, three types of sRNAs are known to activate corresponding RNAi pathways, including small interfering RNAs (siRNAs), which mediate the siRNA pathway; microRNAs (miRNAs), which mediate the miRNA pathway; and piwi-interacting RNAs (piRNAs), which mediate the piRNA pathway (Zhou et al., 2008) (Figure 1-1). Different RNAi pathways play critical roles in insect control. In general, the siRNA pathway mainly defends against viral infections and suppresses transposable elements (Mondal et al., 2018; Swevers et al., 2018), whereas the miRNA pathway regulates gene expression (Lucas et al., 2015), and the piRNA pathway suppresses germline transposon expression (Mondal et al., 2018).

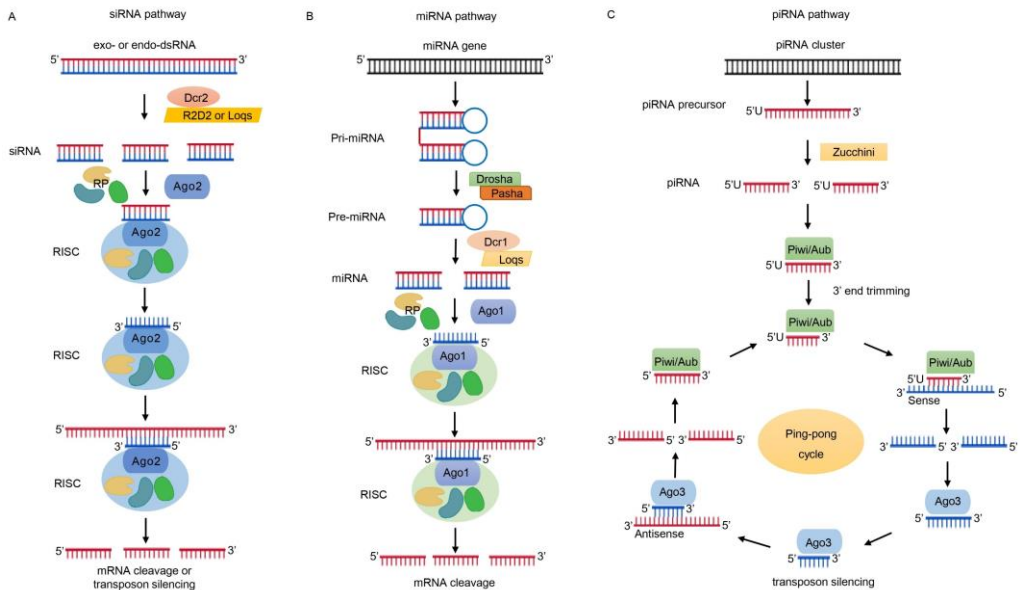


Figure 1-1. Mechanisms of potential RNAi pathways in insects.

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

2 Factors influencing RNAi efficacy in insects

2.1 Differential response of insects to RNAi

Not all insect species respond equally to ingested dsRNA and RNAi. Among them, insects belong to Coleoptera is typically highly susceptible to RNAi, both injected and ingested dsRNA can induce widespread and potent RNAi effects in important coleopteran species (Laudani et al., 2017; Prentice et al., 2017). A dsRNA-binding protein, Staufien C, has been identified as a crucial factor for effective RNAi in coleopterans such as *Leptinotarsa decemlineata*. This gene is detected only in Coleoptera and is essential for the processing of dsRNA into siRNA in these insects (Yoon et al., 2018). Actually, RNAi effectiveness varies across different insect orders, such as Hemiptera, Orthoptera, Diptera, Hymenoptera, and Lepidoptera show varying sensitivity to dsRNA (Christiaens et al., 2014; Guo et al., 2018; Miller et al., 2008; Shukla et al., 2016; Terenius et al., 2011). RNAi effectiveness varies even within the

same insect order (Christiaens et al., 2014; Hughes and Kaufman, 2000; Zhu et al., 2013).

RNAi efficacy also varies depending on the delivery routes of dsRNA. The most important factors influencing the RNAi efficacy through oral delivery include the stability of dsRNA and its uptake within digestive systems. For instance, the midgut environment is alkaline in Lepidoptera species, several Orthoptera species and Hymenoptera species, which destabilizes dsRNA and accelerates its degradation (Ortego, 2012; Teo, 1997; Wu et al., 2016). In many cases, dsRNA is removed from the hemolymph in Lepidoptera and Orthoptera or from the saliva in Hemiptera with nucleases, which is the main contributor to RNAi resistance (Christiaens et al., 2014; Garbutt et al., 2013; Shukla et al., 2016; Spit et al., 2017). Particularly, dsRNA can be trapped in endosomes of *Spodoptera frugiperda*, and of *Heliothis virescens*, hindering its cleavage into siRNAs, which contributes to inefficient RNAi response in Lepidoptera insects (Shukla et al., 2016; Yoon et al., 2017). What's more, tissue-dependence in the RNAi response may lead to inconsistent outcomes. For example, while *D. melanogaster* is generally resistant to external dsRNA, hemocytes of *D. melanogaster* can absorb dsRNA from the extracellular environment, leading to a functional RNAi response (Whyard et al., 2009). Similarly, the *D. melanogaster* S2 cell line, derived from hemocytes, exhibits a high sensitivity to dsRNA and is commonly used for *in vitro* RNAi studies (Clemens et al., 2000). In addition, the mechanisms underlying the tissue-specific RNAi responses remain largely unexplored, and these mechanisms may vary significantly across species and even among tissues within the same species.

2.2 Cellular uptake of dsRNA and systemic RNAi

Two distinct pathways for dsRNA uptake in insect have been described: transmembrane channel-mediated uptake and the endocytosis-mediated uptake. The transmembrane protein system RNA interference defective 1 (Sid1), which is highly conserved and ubiquitously expressed (except in neurons), mediates the release of endosomal dsRNA into the cytoplasm during environmental RNAi, which is caused by dsRNA taken up by a cell from the environment, and systemic RNAi, which refers to the extension of the silencing signal from the cell in which the dsRNA is applied to other cells and tissues (Feinberg and Hunter, 2003; Winston et al., 2002). Sid1 homologous genes have been identified in most insects, except for those of the superorder Antliophora such as Diptera, Mecoptera and Siphonaptera (Dowling et al., 2016). The insect Sid1-like protein shows high homology with *C. elegans* Tag 130/ChUP1 protein (Tomoyasu et al., 2008), and are involved in dsRNA uptake in certain insect species, such as the brown planthopper (*Nilaparvata lugens*) and the Colorado potato beetle (CPB, *L. decemlineata*) (Cappelle et al., 2016; Xu et al., 2013). In contrast, the Sid1-like proteins in *Schistocerca gregaria* or *Plutella xylostella* appear to play no role at all in RNAi efficacy (Wang et al., 2014; Wynant et al., 2014).

The clathrin-dependent endocytic pathway is the most well-documented mechanism of dsRNA uptake in insects. It was first characterized in *Drosophila* S2 cells, which lack Sid-like proteins (Ulvila et al., 2006). Clathrin-dependent, receptor-mediated endocytosis can facilitate dsRNA uptake in several insect species (Pinheiro et al., 2018b; Saleh et al., 2006). Studies both *in vitro* and *in vivo* have shown that pharmacological suppression of this pathway in gut epithelial cells reduces dsRNA

uptake, particularly in Coleoptera species such as the CPB. Additionally, phagocytosis, another major endocytosis pathway, is primarily active in specialized hemocytes, such as plasmatocytes (Cappelle et al., 2016). Pattern recognition receptors (PRRs) and scavenger receptors are crucial for identifying dsRNA and facilitating its uptake via phagocytosis. In *D. melanogaster*, and scavenger receptors, such as SR-CI and Eater together accounted for more than 90% of the dsRNA uptake into S2 cells, indicating that receptor-mediated endocytosis plays a key role in dsRNA uptake in this species (Ulvila et al., 2006). However, several studies indicate that inhibiting or blocking these receptors does not completely stop the absorption process, indicating that dsRNA internalization is a complex process (Abbasi et al., 2020; Pinheiro et al., 2018a).

Systemic RNAi is the process by which RNAi spreads between cells and can be transmitted to the offspring after dsRNA is introduced into a specific tissue or area (Jose and Hunter, 2007). In a dsRNA artificial diet feeding assay, the accumulation of Cyt3-labeled deoxycytidine triphosphate (dCTP) was initially found in the mouthparts of grain aphids. Subsequently, the labeled signal centralized in the midgut before spreading throughout the entire body when the compound was added with the synthesized dsRNA (Wang et al., 2015; Zhang et al., 2013). Combined with the fact that dsRNA-mediated gene silencing typically extends beyond the site of dsRNA delivery, the diffusion of fluorescence signal across body tissues suggests the presence of sustained systemic RNAi effects in aphid species (Yu et al., 2016). Oral delivery of dsRNA targeting the ecdysone receptor (EcR) and ultraspiracle protein (USP) markedly decreased the survival rates of aphids, impacting both the treated generation and their progeny, indicating that the silencing effect was persistent and transgenerational (Yan et al., 2016). In *C. elegans*, Sid1 is required for dsRNA transport between cells to enable systemic RNAi (Shih and Hunter, 2011). However, while systemic RNAi has been observed in various insect species, the underlying mechanisms remain largely unclear (Wynant et al., 2012). Studies in *D. melanogaster* have identified nanotube-like structures that facilitate systemic RNAi by transporting dsRNA and RNAi components between neighboring cells (Karlikow et al., 2016). Furthermore, exosome-like vesicles produced by immune cells (hemocytes) involved in immune priming have been shown to transport siRNAs, mediating systemic RNAi in response to viruses (Tassetto et al., 2017). Despite these findings, further research is needed to explore the underlying mechanisms in other insect species.

2.3 Differences in RNAi machinery components in three pathways

Genes encoding core enzymes for three RNAi pathways have been identified across numerous species across different insect orders (Cooper et al., 2019) (Table 1-1). These core elements/genes involved in RNAi pathways undergo diverse patterns of duplication and deletion across different insect species (Dowling et al., 2016). In the siRNA pathway, gene duplications have been reported for Ago2, Dcr2, and R2D2. For instance, the CPB has a duplicated *Ago2* gene, and both copies are essential for the RNAi response (Yoon et al., 2016). The enhanced RNAi efficiency observed in the red flour beetles (*Tribolium castaneum*) when compared to *D. melanogaster*, is due to the presence of extra copies of Ago2 and R2D2 in *T. castaneum*, while *D. melanogaster* possesses only a single copy of each (Lewis et al., 2016; Marques et al.,

2010). Notably, the Asian citrus psyllid (*Diaphorina citri*), despite its high RNAi sensitivity, lacks the *R2D2* gene (Taning et al., 2016). In the miRNA pathway, only a single gene copy of *Ago1* is present in mosquitoes *Anopheles gambiae* and *Culex pipiens*, but *Aedes aegypti* possesses two copies of the *Ago1* gene (Campbell et al., 2008). The pea aphid (*Acyrtosiphon pisum*) possesses two copies of *Ago1*, *Loqs*, and *Dcr1*, along with four copies of *Pasha* and one *Drosha* (Jaubert-Possamai et al., 2010). In the piRNA pathway, *A. pisum* and *Glossina morsitans* possess two to three copies of *Ago3* (Christiaens et al., 2014; Dowling et al., 2016). Besides, 2-3 copies of *Aubergine* (*Aub*) genes, belonging to the Piwi family and crucial for silencing transposons and maintaining genome stability in animal germline cells, have been reported in species such as the parasitoid wasp (*Nasonia vitripennis*) and the Indian jumping ant (*Harpegnathos saltator*) (Mongelli and Saleh, 2016). And 5-8 copies of *Piwi* genes have been identified in several insect species, including *A. pisum*, *A. aegypti*, *A. gambiae*, and the Russian wheat aphid *Diuraphis noxia* (Dowling et al., 2016; Lewis et al., 2016; Mongelli and Saleh, 2016). Moreover, in systemic RNAi, the number of *Sid1* genes varies among insect orders. Most orders possess a single *Sid1* gene, while some coleopterans have two or three copies, and dipterans lack *Sid1* genes entirely (Cappelle et al., 2016; Miyata et al., 2014; Tomoyasu et al., 2008). Duplications of core RNAi elements/genes could result in sub-functionalization or neofunctionalization within RNAi pathways, which potentially enhance RNAi efficacy in insects. In contrast, the loss of these genes may reduce RNAi efficacy in specific insect lineages (Cooper et al., 2019; Dowling et al., 2016).

Table 1-1 The so far identified key components in insects involved in RNAi

RNAi pathway	Protein	Order	Species	References
siRNA	Argonaute2	Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. (Yoon et al., 2016)
		Diptera	<i>Drosophila melanogaster</i>	Lewis et al. (Lewis et al., 2016)
		Hemiptera	<i>Nilaparvata lugens</i>	Xu et al. (Xu et al., 2013)
		Lepidoptera	<i>Bombyx mori</i>	Wan et al. (Wang et al., 2013)
	Dicer2	Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. (Yoon et al., 2016)
		Diptera	<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
		Hemiptera	<i>Bemisia tabaci</i>	Upadhyay et al. (Upadhyay et al., 2013)
			<i>Nilaparvata lugens</i>	Xu et al. (Xu et al., 2013)
	R2D2	Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. (Wynant et al., 2012)
		Coleoptera	<i>Agrilus planipennis</i>	Zhao et al. (Zhao et al., 2015)
		Diptera	<i>Drosophila melanogaster</i>	Marques et al. (Marques et al., 2010)
		Hemiptera	<i>Diaphorina citri</i>	Taning et al. (Taning et al., 2016)
	Staufen Translin/Trax		<i>Bemisia tabaci</i>	Upadhyay et al. (Upadhyay et al., 2013)
		Coleoptera	<i>Leptinotarsa decemlineata</i>	Yoon et al. (Yoon et al., 2018)
		Coleoptera	<i>Tribolium castaneum</i>	Garbutt et.al. (Garbutt and Reynolds, 2012)
miRNA	Argonaute1	Diptera	<i>Manduca sexta</i>	Garbutt et.al. (Garbutt and Reynolds, 2012)
			<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
			<i>Culex pipiens</i>	Campbell et al. (Campbell et al., 2008)
			<i>Anopheles gambiae</i>	Campbell et al. (Campbell et al., 2008)
	Dicer1	Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. (Jaubert-Possamai et al., 2010)
		Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. (Wynant et al., 2015)
		Diptera	<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
		Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. (Jaubert-Possamai et al., 2010)
			<i>Nilaparvata lugens</i>	Xu et al. (Xu et al., 2013)
		Orthoptera	<i>Schistocerca gregaria</i>	Wynant et al. (Wynant et al., 2015)
	Drosha	Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. (Jaubert-Possamai et al., 2010)

piRNA	Loquacious	Orthoptera	<i>Diaphorina citri</i>	Taning et al. (Taning et al., 2016)
		Diptera	<i>Schistocerca gregaria</i>	Wynant et al. (Wynant et al., 2015)
		Hemiptera	<i>Drosophila melanogaster</i>	Marques et al. (Marques et al., 2010)
			<i>Diaphorina citri</i>	Taning et al. (Taning et al., 2016)
	Pasha		<i>Acyrtosiphon pisum</i>	Jaubert et al. (Jaubert-Possamai et al., 2010)
		Diptera	<i>Drosophila melanogaster</i>	Denli et al. (Denli et al., 2004)
		Hemiptera	<i>Acyrtosiphon pisum</i>	Jaubert et al. (Jaubert-Possamai et al., 2010)
	Argonaute3	Diptera	<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
			<i>Glossina morsitans</i>	Dowling et al. (Dowling et al., 2016)
		Hemiptera	<i>Acyrtosiphon pisum</i>	Christiaens et al. (Christiaens et al., 2014)
			<i>Nilaparvata lugens</i>	Xu et al. (Xu et al., 2013)
	Aubergine	Lepidoptera	<i>Bombyx mori</i>	Wang et al. (Wang et al., 2013)
		Diptera	<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
		Hemiptera	<i>Diaphorina citri</i>	Taning et al. (Taning et al., 2016)
		Hymenoptera	<i>Nasonia vitripennis</i>	Mongelli et al. (Mongelli and Saleh, 2016)
Systemic RNAi	Piwi	Diptera	<i>Aedes aegypti</i>	Lewis et al. (Lewis et al., 2016)
		Hemiptera	<i>Acyrtosiphon pisum</i>	Dowling et al. (Dowling et al., 2016)
	Zucchini	Diptera	<i>Drosophila melanogaster</i>	Nishimasu et al. (Nishimasu et al., 2012)
	Sid 1	Hemiptera	<i>Aphis glycines</i>	Bansal et al. (Bansal and Michel, 2013)
			<i>Sitobion avenae</i>	Wang et al. (Wang et al., 2015)
		Coleoptera	<i>Diabrotica virgifera virgifera</i>	Miyata et al. (Miyata et al., 2014)
			<i>Leptinotarsa decemlineata</i>	Cappelle et al. (Cappelle et al., 2016)

The expression levels of RNAi pathway genes may also play a crucial role in affecting RNAi efficacy. For example, overexpression of *Dcr2* has been shown to significantly enhance RNAi efficacy in transgenic *D. melanogaster* engineered to express hairpin RNAs (Dietzl et al., 2007). Increased RNAi efficacy was observed in a Lepidoptera species, *Bombyx mori*, upon overexpression of the *Ago2* gene (Li et al., 2015). In the western corn rootworm (WCR), the RNAi response is blocked when *Ago2*, *Ago1*, and *Aub* genes are silenced (Yoon et al., 2016). Besides, many studies have documented the basal expression of core siRNA enzymes across various life stages and tissues. In *L. decemlineata*, the expression of core siRNA enzymes is highest in young larvae, coinciding with higher RNAi efficacy at that developmental stage (Guo et al., 2015; Yoon et al., 2016). The expression of core siRNA elements/genes is influenced by various factors, such as repeated exposure to dsRNA, environmental conditions, and pathogens. These factors may account for the variations in RNAi efficacy even within the same insect species (Ulvila et al., 2006).

2.4 Delivery methods of dsRNA

In the past, the major dsRNA delivery methods include microinjection, feeding, and soaking (Zhu and Palli, 2020). Microinjection involves precise injection of a small volume of dsRNA into the insect body or embryo (Joga et al., 2016). The first successful application of dsRNA microinjection to silence the frizzled and frizzled 2 genes was reported in *D. melanogaster* (Kennerdell and Carthew, 1998). While microinjection is a reliable method for functional genomic studies, it is not suitable for controlling insect pests in the field. Moreover, this approach is limited by high technical requirements and application on small or aquatic species (Nunes and Simões, 2009; Walshe et al., 2009). Another basic delivery method is orally, which insects are fed on a diet enriched with synthetic or microorganisms-expressed dsRNA (Yu et al., 2013). This approach is less labor-intensive and more practical for pest control, which is particularly effective in coleopterans (Baum et al., 2007). However, the efficacy of RNAi via oral delivery is relatively lower in many insect species. A typical example is *L. migratoria*, where RNAi is highly effective after injection but minimally effective with feeding. The main difference between the two dsRNA delivery methods is the degradation of dsRNA by double-stranded ribonucleases (dsRNases) in the insect gut (Song et al., 2019). In addition, the soaking delivery approach is commonly applied to insect cell lines by introducing dsRNA into the culture medium (Clemens et al., 2000; Johnson et al., 2010). Studies have explored the potential of topically applied dsRNA/siRNA formulations to breach the insect cuticle and cause mortality (Pridgeon et al., 2008; San Miguel and Scott, 2016).

Recently, several advanced technologies have been developed to improve delivery methods, such as cationic liposome-assisted, nanoparticle-enabled, and microbe-mediated delivery systems. Cationic liposomes serve as transfection agents, enabling the effective transfer of dsRNA into animal cells through a process called lipofection (Lechanteur et al., 2018). Positively charged cationic lipids interact with negatively charged dsRNA to form lipoplexes through electrostatic forces, which is widely used for dsRNA delivery in insect cell cultures (Zuhorn et al., 2007). In addition, combining RNAi with nanotechnology presents an alternative innovative methodology for pest control, as recently reviewed (Xing et al.). In a pioneering study on nanoparticle-mediated dsRNA delivery, chitosan nanoparticles were used to silence chitin synthase

gene in *A. gambiae*, resulting in increased RNAi efficacy (Zhang et al., 2010). To date, chitosan along with other dsRNA nanoparticles, have been used to enhance RNAi efficacy in various insect species (Ramesh Kumar et al., 2016; Zhang et al., 2015b). Moreover, microbe-based dsRNA delivery, including the symbiont-mediated and pathogen-mediated RNAi, has emerged as a promising strategy. Symbiont-mediated RNAi exploits the symbiotic bacteria to continuously produce dsRNA, inducing RNAi in the insect hosts. Researchers have developed RNase III-deficient bacterial strains derived from the symbionts of the kissing bug (*Rhodnius prolixus*) and the western flower thrips (WFT, *Frankliniella occidentalis*), enabling stable generation of specific dsRNA molecules that effectively suppress targeted insect genes (Whitten et al., 2016). In addition, pathogen-mediated RNAi involves engineering entomopathogenic fungi to express miRNAs targeting host immune genes, dramatically increasing fungal virulence against *Aedes aegypti* and *Galleria mellonella* (Cui et al., 2022). Collectively, these microbial-based RNAi delivery methods represent alternative promising tools for effective and sustainable insect pest management.

3 Applications of RNAi for insect control in crop protection

The application of RNAi in agricultural pest control is mainly divided into three categories: host-induced gene silencing (HIGS), virus-induced gene silencing (VIGS), and spray-induced gene silencing (SIGS) (Figure 1-2; Table 1-2). HIGS was first demonstrated by enabling transgenic crops to produce pathogen-targeting dsRNA, thereby silencing fungal virulence genes during infection (Nowara et al., 2010). The first proof-of-concept of HIGS for insect pest control was demonstrated in WCR. Transgenic maize plants expressing hairpin RNA (hpRNA) targeting the *snf7* gene in WCR were shown to protect the plants from larval feeding damage (Bolognesi et al., 2012). Subsequently, HIGS was employed to protect a variety of crops against insect pests. For instance, HIGS targeting *CYP6AE14*, a cytochrome P450 gene, was effective against the cotton bollworm *Helicoverpa armigera* in transgenic cotton (Mao et al., 2011). Expression of dsRNA homologous to the *shp* gene in transgenic barley significantly reduced the reproduction and survival rates of grain aphids (*Sitobion avenae*) (Abdellatef et al., 2015). Transgenic cotton expressing dsRNA targeting *FATTY ACYL-COA REDUCTASE (AsFAR)* showed reduced predation by cotton aphids (*Adelphocoris suturalis*), and diminished insect fertility (Luo et al., 2017). Transgenic wheat expressing the dsRNA targeting a zinc finger protein encoding gene *SaZFP* and the salivary protein gene *SmDSR33*, respectively, led to increased mortality and reduced fecundity of aphids (Sun et al., 2019; Zhang et al., 2023a). Moreover, dsRNA can be expressed at high levels in plant plastids that lack RNAi mechanisms. This transplastomic approach has been successful in protecting plants from chewing insects like the CPB, the WFT, and willow leaf beetle *Plagiodera versicolora* (Wu et al., 2022; Zhang et al., 2015a), as well as sap-sucking insects like the green peach aphid *Myzus persicae* (Dong et al., 2022). For example, transplastomic poplar that express dsRNAs targeting the β -Actin and *Srp54k* genes of *P. versicolora* show significantly higher larval mortality compared to the nuclear transgenic plants (Zhang et al., 2024).

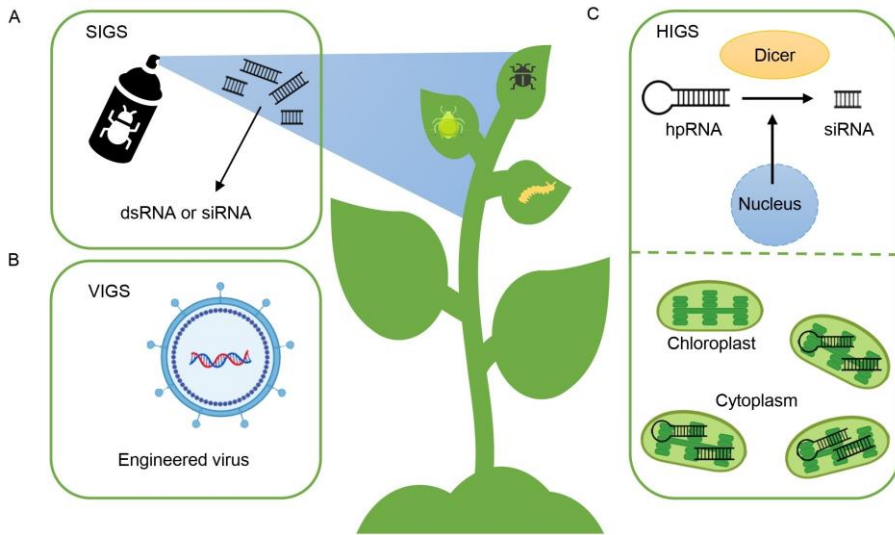


Figure 1-2. Diverse methods for RNAi application.

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

Table 1-2 Insect control in crops via RNAi using HIGS, VIGS, and SIGS methods

Application methods	Crop	Insect species	Target genes	references
HIGS	Barley (<i>Hordeum vulgare</i>)	<i>Sitobion avenae</i>	<i>Shp</i>	Abdellatef et al. (Abdellatef et al., 2015)
	Cotton (<i>Gossypium hirsutum</i>)	<i>Adelphocoris suturalis</i>	<i>fatty acyl-coenzyme A reductase;</i> <i>hormone receptor 3</i>	Luo et al. (Luo et al., 2017); Han et al. (Han et al., 2017)
	Maize (<i>Zea mays</i>)	<i>Helicoverpa armigera</i>	<i>CYP6AE14</i>	Mao et al. (Mao et al., 2011)
		<i>Apolygus lucorum</i>	<i>AlucV-ATPase-E</i>	Liu et al. (Liu et al., 2019)
		<i>Chilo partellus</i>	<i>Chitinase</i>	Adeyinka et al. (Adeyinka et al., 2023)
		<i>Diabrotica virgifera virgifera</i>	<i>vitellogenin receptor, boule;</i> <i>SSJ1;</i> <i>Snf7;</i> <i>wings up A</i>	Niu et al. (Niu et al., 2017); Hu et al. (Hu et al., 2020); Bolognesi et al. (Bolognesi et al., 2012); Fishilevich et al. (Fishilevich et al., 2019)
	Potato (<i>Solanum tuberosum</i>)	<i>Diabrotica virgifera virgifera</i>	<i>ecdysone receptor</i>	Hussain et al. (Hussain et al., 2019)
		<i>Halyomorpha halys,</i> <i>Phenacoccus madeirensis</i>	<i>v-ATPase</i>	Kaplanoglu et al. (Kaplanoglu et al., 2022)
		<i>Henosepilachna vigintioctopunctata,</i> <i>Myzus persicae,</i> <i>Spodoptera litura</i>	<i>β-actin</i>	Ren et al. (Ren et al., 2021)
		<i>Leptinotarsa decemlineata</i>	<i>cuticular protein, glutathione synthetase, cytochrome P450 monooxygenases;</i> <i>v-ATPase;</i>	Naqqash et al. (Naqqash et al., 2020); Kaplanoglu et al. (Kaplanoglu et al., 2022); Hussain et al. (Hussain et al., 2019)

Rice (<i>Oryza sativa</i>)	<i>Myzus persicae</i>	<i>Ecdysone receptor macrophage inhibitory factor</i>	Murtaza et al. (Murtaza et al., 2022)
	<i>Chilo suppressalis</i>	<i>Krüppel homologue 1; heat shock protein 19; fatty acyl-coenzyme A reductase; disembodied; Spook and Ecdysone receptor; small heat shock protein gene</i>	Tang et al. (Tang et al., 2024); Wu et al. (Wu et al., 2023b); Sun et al. (Sun et al., 2022); Zheng et al. (Zheng et al., 2021); He et al. (He et al., 2019); Mao et al. (Mao et al., 2022)
Soybean (<i>Glycine max</i>)	<i>Nilaparvata lugens</i>	<i>glutathione S- transferas</i>	Yang et al. (Yang et al., 2021)
	<i>Apolygus lucorum</i>	<i>AlucV-ATPase-E</i>	Liu et al. (Liu et al., 2019)
	<i>Leguminivora glycinivorella</i>	<i>SpbP0</i>	Meng et al. (Meng et al., 2017)
Tobacco (<i>Nicotiana tabacum</i>)	<i>Tetranychus urticae</i>	<i>coatomer subunit α, aquaporin 9</i>	Dubey et al. (Dubey et al., 2017)
	<i>Bemisia tabaci</i>	<i>Acetylcholinesteras e; ecdysone receptor, aquaporin, α- glucosidase; β-actin</i>	Malik et al. (Malik et al., 2016); Raza et al. (Raza et al., 2016); Dong et al. (Dong et al., 2020)
	<i>Chilo partellus, Maruca vitrata, Spodoptera litura</i>	<i>chitin synthase A</i>	Rana et al. (Rana et al., 2020)
	<i>Frankliniella occidentalis</i>	<i>β-actin, Tubulin v- TPase B, Snf7</i>	Wu et al. (Wu et al., 2022)
	<i>Helicoverpa armigera</i>	<i>Chitinase acetylcholinesteras e 1</i>	Mamta et al. (Mamta et al., 2016) Saini et al. (Saini et al., 2018)

	Tomato (<i>Solanum lycopersicum</i>)	<i>Leptinotarsa decemlineata</i>	β -actin, Shrub	Zhang et al. (Zhang et al., 2015a)
		<i>Manduca sexta</i>	CYP6B46	Kumar et al. (Kumar et al., 2012)
		<i>Myzus persicae</i>	v-ATPaseA	Poreddy et al. (Poreddy et al., 2017)
			CYP82E4v1;	Zhao et al. (Zhao et al., 2016);
			cathepsin L;	Rauf et al. (Rauf et al., 2019);
		<i>Bemisia tabaci</i>	Dhc64C	Dong et al. (Dong et al., 2022)
			Phenolic glucoside malonyltransferase 1;	Xia et al. (Xia et al., 2021);
			vacuolar ATP synthase catalytic subunit A	Pizetta et al. (Pizetta et al., 2022)
		<i>Frankliniella occidentalis</i>	coatamer protein subunit epsilon	Venkatesh et al. (Venkatesh et al., 2023)
		<i>Helicoverpa armigera</i>	Chitinase;	Mamta et al. (Mamta et al., 2016);
VIGS	Wheat (<i>Triticum aestivum</i>)	<i>Tetranychus evansi</i>	ecdysone receptor	Yogindran et al. (Yogindran and Rajam, 2021)
		<i>Schizaphis graminum</i>	β -Actin	Wu et al. (Wu et al., 2023a)
		<i>Sitobion avenae</i>	Sg2204	Zhang et al. (Zhang et al., 2022)
			lmf2-like gene;	Xu et al. (Xu et al., 2017);
			Chitinase;	Zhao et al. (Zhao et al., 2018);
		<i>Sitobion miscanthi</i>	Gqa;	Hou et al. (Hou et al., 2019);
			ZFP	Sun et al. (Sun et al., 2019)
			DSR33	Zhang et al. (Zhang et al., 2023a)
	Cotton (<i>Gossypium hirsutum</i>)	<i>Aphis gossypii</i>	callose synthase 5	Mbiza et al. (Mbiza et al., 2022)
		<i>Helicoverpa armigera</i>	peptidyl prolyl trans-isomerase 5	Wang et al. (Wang et al., 2024b)
VIGS	Maize (<i>Zea mays</i>)	<i>Ostrinia furnacalis</i>	EXP-A20	Batool et al. (Batool et al., 2025)
	Tobacco (<i>Nicotiana attenuate</i>)	<i>Manduca sexta</i>	CYP6B46	Kumar et al. (Kumar et al., 2012)
	Tobacco	<i>Diaphorina citri</i>	phytoene	Hajeri et al. (Hajeri et al., 2014)

	<i>(Nicotiana benthamiana)</i>		<i>desaturase, Awd</i>	
		<i>Planococcus citri</i>	<i>Actin, chitin synthase 1, V-ATPase</i>	Khan et al. (Khan et al., 2013)
		<i>Tetranychus evansi</i>	<i>protein disulfide isomerase, APX3-1</i>	Cui et al. (Cui et al., 2023)
	Tobacco <i>(Nicotiana tabacum)</i>	<i>Phenacoccus solenopsis</i>	<i>Bursicon, V-ATPase</i>	Khan et al. (Khan et al., 2018)
	Wheat <i>(Triticum aestivum)</i>	<i>Sitobion miscanthi</i>	<i>chemosensory protein 4</i>	Zhang et al. (Zhang et al., 2023b)
	Barley <i>(Hordeum vulgare)</i>	<i>Sitobion avenae</i>	<i>Shp; migration inhibitory factors</i>	Biedenkopf et al. (Biedenkopf et al., 2020); Liu et al. (Liu et al., 2021)
	Potato <i>(Solanum tuberosum)</i>	<i>Leptinotarsa decemlineata</i>	<i>Actin</i>	San et al. (San Miguel and Scott, 2016)
SIGS	Soybean <i>(Glycine max)</i>	<i>Aphis glycines</i>	<i>TREH, ATPD, ATPE and CHS1</i>	Yan et al. (Yan et al., 2020)
	Tomato <i>(Solanum lycopersicon)</i>	<i>Myzus persicae, Tetranychus urticae, Trialeurodes vaporariorum,</i>	<i>helper component-proteinase</i>	Gogoi et al. (Gogoi et al., 2017)
	Wheat <i>(Triticum aestivum)</i>	<i>Schizaphis graminum</i>	<i>Sg2204</i>	Zhang et al. (Zhang et al., 2022)

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

VIGS is an RNA-based reverse genetics technology that has been developed to produce insect-specific dsRNAs in plants for pest management. For example, tobacco with Tobacco rattle virus (TRV) targeting the hornworm *Manduca sexta* Cytochrome P450 gene *CYP6B46* made *M. sexta* more susceptible to nicotine damage (Kumar et al., 2012). Similarly, recombinant Tobacco mosaic virus (TMV) targeting the citrus mealybug (*Planococcus citri*) in tobacco resulted in reduced fecundity and increased mortality of *P. citri* (Khan et al., 2013). In addition to phytovirus, VIGS can also directly use recombinant viruses to infect insects, inducing RNAi-mediated gene silencing in insect cells (Kolliopoulou et al., 2017). The recombinant Citrus tristeza virus (CTV)-RNAi vector, engineered with the *abnormal wing disc* (*Awd*) gene of Asian citrus psyllid (*D. citri*), resulted in malformed-wing phenotype in adults and increased mortality (Hajeri et al., 2014).

SIGS is a non-transgenic method in which dsRNAs or sRNAs targeting pest and pathogen genes are sprayed directly onto plants. These RNAs are then absorbed by the pest and pathogen, inducing the silence of target genes (Qiao et al., 2021; Wang et al., 2024a). SIGS can also be applied through root absorption and trunk injection, enabling insects to ingest dsRNA via sucking or chewing (Joga et al., 2016). The first study demonstrating SIGS was using targeting the acetylcholine esterase gene of the diamondback moth (*P. xylostella*). The second-instar larvae fed on treated leaves showed a mortality rate of 89% (Gong et al., 2013). Another study used a sprayable dsRNA formulation against the CPB. Foliar application of naked dsRNA targeting the actin gene was sufficiently stable under greenhouse conditions for at least 28 days, resulting in effective insect control (San Miguel and Scott, 2016). Moreover, SIGS targeting the salivary effector Sg2204 in the greenbug (*Schizaphis graminum*) led to induced plant defense responses, altered feeding behavior and decreased aphid survival (Zhang et al., 2022).

4 Advantage variability of RNAi-based insect control

While the application of RNAi holds great promise in insect control, field use will require careful consideration of advantages and inconvenients. HIGS shows immense potential for controlling species within the order Hemiptera. Many hemipteran insects, such as aphids, are economically significant agricultural pests, making HIGS a promising strategy for pest management (Cao et al., 2018). Furthermore, the transplastomic approach can overcome the limitations of nuclear transformation in which most of the dsRNAs are processed into siRNAs by Dicer enzymes, while these are absent in plastids. The transplastomic resistant crops do not transmit their genetic material via pollen, thereby benefit ecological sustainability (Li et al., 2022). However, due to the limitation of plastid transformation technology, only a few plant species can be subjected to this method. Moreover, genetically modified plants expressing dsRNAs may take many years to develop and require millions of dollars to commercialize due to the stringent regulations. Also, the general lack of public acceptance toward genetically modified crops is a significant barrier mainly in Europe, delaying the broader application of HIGS in agriculture (Zhao et al., 2024). However, due to its more effectiveness, HIGS has been approved for commercial use. In 2023, the transgenic corn variety SmartStax® Pro was launched in the U.S. market. This variety expresses the dsRNA targeting the *snf7* gene in the WCR and is combined with

two *Bacillus thuringiensis* (Bt) Cry proteins (Cry3Bb1 and Cry34/35Ab) to delay the development of resistance to Bt proteins. In 2024, the U.S. Environmental Protection Agency (EPA) gave the green light to DP23211, an RNAi-based maize product that is both herbicide-tolerant and insect-resistant. This genetically modified maize contains DvSSJ1 dsRNA and the IPD072Aa protein, which are specifically designed to combat corn rootworm (*Diabrotica* spp.) infestations. Additionally, the application of HIGS goes beyond pest control. For instance, Bayer's "Vistive Gold" high-oleic soybeans (Mon87705), targeting a gene in the fatty acid biosynthesis pathway, have been approved for food, feed, and cultivation in the USA, Canada, and Japan, respectively (De Schutter et al., 2022).

The advantage of VIGS lies in its high efficiency even in refractory cells, because viruses can naturally penetrate cells, bypassing the obstacles to dsRNA uptake in insects. Additionally, the host specificity of viruses ensures that dsRNA plays a role in the intended target species, reducing off-target effects and minimizing potential ecological disruptions (Christiaens et al., 2020). However, the use of recombinant viruses for dsRNA delivery may be constrained by virally encoded RNAi inhibitors, which can modulate the silencing effect. Furthermore, the ecological consequences of releasing recombinant viruses into the environment must be carefully evaluated to avoid unintended impacts on non-target organisms and ecosystems (Kolliopoulou et al., 2017).

The application of SIGS minimizes the risk of toxicity to animals following ingestion or exposure. In addition, the topical application of dsRNA is unlikely to pose an environmental hazard, as dsRNAs degrade rapidly in soil and water (Fischer et al., 2017). Moreover, the instability of dsRNA is also a challenge for SIGS application in agriculture, which reduces its effectiveness compared to genetically modified HIGS plants that produce dsRNA continuously. Therefore, SIGS may require specialized formulations to enhance dsRNA stability and improve RNAi efficacy in target insects. Foliar application may be more effective against insects that are particularly sensitive to dietary dsRNA intake (Christiaens et al., 2020). Compared to chewing insects, delivering dsRNA through SIGS to piercing-sucking insects belong to Hemiptera such as aphids remains challenging. These insects consume minimal plant tissue while feeding on phloem sap, making foliar application of dsRNA unlikely to elicit an effective RNAi response (Liu et al., 2020). Microinjection is an effective method for delivering dsRNA into various insect orders, including Hemiptera (Scott et al., 2013). However, it is impractical as a strategy for crop protection in the field. A promising SIGS application is the sprayable biopesticide Ledprona, which contains long dsRNAs that target the *psmb5* gene of CPB (Rodrigues et al., 2021). This product is approved for commercialization by EPA in 2023, marking a significant step toward the commercial use of SIGS in pest management.

5 Strategies for improving the RNAi effectiveness in pest control

Numerous strategies have been devised to meet the challenges that hinder the effectiveness of RNAi and its application in insect pest control (Figure 1-3). One is using chemically modified molecules to enhance dsRNA delivery, which can significantly increase RNAi efficacy (Kurreck, 2003). For instance, siRNAs modified

with two 2'-methoxyl-nucleotides at both terminuses have shown effective silencing in the diamondback moth (*P. xylostella*) (Gong et al., 2013; Gong et al., 2011). Studies in vertebrate RNAi indicates that chemical modifications can also reduce off-target effects of short siRNAs (Jackson et al., 2006), indicating this approach is a potential more promising strategy for pest control. In addition, combining RNAi with other pest management strategies, such as Bt toxins, biological agents and chemical treatments, is a more comprehensive approach for insect control. For example, transgenic cotton that integrates Bt toxins with RNAi targeting insect *JHMT* and *JHBP* genes has shown promise in delaying resistance development to Bt toxins in pests (Ni et al., 2017). RNAi targeting the *IAP1* gene of *L. migratoria* combined with entomopathogenic fungi *Metharhizium acridum*, resulted in a lethal effect in *L. migratoria* and made them more susceptible to *M. acridum* infection (Zhang et al., 2020). The chemical treatments ethylenediaminetetraacetic acid (EDTA), which can inhibit metalloenzymes and enhance dsRNA stability, has been used as a co-formulant to enhance RNAi effectiveness in the Neotropical stinkbug (*Euschistus heros*) (Castellanos et al., 2019).

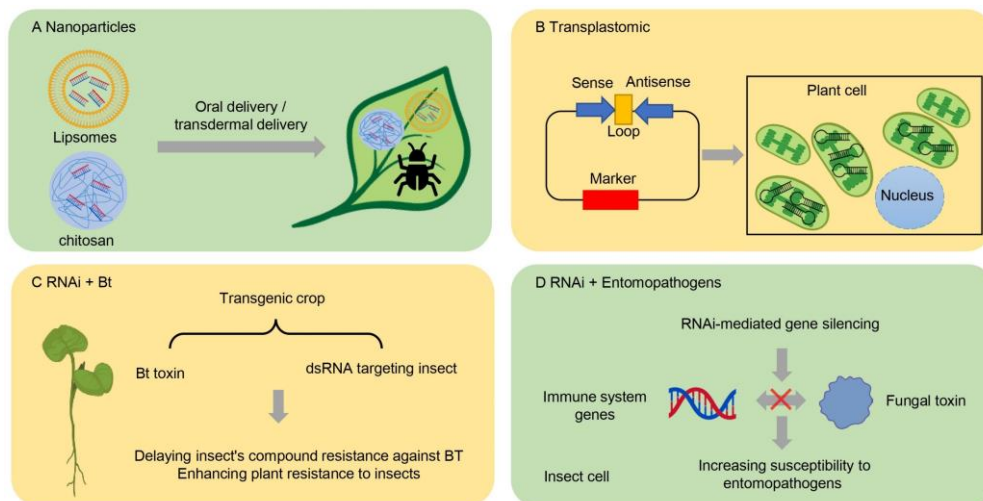


Figure 1-3. Improved strategies using RNAi for insect control.

(A) Synthetic nanoparticles can be employed to enhance dsRNA delivery by insect feeding or spraying. (B) Transplastomic plants effectively enhance the accumulation of long dsRNA in the encapsulated chloroplast. (C) Delaying insect's compound resistance against *Bacillus thuringiensis* (Bt) toxins. (D) Enhancing plant resistance to insects through the combination of RNAi and entomopathogenic fungi.

Recently, several new delivery strategies of dsRNA in insects have been reported, offering promising alternatives to enhance RNAi efficacy for fundamental research and pest control applications. For example, viral-like particles (VLPs), which can be produced in micro-organisms and have the ability to self-assemble *in vitro*, allowing the production of the dsRNA inside the particle (Hoffmann et al., 2016). VLPs are

considered biologically safe due to the lack of reproducing viral genetic material. VLPs were already described in several insect viruses (Xue et al., 2023). Novel VLPs derived from the *Drosophila* X virus (DXV) enable delivery of dsRNA both *in vitro* and *in vivo* in insects (Xue et al., 2024). The finding suggests that insect virus-based VLPs can serve as nano-vehicles for dsRNA delivery. Furthermore, combining the transplastomic RNAi technology with a bacteriophage MS2 VLP-based RNAi delivery system could enhance plant resistance to cotton bollworm (Jiang et al., 2024). This approach effectively controlled lepidopteran pests through both bacteria- and plastid-mediated RNAi. In addition, a study pioneered the use of bacteria-expressed and nanocarrier-delivered RNA pesticides for pest control in a greenhouse trial (Ma et al., 2023). In this study, the V-type proton ATPase subunit d (ATP-d) and ATP-G were selected as the RNAi target genes, and the hairpin RNAs (hpRNAs) targeting these two genes were fused together to be simultaneously expressed in *E. coli* BL21(DE3). Through spray application, the RNA pesticide achieved a promising control effect on *M. persicae* in the greenhouse. Besides, encapsulating dsRNA within polymeric nanocarriers represents a promising strategy. Polymeric systems such as polycations, chitosan, guanylated polymers (GNPs), and star-polycation (SPc) have shown potential in protecting dsRNA from degradation, enhancing cellular uptake, and ensuring controlled and precise release (Quilez-Molina et al., 2024). Examples include the BioClay nanocarrier platform, which uses the molecular complexation with layered double hydroxides (LDHs) to enhance the environmental stability and biocidal efficacy of topically applied dsRNA on phylloplane surfaces (Jain et al., 2022). Another study demonstrates that guanidinium-containing disulfide assembled sRNA nanoparticles (Gu⁺-sRNA NPs) facilitate the long-distance translocation of siRNAs through the vascular system of plants, which is essential for enhancing whole-plant resistance to insect pests (Lin et al., 2025). Furthermore, to enhance dsRNA delivery and improve RNAi efficacy in lepidopterans, a study used a fusion protein combining *Galanthus nivalis* agglutinin (GNA) and a dsRNA binding domain (dsRBD) to create a lectin-based dsRNA delivery system (Martinez et al., 2021). GNA associated dsRBD increased dsRNA uptake and transfection efficiency in lepidopteran midgut cells, leading to significant RNAi enhancement.

6 Conclusions and perspectives

The application of RNAi for insect control heralds a new era in ecologically sustainable agriculture development. Some barriers need to be got over before the application of RNAi technology for pest control becomes a routine practice in the field. For example, the mechanisms of dsRNA uptake and transport, the systemic RNAi and different RNAi responses in insects remains largely unknown. Besides, developing strategies that can delay or even prevent the occurrence of insect resistance would be very important for the RNAi application. A combined approach involving gene pyramiding can be employed. For instance, combining genes with distinct functions, such as Bt toxins and dsRNAs targeting RNAi pathways, could effectively control lepidopteran, coleopteran, and hemipteran pest species simultaneously (Yu Xiu et al., 2016). Moreover, the synergistic effects of targeting multiple essential genes can be effective in enhancing the ability of RNAi to control aphids (Dong et al., 2024). Furthermore, according to the behaviors of the pests, dsRNAs can be designed for

developmental stage- or tissue-specific expression with specific promoters in RNAi plants. Aphid feeding behavior indicates that phloem-specific promoters are particularly useful as they drive high expression of exogenous genes in the phloem, which can strengthen the resistance of RNAi plants against phloem-feeding aphids by increasing defensive dsRNAs in the phloem sap while minimizing the exposure of non-target insects to these compounds (Yu et al., 2014). At last, it is foreseeable that insect-specific RNAi-based biopesticides hold significant potential for wide applications in pest control in agricultural practice, avoiding societal and public concerns about chemical pesticides and the widespread reluctance toward GMOs. However, the productions of cost-effective dsRNA pesticides remain a major challenge. Current dsRNA synthesis methods mainly include *in vitro* transcription (IVT), microbial expression in bacteria or fungi, and cell-free synthesis (Kim and Zhang, 2022). Each method has trade-offs regarding scalability, cost, and complexity. For instance, while IVT kits allow small-scale dsRNA synthesis for initial RNAi screening, their high cost and low yield make them impractical for field use (Ahn et al., 2019). In contrast, microbial expression systems, such as the L4440-HT115 (DE3) strain, offer a sustainable and scalable alternative (Ma et al., 2020). Recent advancements in microbial expression system include optimized strains, genetic engineering, improved expression vectors, fermentation techniques, and dsRNA extraction methods (Abbasi et al., 2020; Ahn et al., 2019; Figueiredo Prates et al., 2023; Hashiro et al., 2021; Islam et al., 2021). Additionally, cell-free systems could eliminate the need for cell viability, simplifying the dsRNA production process (Carlson et al., 2012; Garamella et al., 2016). Recently, GreenLight Biosciences has recently reported the utilization of cell-free transcription to produce dsRNA on a large scale, effectively reducing production costs (<https://www.greenlightbiosciences.com/in-the-pipeline-colorado-potato-beetle/>).

Nevertheless, it is expected the RNAi crops, especially the insect-specific RNAi-based biopesticides will have significant potential for wide applications in pest control in agricultural practice on a global scale to ensure food security and sustainable agricultural development.

7 References

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Chapter II

Research background, objectives and outline

1 Research background

Since its discovery, RNAi has been harnessed as a powerful tool for functional genomics and pest management (Chaudhary et al., 2024; Kumar et al., 2025; Mahanta et al., 2025). Among various delivery strategies, HIGS has emerged as a particularly promising approach for agricultural pest control. In this strategy, transgenic plants can be engineered to express dsRNA targeting genes that are essential for the survival, development, or reproduction of insect pests. When the pest feeds on the transgenic plant, it ingests the dsRNA and experiences knockdown of the targeted gene (Mahanta et al., 2025). This approach offers remarkable species-specificity, minimizing harm to beneficial insects and pollinators, and it aligns well with integrated pest management (IPM) by reducing reliance on chemical sprays (Guan et al., 2025; Zhang et al., 2025). However, successful translation of HIGS from laboratory models to commercial agriculture requires careful target selection and a deep understanding of the pest's physiology and feeding behavior (Thanvarshni et al., 2026).

Aphids constitute one of the most destructive pest groups affecting cereal crops worldwide. Their phloem-feeding activity not only drains nutrients and causes leaf chlorosis but also transmits devastating viral diseases, leading to yield losses (Khan and Naveed, 2022; Sarwar, 2020). Climate change is further expanding the geographical range and activity periods of these vectors, exacerbating the threat (Ryalls and Harrington, 2017; Wu et al., 2020). Current control relies heavily on synthetic insecticides, but the rapid evolution of resistance, together with environmental contamination and harm to natural enemies, underscores an urgent need for sustainable alternatives (Bass and Nauen, 2023). HIGS offers a species-specific and environmentally benign solution. Among the key factors determining its success is the choice of target genes, ideally those that are essential for pest survival and highly conserved across species (Cedden and Bucher, 2025).

Aphids rely heavily on endosymbionts, particularly the primary symbiont, *Buchnera aphidicola*, to fulfil the nutritional gap by providing essential amino acids, nutrients that are normally deficient in phloem sap (Akman Gündüz and Douglas, 2009). This ancient association, which dates back 160 to 280 million years, has made *Buchnera* an irreplaceable partner for aphid survival and reproduction (Baumann, 2005; Moran, 2021). Evidence from several investigations has demonstrated the potential of indirect pest suppression through interference with insect-symbiont interactions (Chaudhary et al., 2014; Chung et al., 2018; Leonard et al., 2020; Li et al., 2022; Xu et al., 2021). Such observations highlight that destabilizing the obligate association between aphids and their primary symbiont could offer a viable route for biological control. However, whether plant-mediated RNAi can be effectively deployed to silence aphid genes that underpin this mutualism is still an open question.

Aphids feed on phloem sap by using their specialized piercing-sucking stylets to penetrate host plants (Shih et al., 2023). During feeding, aphids secrete saliva that contains a suite of effector molecules capable of modulating host plant defense responses. These effectors are essential for aphids to overcome plant immunity and establish successful infestations (Mondal, 2020). Studies suggest that aphid salivary protein genes are promising targets for plant-mediated RNAi-based aphid control (Wang et al., 2015; Zhang et al., 2023; Zhang et al., 2022a; Zhang et al., 2022b). These studies collectively establish salivary effectors as ideal RNAi targets. The value of

targeting salivary effectors lies in their direct contribution to virulence: gene silencing does not act as a direct toxin but rather reduces the aphid's capacity to manipulate the plant, thereby enabling the host's natural defenses to function effectively (Huang et al., 2026; Shang et al., 2026; Xu et al., 2019).

In summary, the obligate symbiosis with *B. aphidicola* makes aphids nutritionally dependent and thus highly vulnerable. When RNAi interferes with this mutualism, aphids lose access to essential amino acids, which causes a marked reduction in their overall fitness. This penalty is not easily bypassed through resistance evolution. Simultaneously, salivary effector proteins serve as the primary tools through which aphids overcome plant immunity. Silencing the corresponding genes impairs the pest's capacity to interfere with host defenses, thereby allowing the plant to mount a more robust defense reaction. Critically, the two target categories are functionally conserved across a range of cereal aphids, which offers a viable strategy against the mixed infestations commonly seen in wheat. Yet, despite these promising prospects, the application of plant-mediated RNAi targeting these aphid genes in wheat has so far been scarcely investigated.

2 Research objectives

This study aims to identify and validate candidate genes from aphid symbiont-related and salivary effector categories that can serve as effective targets for plant-mediated RNAi-based wheat protection. The specific objectives are as follows:

(1) To screen transcriptomic datasets of grain aphid (*S. miscanthi*) for candidate genes involved in symbiont and salivary effector function (*Smrrl* and *SmDSR32*), and to validate their silencing efficiency through artificial-diet assays.

(2) To construct hairpin RNAi expression vectors targeting *Smrrl* and *SmDSR32*, and to generate stable transgenic wheat lines via particle bombardment transformation.

(3) To evaluate the aphid resistance performance of the generated transgenic lines through comprehensive bioassays measuring aphid fecundity, survival, and feeding behavior.

3 Research outline

3.1 Research roadmap

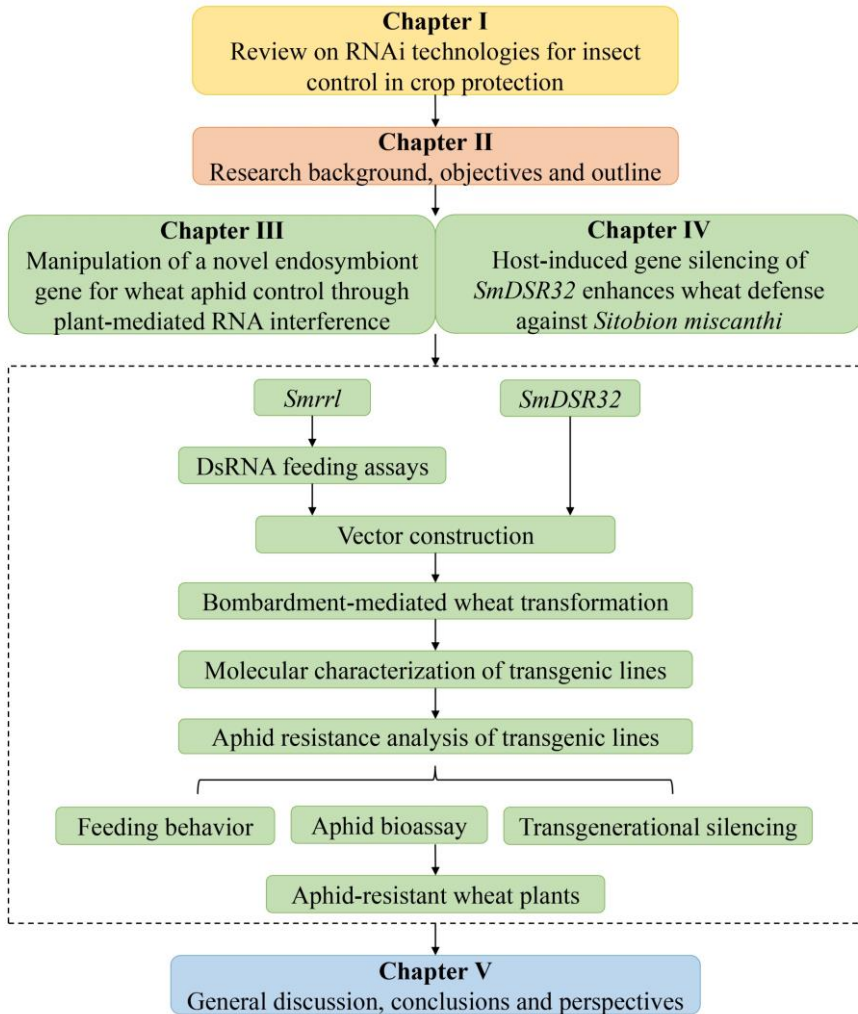


Figure 2-1. The research roadmap of thesis.

3.2 Thesis outline

Chapter I reviews RNAi technologies for insect control in crop protection.

Reference: Xue Zhong[#], Xiudao Yu[#], Jiahui Zhang[#], Jiajing Xu, Mengchao Qin, Mingxin Cao, and Frédéric Francis and Lanqin Xia, RNAi technologies for insect control in crop protection. *The Crop Journal*, 2025.

Chapter II provides the research background on plant-mediated RNAi, defines the study objectives, and outlines the research roadmap.

Chapter III develops aphid-resistant wheat plants through RNA interference of an

endosymbiont gene *Smrrl*.

Reference: Xue Zhong[#], Yongwei Sun[#], Jiajing Xu, Mengchao Qin, Mingxin Cao, and Frédéric Francis and Lanqin Xia, Manipulation of a novel endosymbiont gene for wheat aphid control through plant-mediated RNA interference (Under review).

Chapter IV develops aphid-resistant wheat plants through RNA interference of a key putative salivary peptide encoding gene *SmDSR32*.

Reference: Jiahui Zhang[#], Xue Zhong[#], Mingxin Cao, Jiajing Xu, Mengchao Qin, and Frédéric Francis and Lanqin Xia, Host-induced gene silencing of *SmDSR32* enhances wheat defense against *Sitobion miscanthi*. *Current Issues in Molecular Biology*, 2026.

Chapter V offers a summary and discussion of the major findings. It further outlines the research implications, acknowledges the limitations, and suggests directions for subsequent investigations.

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Chapter III

**Manipulation of a novel endosymbiont gene
for wheat aphid control through plant-
mediated RNA interference**

Abstract

Aphids (Aphididae) are major agricultural pests, relying on an obligate mutualism with the endosymbiotic bacterium *Buchnera aphidicola* for essential nutrients. Disrupting this symbiont's function represents a promising way for crop aphid control. Nevertheless, this has not been documented in plants yet.

We identified and characterized *Smrrl*, a 23S rRNA gene from *B. aphidicola* which upregulated during aphid feeding on wheat. Silencing *Smrrl* via artificial diet or stable transgenic wheat lines triggered high aphid mortality, reaching 51% and 80% respectively. In aphids feeding on RNAi plants, *Smrrl* transcript levels were reduced by 2.5- to 4-fold compared to wild-type controls, leading to a 1.6-fold prolongation of probing and a 31% reduction in sustained phloem ingestion as measured by electrical penetration graph (EPG). This resulted in significantly reduced aphid fecundity and population growth, including a 10-day shortening of lifespan and a reduction of 43 to 48 individuals in progeny production. This silencing effect persisted for up to three generations in offspring.

Our findings identify a novel RNAi target and provide a proof-of-concept framework for plant-mediated RNAi against genes in aphid symbionts, offering an alternative and effective strategy in crop aphid management.

Keywords: Wheat (*Triticum aestivum* L.), Grain aphid (*Sitobion miscanthi* F.), RNA interference (RNAi), *Buchnera aphidicola*, Pest management

1 Introduction

Aphids are major global agricultural pests, causing substantial economic losses across various crop species through phloem-feeding damage and transmission of plant pathogenic viruses (Gadhave et al., 2020; Shih et al., 2023). Although chemical insecticides remain as primary and effective strategy for pest management, their continuous and excessive applications have led to the development of resistance in aphid populations, reduced field efficacy and increasing environmental concerns (Nicolopoulou Stamati et al., 2016; Sanahuja et al., 2011; Tang et al., 2021; Tang et al., 2017). In recent years, plant-mediated RNA interference (RNAi) targeting essential aphid genes has emerged as a promising, environmentally friendly alternative to conventional insecticides (Guo et al., 2025; Xu et al., 2026; Zhang et al., 2024). The RNAi process is triggered upon insect ingestion of double strand RNA (dsRNA), which must be taken up by cells and processed by the RNAi machinery to specifically downregulates target gene transcripts, ultimately inhibiting pest development or causing mortality (Mahanta et al., 2025).

Aphids harbor various endosymbionts, particularly the obligate γ -proteobacterium *Buchnera aphidicola*, which has co-evolved with inseparably for 160-280 million years (Baumann, 2005; Moran, 2021). This endosymbiont can provide essential amino acids which are (Akman Gündüz and Douglas, 2009), influence aphid heat tolerance (Zhang et al., 2019), support aphid developmental differentiation (Perreau et al., 2021), and has been indirectly associated with insecticide resistance (Guo et al., 2020). The potentials of indirect insect control via manipulating the insect-symbiont mutualistic relations have been demonstrated in several studies (Chaudhary et al., 2014; Chung et al., 2018; Leonard et al., 2020; Li et al., 2022a; Xu et al., 2021). For example, heterologous expression of *GroEL* and *GroES* genes derived from *B. aphidicola* in plants can trigger plant defense responses and enhance plant resistance against aphids (Chaudhary et al., 2014; Li et al., 2022a). The honeybee gut symbiont *Snodgrassella alvi* was genetically engineered to continuously produce targeted dsRNA which activated RNAi defenses in bees, and then effectively suppressing both viral infections and ectoparasitic mite infestations (Leonard et al., 2020). Furthermore, oral of dsRNA targeting *amiD* and *ldcA1* genes from *B. aphidicola* in pea aphid (*Acyrtosiphon pisum*) led to a significant reduction in *Buchnera* abundance and activity, along with decreased aphid growth rates in an *in vivo* dsRNA assay (Chung et al., 2018). These findings highlight disrupting the aphid-symbiont coupling as an alternative aphid biocontrol strategy. However, the application of plant-mediated RNAi targeting aphid genes remains to be explored.

Here, we identified a novel RNAi target gene *Smrrl*, which corresponds the 23S ribosomal RNA (rRNA) locus of *B. aphidicola* (a non-coding RNA gene), based on our transcriptome profiling of the alimentary canal of grain aphid (*Sitobion miscanthi*) after its feeding on wheat plants (Zhang et al., 2013). Ribosomal RNAs are essential, non-protein-coding components of the ribosome. We found this gene was significantly up-regulated after feeding on wheat plants ($P<0.01$). We hypothesized that this gene may play important roles in aphids' feeding, and knocking-down the expression of this gene through plant-mediated RNAi will alleviate aphid infestation on wheat plants. Here, in order to evaluate if this gene from *B. aphidicola* could serve as an effective RNAi target for aphid control in wheat, we first investigated *Smrrl*-dsRNA

could reduce *Smrrl* expression levels, thereby resulting aphid mortality in an *in vitro* dsRNA feeding bioassay. We then generated stable RNAi wheat lines expressing *Smrrl*-dsRNA and evaluated their effects on aphid feeding behavior, life span, population growth and mortality, respectively. We also investigated the persistence of this RNAi effects across generations in aphids' progeny. Our aim is to determine if manipulation of this symbiosis through plant-mediated RNAi against endosymbiont genes could serve as an effective aphid control strategy in crops.

2 Materials and methods

2.1 Plants and insects

The hexaploid wheat variety cv. ZM7698 was used. Seeds (30-35) from both wild-type and transgenic lines were sown in pots and cultivated under controlled conditions in a climate chamber. The environment was maintained at $21 \pm 1^\circ\text{C}$ with a 16-hour light photoperiod and $50 \pm 10\%$ relative humidity.

Grain aphids (*S. miscanthi*) were reared on wheat seedlings at the two-leaf stage under the same climate chamber conditions as plant growth. Apterous adult aphids from a single clonal lineage were cultured on seedlings for 24 hours to generate synchronized nymphs, after which the adults were removed. The progeny was used for subsequent experiments. All experiments were conducted under the same climate chamber conditions as described above.

2.2 Sequences analysis of *Smrrl*

Homologs of *Smrrl* in other aphid species were identified using the Basic Local Alignment Search Tool (BLAST) against the AphidBase database (<http://bipaa.genouest.org/is/aphidbase/>). Multiple sequence alignment was performed using DNAMAN v10 (<https://www.dnaman.net/>). Phylogenetic trees for *Smrrl* across 10 aphid species were constructed in MEGA 11 (www.megasoftware.net) using nucleotide sequences as a matrix. Branch strength was assessed via the maximum likelihood method with 1000 bootstrap replications.

2.3 RNA extraction and qRT-PCR analysis

For the expression level of *Smrrl* in green aphids at different development stages, total RNAs were isolated from the first, second, third, fourth instar nymphs and adults of grain aphid reared on wheat. For the expression level of *Smrrl* in aphids fed with different transgenic wheat and wild-type plants, the 2nd instar aphids were collected and used for total RNA extraction and further experiments. For the expression levels of the hairpin RNA construct (hp-*Smrrl*) of transgenic wheat, total RNAs were isolated from four-week-old leaves of wild type and different transgenic wheat plants.

qRT-PCR was used to analyze *Smrrl* expression. RNA was extracted from aphids using TransZol Up (TransGen Biotech, Beijing, China) and converted to cDNA with the FastKing RT Kit (Tiangen, Beijing, China). qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix (Tiangen, Beijing, China) on an ABI 7300 system, with cDNA as the template. The aphid actin gene was used as internal control, with specific primers designed for *Smrrl* normalization (Table 3-S1). All qRT-PCR experiments were conducted in triplicate. Relative expression levels of target genes were calculated using the mean values of the reference genes, employing the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

2.4 The dsRNA design and synthesis

Before dsRNA synthesis, the targeted *Smrrl* dsRNA region was analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) to evaluate the specificity and potential off-target effects. The analysis compared sequences with the genomes of aphid non-target organisms, including wheat (taxid: 4565), human (taxid: 9606), and key aphid natural enemies. These natural enemies comprised *Chrysopa* (taxid: 76806), *Asaphes vulgaris* (taxid: 338020), *Coccinellidae* (taxid: 7080), *Reduviidae* (taxid: 27479), flower flies (taxid: 34680), *Araneae* (taxid: 6893), and *Mantodea* (taxid: 7504).

Smrrl-dsRNA was synthesized and incorporated into an artificial diet. *GFP*-dsRNA was used as a negative control. The experiment was conducted at a 7.5 ng/μL dsRNA concentration to assess the mortality of second instar grain aphids feeding on the dsRNA-enriched artificial diet. The dsRNAs were synthesized using the Q5® High-Fidelity PCR Kit (New England Biolabs, Inc) (Table S1). For *GFP*-dsRNA, a 360-nt amplicon containing a T7 promoter sequence was generated via PCR using star plasmids as templates. For *Smrrl*-dsRNA, total RNA was extracted from aphids. Retrotranscription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) to synthesize cDNA. A 289 nt *Smrrl* amplicon containing a T7 promoter sequence was then amplified by PCR. Both *GFP* and *Smrrl* dsRNAs were synthesized from the purified PCR products using the MEGASCRIP® RNAi Kit (Invitrogen, Waltham, MD, USA). The quality and quantity of the synthesized dsRNAs were verified using a NanoDrop spectrophotometer and electrophoresis on a 1% agarose gel. The dsRNAs were subsequently stored at -20°C for further use.

2.5 Artificial diet bioassays

The liquid artificial diet and dsRNA artificial diet bioassays were performed following Zhang and colleagues (Zhang et al., 2013). Tubular glass vials (2 cm in diameter) used for the artificial feeding assay were sterilized. One opening of each vial was sealed with parafilm before sample application. An aliquot (200 μL) of the artificial diet was pipetted onto the parafilm, and another layer of parafilm was stretched over the top to form a sachet, ensuring even distribution of the liquid diet. After one day of pre-feeding on the artificial diet under controlled conditions (same as described before), 20 age-synchronized first instar nymphs were transferred using a fine paintbrush to a new vial containing an artificial diet supplemented with dsRNA. Controls included aphids fed with *GFP*-dsRNA (negative control) and those on a diet without dsRNA (blank control). The other opening of the vial was then covered with a piece of sterilized gauze to enclose the aphids. Aphid survival was recorded every two days over an eight-day period to assess dsRNA effects on mortality. Each treatment was performed in triplicates. To evaluate the RNAi effect, RNA was extracted from dsRNA-treated surviving aphids at different time points during the eight-day feeding period. qRT-PCR was performed as previously described, with at least three technical replicates per sample.

2.6 Construction of vector and generation of transgenic wheats

Specific primers were designed to amplify a 249 bp *Smrrl* target sequence, while a 320 bp *GFP* fragment served as a control for aphid bioassays (Table S1). PCR products were purified and cloned as inverted repeats into the pEasy-Blun-Zero-*Adhl*

vector at *SpeI*/*EcoRV* and *SacI*/*HpaI* sites, generating hairpin RNAi structures Bzero-*Smrrl-F* fragment (249 bp)-*AdhI-Smrrl-R* fragment (249 bp) (*dsSmrrl*) and Bzero-*GFP-F* fragment (320 bp)-*AdhI-GFP-R* fragment (320 bp) (*dsGFP*). The expression cassettes were then isolated by digestion with *SspI* and *BsrGI* and driven by the maize *Ubi* promoter. Wheat transformation was conducted via particle bombardment into immature embryos of the ZM7698 variety. The induced somatic embryos were then transferred to Murashige and Skoog (MS) regeneration medium and incubated under a 16-h light/8-h dark photoperiod at $25 \pm 1^\circ\text{C}$ for 2-3 weeks. Somatic embryos were induced on medium, and regenerated plants were grown and selected. Healthy seedlings were transplanted into soil and grown to maturity.

2.7 Southern blot analysis

Genomic DNA was extracted from T3 plant leaf tissues using the CTAB method as previously described (Sambrook, 1989). A total of 35 μg DNA was digested overnight with a restriction enzyme *SpeI* and separated by electrophoresis on a 0.8% agarose gel in $1\times\text{TBE}$ buffer at 60 V for 12-16 hours. The DNA was then transferred to Hybond-N+ membranes (Amersham, UK) via blotting. Membrane prehybridization, hybridization, washing, and detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit I protocol (Roche, Germany). The DNA probes were synthesized with the *Smrrl-F/R* primer set (Table 3-S1).

2.8 Electrical penetration graph (EPG) technique analysis

A Giga-8 DC EPG amplifier (EPG-Systems, Wageningen, Netherlands) coupled with a Faraday cage was employed to monitor the probing and feeding behaviors of aphids on wheat. Initially, synchronized first-instar aphids were placed on both transgenic and control plants for two days. Following this inoculation period, the aphids underwent a two-hour starvation. Each aphid was then individually tethered by applying water-soluble silver conductive paint to attach a flexible gold wire (18 μm diameter $\times$ 2 cm length) onto its dorsal thorax. The tethered insects were carefully positioned on the adaxial surface of leaves from either transgenic or wild-type wheat plants at the three-leaf stage. The free end of each gold wire was connected to a copper wire using conducting silver glue, and these copper wires were linked to the DC amplifier, while a plant electrode inserted into the soil completed the electrical circuit for signal detection. This setup allowed for precise, continuous monitoring of each aphid's feeding behavior over an 8-hour period under light conditions. In total, 12 behavioral recordings were obtained for each treatment, and the resulting EPG signals were analyzed using the Stylet+ software (EPG-Systems). According to the methodology described by Tjallingii distinct EPG waveforms correspond to specific aphid feeding behaviors (Tjallingii, 1985). The non-probing waveform indicates that the stylet is positioned outside the wheat leaf tissue, signifying an absence of probing activity. During the pathway phase, two waveforms are observed: Waveform C reflects the intercellular movement of the stylet, while potential drops (pd) represent brief intracellular punctures occurring along this trajectory. Waveform G, known as the xylem phase, corresponds to active sap ingestion from xylem elements. The phloem phase is subdivided into two parts: the E1 waveform marks the initiation of the phloem phase and denotes saliva secretion into the sieve elements, and the subsequent E2 waveform indicates the passive ingestion of phloem sap. Finally, the

recorded EPG data were processed and analyzed using the EPG-Excel data workbook developed by Sarria et al. (Sarria et al., 2009).

2.9 Aphid bioassays

A single clonal lineage of apterous adult grain aphids was reared on wheat seedlings in cages for 24 hours to produce synchronized nymphs, which were then transferred to fresh transgenic wheat plants. Homozygous T3 wheat plants expressing *Smrrl*-dsRNA were selected to evaluate their effects on aphid survival and fecundity. At the 3-4 leaf stage, 20 first-instar *S. miscanthi* nymphs were placed on each plant. Aphid mortality was monitored daily. Ten plants per transgenic line were used, and the entire experiment was repeated three times.

Life cycle parameters were calculated as follows: the net reproductive rate, $R_0 = \sum l_x \cdot m_x$, the mean generation time, $T = (\sum x \cdot l_x \cdot m_x) / (\sum l_x \cdot m_x)$, the intrinsic rate of increase, $r_m = (\ln R_0) / T$, and the finite rate of increase, $\lambda = e^{r_m}$. In the equations, l_x is the surviving rate to a specific age x , and m_x is the number of new-born nymphs produced by per live adult for a specific age x (Biondi et al., 2013).

2.10 Statistical analysis

Statistical differences between wild-type and transgenic wheat lines were assessed using a two-tailed Student's *t*-test. Significance was determined at the 1% or 5% level ($*P < 0.05$ or $**P < 0.01$). Standard error of the mean (SEM) was calculated from three biological replicates for each treatment. In the EPG assays, mean values and SEMs were derived from individual aphid recordings, and statistical comparisons were similarly conducted using Student's *t*-test. All data are presented as means $\pm$ SEM.

3 Results

3.1 Identification of RNAi target gene *Smrrl* and effects on aphid biology

We first identified a candidate gene *Smrrl*, an endosymbiont *B. aphidicola* derived gene encoding a 23S rRNA subunit, based on our transcriptome profiling of the alimentary canal of grain aphid after its feeding on wheat plants (Zhang et al., 2013). We found this gene was significantly up-regulated after feeding on wheat plants (Figure 3-1A). The *Smrrl* gene is 2916 bp in length (GenBank accession number: CP084934) (Figure 3-S1). Phylogenetic analysis revealed that *Smrrl* orthologs from 10 various aphid species formed distinct clusters (Figure 3-S2). These 10 aphid species include wheat aphid (*S. miscanthi*, CP084934), pea aphid (*A. pisum*, AP036055.1), bird cherry-oat aphid (*Rhopalosiphum padi*, CP034858.1), cotton aphid (*Aphis gossypii*, CP042426.1), soybean aphid (*Aphis glycines*, CP009253.1), sugarcane aphid (*Melanaphis sacchari*, CP029161.1), corn aphid (*Rhopalosiphum maidis*, CP032759.1), peach aphid (*Myzus persicae*, CP002697.1), groundnut aphid (*Aphis craccivora*, CP034897.1) and turnip aphid (*Lipaphis pseudobrassicae*, CP034870.1), respectively (Figure 3-S2). As indicated in Figure 3-S2, *Smrrl* has closer evolutionary relationships with its orthologs in pea aphid, peach aphid and turnip aphid. We also evaluated the expression levels of *Smrrl* in grain aphid at different developmental stages by quantitative real-time PCR (qRT-PCR). Our results showed that *Smrrl* expressed throughout all developmental stages, with the highest

expression level observed in the second-instar aphids (Figure 3-1B).

To assess the potential application of *Smrrl* as an RNAi target for aphid control, a 249 bp fragment of the gene was selected as a RNAi target for *in vitro* dsRNA feeding assays (Figure S1). There were three continuous 21 bp sequences within the selected 249 bp target sequences among 11 aphid species (Figure 3-S3), this conservation provides a molecular basis for the potential use of this target fragment in cross-species RNAi strategies (Bachman et al., 2013). Moreover, the BLAST analysis showed that the 249 bp fragment of *Smrrl* did not share any contiguous 21-nucleotide sequences with known genetic sequences of aphid non-target organisms, indicating a low potential for off-target RNAi effects based on sequence homology.

We also employed a 320 bp fragment of the *green fluorescent protein (GFP)* gene as a control in *in vitro* dsRNA feeding assays. The relative expression levels of *Smrrl* in aphids after feeding on diet were quantified by qRT-PCR. Ingestion of *Smrrl*-dsRNA resulted in a significant reduction in *Smrrl* expression starting from day 2 ($P < 0.01$) and continuing through day 4, day 6, and day 8 ($P < 0.01$) (Figure 3-1C). By day 8, *Smrrl* expression levels had decreased substantially (to 0.48) compared to those in aphids fed on either a dsRNA-free diet (blank control) or diet containing *GFP*-dsRNA (control). In contrast, the artificial diet containing *GFP*-dsRNA (control) showed no effect on *Smrrl* expression levels relative to the blank control, indicating that the observed reduced expression level was caused by a sequence-specific effect of *Smrrl*-dsRNA, rather than the nonspecific effects related physical or chemical characteristics of the dsRNA itself (Figure 3-1C). Furthermore, to investigate the relationship between the expression level of *Smrrl* and aphid mortality, we quantified survivorship following dsRNA feeding. Aphids fed with *Smrrl*-dsRNA displayed a progressively increasing mortality over time, reaching 16.7% on day 2, 25% on day 4, 31.7% on day 6, and 51.7% on day 8. These values were significantly higher than those of aphids fed with *GFP*-dsRNA control or blank control at all corresponding time points (Figure 3-1D). These findings highlight the effectiveness of the 249 bp fragment of *Smrrl* as a target for RNAi.

3.2 RNAi lines expressing *Smrrl*-dsRNA induce *Smrrl* silencing in aphids upon feeding

To further assess the feasibility of utilizing *Smrrl* as a target for plant-mediated RNAi, we constructed RNAi vectors with a hairpin structure of *dsSmrrl*, which could potentially produce *Smrrl* dsRNA for RNAi silencing, and a hairpin structure of *dsGFP* as control (Figure 3-1E). After the transformation of immature embryos from the wheat cultivar cv. Zhengmai 7698 (ZM7698), we obtained three independent transgenic wheat lines, namely rrl-50, rrl-64 and rrl-154 (hereafter referred as RNAi lines), respectively. We also generated *dsGFP* RNAi lines as control. Southern blot analysis confirmed the stable integration of the *dsSmrrl* expression cassette into the wheat genome with copy numbers ranging from two to three (Figure 3-1F), and the qRT-PCR results confirmed the expression of hp-*Smrrl* in RNAi lines (Figure 3-S4). To evaluate whether the expression of the target *Smrrl* gene in aphids was suppressed during feeding on RNAi lines, one-day-old synchronized nymphs were individually transferred onto RNAi lines and wild-type plants, respectively. The relative *Smrrl* expression levels in second-instar aphids were investigated after feeding on the plants for 2 days. Significant decreased *Smrrl* expression levels were observed in grain

aphids feeding on the three RNAi lines ($P < 0.01$), as compared to those on wild-type plants (Figure 3-1G).

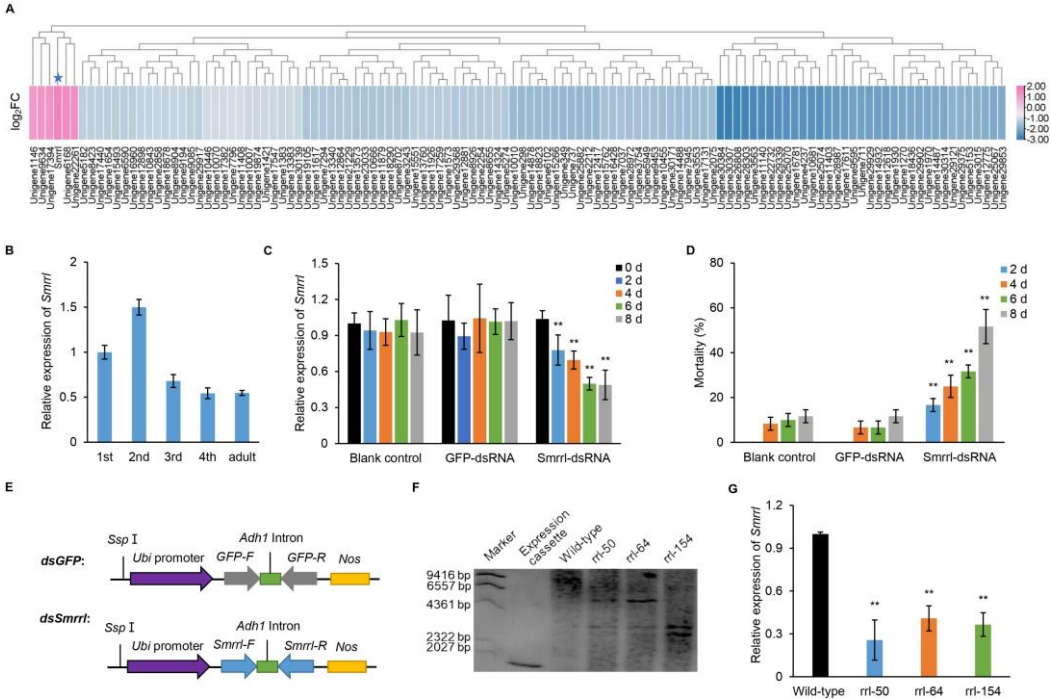


Figure 3-1. Effects of *Smrri1*-dsRNA on aphid mortality in artificial diet assays and generation of wheat RNAi lines.

(A) The relative expression levels of differentially expressed genes in aphid endosymbionts upon feeding. The blue star indicates the target gene, *Smrri1*, selected in this study for further analysis. (B) The relative expression levels of *Smrri1* in grain aphid at different developmental stages. (C) The relative expression levels of *Smrri1* in aphids fed on an artificial diet supplemented with *Smrri1*-dsRNA, *GFP*-dsRNA and blank control at different time points. (D) The mortality of grain aphids fed on artificial diets supplemented with *Smrri1*-dsRNA, *GFP*-dsRNA or blank control at various time points. (E) Schematic shows of the *dsGFP* and *dsSmrri1* hairpin expression cassettes for plant-mediated RNAi, respectively. (F) Southern blot analysis of the integration of *dsSmrri1* hairpin expression cassette in wheat genome in transgenic wheat lines. Genomic DNA was digested with Ssp I and hybridized with a *Smrri1* gene fragment with the expression cassette digested with Ssp I as a positive control. The lanes from left to right are: DNA molecular weight marker (Marker), positive control (Expression cassette), negative control (Wild-type), and the three independent transgenic lines rrl-50, rrl-64, and rrl-154. (G) The relative expression levels of *Smrri1* in grain aphids after fed on wild-type and RNAi wheat lines. 1st-4th: first-instar to fourth-instar aphids. Blank control: artificial diet without dsRNA. Control: artificial diet with *GFP*-dsRNA. Values represent the mean $\pm$ SEM of three independent biological replicates (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

3.3 Effects of *Smrrl* silencing on aphid feeding behavior

To investigate aphid behavior upon feeding, the RNAi line rrl-50 was selected as a representative line for electropenetrography (EPG) analysis. Aphids feeding on these plants exhibited different EPG waveform profiles in comparison to wild-type lines, indicating the aphids feeding on rrl-50 lines exhibited significantly altered behaviors (Figure 3-2A). Aphids feeding on RNAi plants required significantly more time to initiate their first probing activity compared to those on wild-type plants ($P < 0.01$) (Figure 3-2B). While no significant difference was observed in the number of non-probing (np) waveforms between aphids on the rrl-50 line and those on wild-type plants, the total duration of on-probing (np) waveforms and stylet probing (C) phases was significantly longer in aphids on RNAi plants compared to those on the wild-type plants ($P < 0.01$) (Figure 3-2B), suggesting that aphids experienced difficulties in stylet penetration through plant tissues. Notably, aphids feeding on RNAi plants also exhibited an extended duration of phloem salivation (E1) waveforms ($P < 0.05$), whereas the duration of phloem sap ingestion (E2) was significantly shorter compared to aphids fed on wild-type plants ($P < 0.01$) (Figure 3-2A, 2B). The extended duration of the E1 phase is likely attributed to reduced vitality of aphids, decreased salivation of effector proteins which potentially dependent on endosymbiont supplied metabolites such as amino acids essential for protein synthesis, thereby postponing the initiation of sustained phloem sap ingestion E2 phase. The shortened E2 phase reflects a decline in nutrient uptake efficacy from RNAi plants (Figure 3-2B). Consistent with this, the ratio of E1/(E1+E2) was significantly increased in aphids feeding on RNAi line compared to controls ($P < 0.05$) (Figure 3-S5). Collectively, these behavioral shifts further emphasize the pivotal role of *Smrrl* in establishment of the mutualistic relationship between aphid and its endosymbiont in effective feeding on wheat plants.

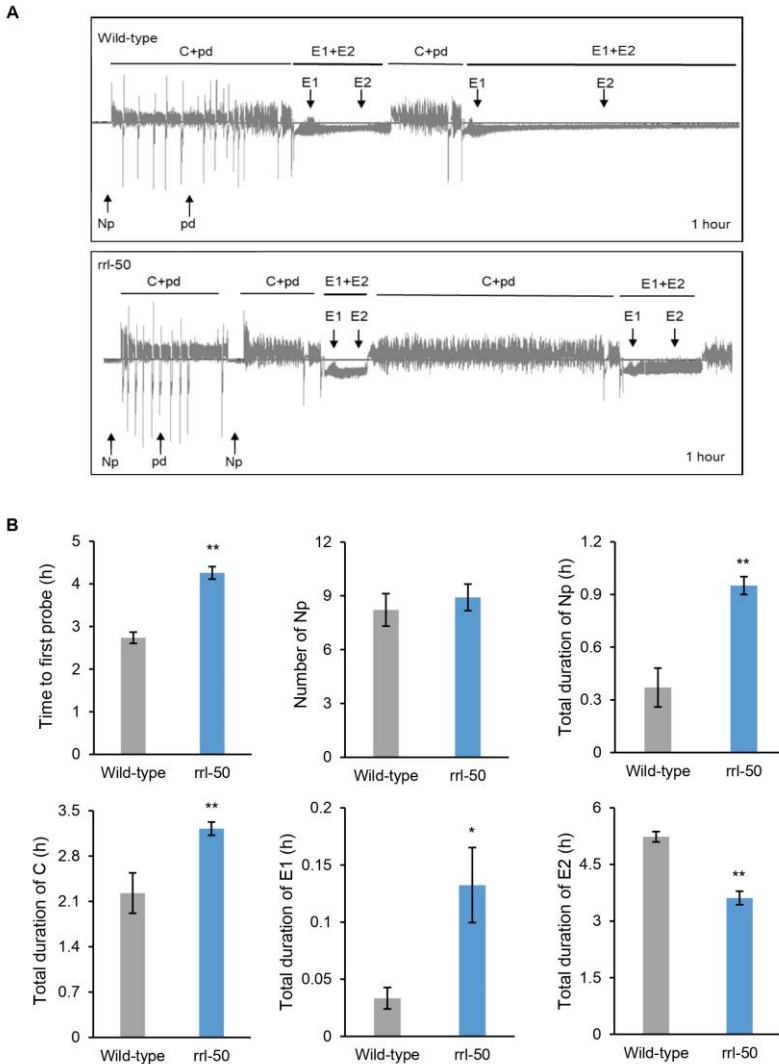


Figure 3-2. Effects of *Smrrl* silencing on feeding behavior of grain aphid based on EPG recordings.

(A) Representative EPG waveforms of grain aphids feeding on wild-type and *rrl-50* wheat plants. (B) Effects of *Smrrl* silencing on feeding behavior of grain aphid based on EPG recordings. Representative parameters of aphid feeding behavior: Non-probing (Np), stylet probing (C), intracellular stylet puncture (pd), phloem salivation (E1), and phloem ingestion (E2). Data shown are mean $\pm$ SEM. Asterisks above bars indicate significant differences between controls and treatments (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

3.4 Fitness of the aphids fed on *Smrrl* RNAi lines

We further assessed the effects of *Smrrl* silencing on aphid fitness parameters upon feeding on RNAi lines, including life cycle duration, mortality rates and reproduction

with both *dsGFP* RNAi lines and wild-type as controls. The developmental duration of aphids from the nymphal stage to adult showed that feeding on *rrl-64* and *rrl-154* RNAi plants led to a significant increase in the duration of the pre-adult stages, respectively, compared to *dsGFP* RNAi lines and wild-type controls ($P < 0.01$) (Figure 3-S6). Although no significant difference was observed in the adult preoviposition period (APOP) or the total preoviposition period (TPOP) among aphids feeding on different lines (Figure 3-S6), the total longevities of aphids feeding on RNAi lines were markedly reduced by 10 days, and both adult lifespan and reproductive period were significantly shortened by 9.2 days and 9.5 days respectively, compared to those on wild-type plants ($P < 0.01$) (Figure 3-3A). The total aphid production was significantly lower on all three RNAi lines compared to wild-type ($P < 0.01$) (Figure 3-3B), albeit no significant difference in daily fecundity was observed for aphids on the *rrl-50* and *rrl-154* RNAi lines. This suggests that the reduction in total production is primarily attributable to shortened reproductive lifespan rather than a decrease in daily fecundity. Additionally, population parameters such as the net reproductive rate (R_0) and the mean generation time (T), significantly decreased in aphids feeding on these three RNAi lines compared to those on wild-type and *dsGFP* plants ($P < 0.01$) (Figure 3-3C). Specifically, the intrinsic rates of increase (r_m), the finite rate of increase (λ) and population doubling time (DT) of aphids on RNAi lines *rrl-64* ($P < 0.01$) and *rrl-154* ($P < 0.05$) were significantly different from those on control plants, while *rrl-50* did not (Figure 3-3C). As a result, aphids feeding on RNAi lines exhibited significantly higher mortality rates compared to those on wild-type plants, with mortality surpassing 80% at 18 days after feeding (DAF) ($P < 0.01$) (Figure 3-3D).

3.5 Feeding on RNAi lines induces transgenerational silencing of *Smrrl* in aphids' progeny

In order to investigate the potential transgenerational RNAi effects, newborn nymphs derived from the aphids feeding on RNAi line *rrl-50* were then transferred onto wild-type plants. Our results indicated that despite being transferred to wild-type plants, *Smrrl* expression remained suppressed across 3 generations in aphid progeny. The decreased expression levels of *Smrrl* in aphids persisted from 1st to 3rd generations ($P < 0.05$) and returned to normal in the 4th generation ($P < 0.05$) (Figure 3-3F). Moreover, the 1st generation offspring continued to exhibit elevated mortality rates ($P < 0.01$) (Figure 3-3G).

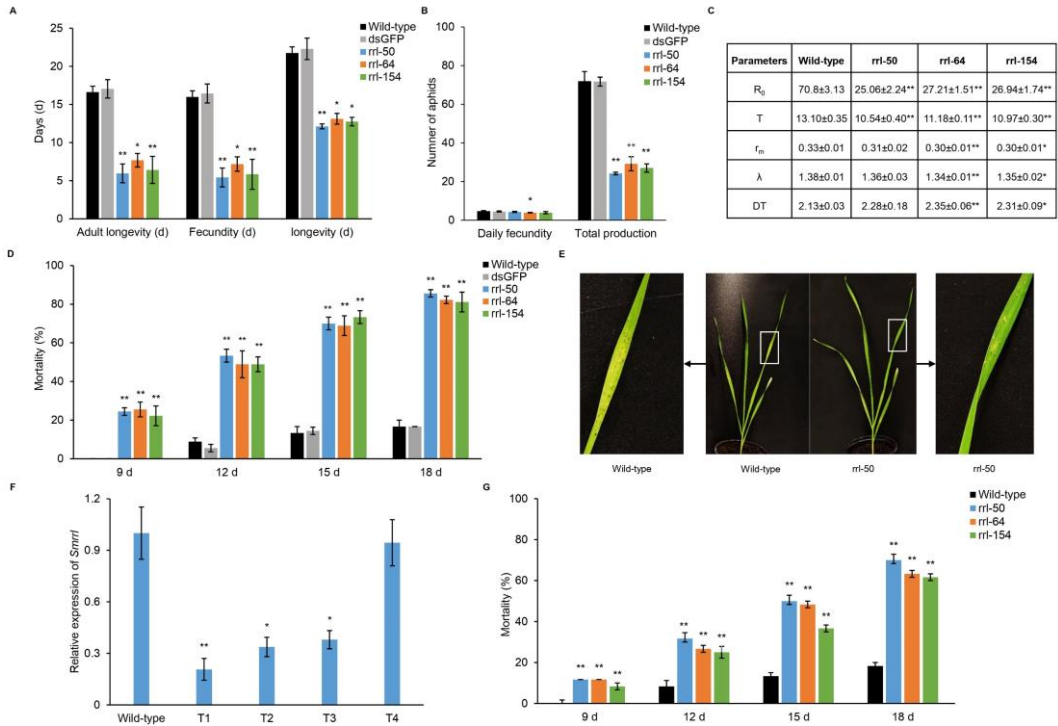


Figure 3-3. Fitness analysis of aphids after feeding on wheat RNAi lines.

(A) The adult longevity, fecundity and the total longevity of aphids fed RNAi and control wheat lines. (B) The reproduction of aphids fed on RNAi and control wheat lines. (C) Life table parameters of aphids fed on RNAi and control wheat lines. R_0 , net reproductive rate; r_m , the intrinsic rate of increase; λ , the finite rate of increase; T, the mean generation time; DT, doubling time (day). (D) Mortality of aphids fed on RNAi and control wheat lines. (E) Phenotypes of aphid resistance in RNAi and wild-type wheat plants. (F) The *Smrri1* levels in second-instar aphids across four successive generations. (G) The mortality of the first-generation offspring of aphids fed on RNAi and wild-type lines at different time points after being transferred to wild-type plants. All experiments were repeated three times, each with 20 synchronous one-day-old nymphs. Values represent the mean $\pm$ SEM (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

4 Discussion

RNAi technology has transitioned from laboratory research to commercial agricultural applications (Stokstad, 2024). The recent approval of the first RNAi biopesticide “Calantha” in USA represents as a milestone, demonstrating the field efficacy and environmental safety of RNAi biopesticide for global crop protection (Pallis et al., 2023). This paradigm shift has been underscored by the recognition of RNAi-based biocontrol as one of the ten most significant scientific breakthroughs in 2024 by Science (<https://www.science.org/content/article/breakthrough-2024>). Yet, its practical application has been largely constrained by the limited availability of

effective target genes. Plant-mediated RNAi is a promising strategy for developing next-generation insect-resistant crops (Zhang et al., 2025). Previous studies mainly focused on silencing genes directly involved in aphid growth and development to achieve plant-mediated RNAi (Adeyinka et al., 2023; Li et al., 2024; Liang et al., 2021; Sun et al., 2019; Tang et al., 2024; Venkatesh et al., 2023; Zhang et al., 2022). The essential role of endosymbionts in insect development makes disruption of the symbiotic mutualism a promising avenue for crop aphid management.

This study provides the first proof-of-concept that a plant-mediated RNAi strategy can effectively target the aphid endosymbiont *B. aphidicola* for pest control. We identified *Smrrl* as a functional target and demonstrated that its silencing, through either artificial diet or stable expression in transgenic wheat, induced profound physiological deficits in the grain aphid. Targeting the 23S rRNA gene (*Smrrl*) is a non-standard RNAi application. However, RNAi works by degrading RNA sequences that match the siRNA. Bacterial rRNA can be targeted this way because it contains accessible regions for siRNA binding. This approach has precedent in other systems, supporting the theoretical basis of our strategy (Shatskikh et al., 2024; Zhou et al., 2017). For this to work, siRNA from the plant must reach *Buchnera* inside the aphid's cells. Our key evidence includes both the specific reduction in *Smrrl* transcript levels in aphids on transgenic plants and the consistent phenotypic effects observed exclusively in the treatment group. Aphids on RNAi plants showed disrupted feeding behavior, severely reduced fecundity, and increased mortality, leading to sustained population suppression. This severe effect matches what is expected when disrupting an essential function like the ribosome. Together, these evidences support the conclusion that the silencing signal is active within the aphid-symbiont system.

The observed decline in aphid fitness following silencing of *Smrrl* may be linked to both functional impairment of the symbiont and potential changes in its population dynamics. As a ribosomal RNA gene, silencing *Smrrl* is predicted to disrupt protein synthesis in *Buchnera*, thereby interfering with its core role in providing essential nutrients to the aphid host (Leśniczak Staszak et al., 2021; Maria Grazia Giuliano, 2021; Sloan et al., 2017). This induced nutritional deficit provides a mechanistic explanation for the specific behavioral alterations we observed: the prolonged probing phase and the reduced sustained ingestion, which are established indicators of an insect struggling to acquire adequate nutrition (Walker et al., 2024). Such sustained functional disruption may also indirectly affect the survival or proliferation of *Buchnera* within bacteriocytes, amplifying the detrimental effects on the aphid at the population level. Consequently, the resulting gradual mortality and decline in fecundity are interpreted as the combined physiological outcome of this targeted disruption to the symbiotic partnership. This finding reinforces the effectiveness of targeting key symbiont genes for pest control.

Notably, sequence alignment revealed that the 249 bp target fragment of *Smrrl* is highly conserved among orthologs from 11 agriculturally important aphid species (Figure 3-S3). However, translating this prediction into a practical broad-spectrum control strategy requires critical validations. For example, the fragment could be tested via artificial diet on representative species to assess mortality and silencing, followed by transient plant assays, given that RNAi efficiency varies among species. In addition, the BLAST analysis showed that the 249 bp fragment of *Smrrl* did not

share any contiguous 21-nucleotide sequences with selected aphid non-target organisms. Yet, the possibility of RNAi effects via partial homology or in untested non-target organisms cannot be excluded. Future non-target risk assessment could include bioassays with representative beneficial insects and expanded sequence-based screening against a broader panel of non-target species to provide a more comprehensive evaluation of environmental safety. The high conservation of the ribosomal target, central to our approach, necessitates particularly careful scrutiny of its potential impact on non-target organisms. The combination of high intra-pest conservation and initial specificity screening identifies *Smrrl* as a candidate target worthy of broader exploration for cross-species RNAi strategies. While target site accessibility has limited some rRNA-targeting RNAi efforts, our empirical data demonstrate that the selected *Smrrl* fragment is effectively silenced, supporting its feasibility as a target. The conserved nature of this ribosomal target is a double-edged sword; while it may increase the scope of potential efficacy, it also necessitates heightened scrutiny regarding specificity.

Moreover, the functional essentiality of rRNA imposes strong evolutionary constraints on its sequence. Mutations that simultaneously evade dsRNA binding while preserving ribosome function are expected to be extremely rare and potentially lethal to the bacterium itself, theoretically lowering the risk of rapid resistance development (Parks et al., 2019; Welfer et al., 2025). We also recognize that compensatory mutations or altered rRNA modifications could potentially influence resistance development, although these possibilities remain to be experimentally tested (Levin et al., 2000; Tian and Wang, 2025). In practice, a “multi-target stacking” strategy could be employed, combining RNAi targeting *Smrrl* with other mechanisms of action, such as targeting essential aphid genes or other critical *Buchnera* genes, thereby significantly raising the genetic hurdle for pest adaptation (Li et al., 2022b; Whitten and Dyson, 2017). Furthermore, crop rotation or intercropping with plants employing different resistance mechanisms, or alternating use with specific chemical insecticides, can effectively reduce selection pressure (Al Musawi et al., 2025; Guinet et al., 2023; Lamzira et al., 2025; Zhou et al., 2024).

In addition to *Buchnera*, aphids also harbor various heritable bacterial endosymbionts, known as “facultative” or “secondary” symbionts, which are not essential for host growth or reproduction, and can occasionally be transmitted horizontally (Russell et al., 2003; Sandström et al., 2001). Several facultative symbionts confer protection to aphids against natural enemies. *Hamiltonella defensa*, for instance, enhances resistance to parasitoid wasps (Oliver et al., 2003; Vorburger et al., 2010), whereas *Regiella insecticola* primarily offers defense against pathogenic fungi (Scarborough et al., 2005), with certain strains also confer protection against parasitoids (Vorburger et al., 2010). Furthermore, aphids also harbor *Serratia symbiotica*, which can compensate for compromised nutritional functions of the obligate endosymbiont, particularly under stress conditions such as heat shock (Koga et al., 2003; Russell and Moran, 2006). Multi-gene targeting may enhance the efficacy of RNAi-based aphid control through synergistic effects (Dong et al., 2024; Fan et al., 2024; Wang et al., 2024). Therefore, identifying additional endosymbiont genes and simultaneously targeting multiple essential genes from different endosymbionts, may represent an important aspect in future research for aphid control (Chong and Moran,

2018; Koga et al., 2003).

In addition, this study found that the growth impairment experienced by parent aphids feeding on transgenic wheat persisted in their offspring (F1 to F3 generations), which were not directly exposed to dsRNA, indicating a transgenerational effect of the intervention. This phenomenon could be explained by several mechanisms: (1) severe physiological compromise of the exposed parents leading to intrinsically weaker offspring; (2) the transmission of a *Buchnera* population with stably impaired function to the progeny. Disentangling these possibilities represents a compelling and complex question for future research. Nevertheless, this transgenerational effect suggests that field deployment of the strategy has significant practical value, offering the potential for durable pest control that extends beyond a single generation.

However, in contrast to the enduring phenotypic effects, the silencing level of the target gene *Smrrl* did not deepen in the offspring, and its expression even showed a trend of recovery by the fourth generation (Figure 3-3F). This phenomenon reveals the complexity concerning the persistence and dynamics of plant-mediated RNAi for agricultural pest control. We speculate that the recovery of *Smrrl* expression may be attributed to the following reasons. First, attenuation in the efficiency of the systemic RNAi machinery could lead to decreased generation or amplification of new siRNAs in the offspring (Chen et al., 2018; Cooper et al., 2019). Second, there is limited transgenerational transmission of the ingested dsRNA/siRNA via germ cells or early embryos in the parent generation, and the carried silencing signal may be gradually diluted or degraded in subsequent generations (Alcazar et al., 2008; Shugarts Devanapally et al., 2025; Vastenhouw et al., 2006). Finally, adaptive regulation of the endosymbiont population may play a role. The *Buchnera* population or the host aphid might compensate for the loss of function of this key gene through the activation of alternative metabolic pathways or other regulatory mechanisms, thereby partially restoring *Smrrl* expression at the transcriptional level, although the full functional recovery may be lagged (Chong and Moran, 2018; Csorba et al., 2024a; Neiers et al., 2021). Elucidating the precise molecular mechanisms behind this transgenerational silencing and recovery, particularly the link between endosymbiont dysfunction and host phenotypic memory, will be an important direction for future research.

It is worth to note that although aphid populations were significantly reduced on *Smrrl* RNAi wheat plants (Figure 3-3E), variability in environmental factors and plant-aphid interactions may influence the overall efficacy of RNAi responses (Ai et al., 2025; Darrington et al., 2025). The effectiveness of this strategy may also vary among aphid species with distinct feeding behaviors or with differing symbiotic complements, including those harboring secondary endosymbionts that could theoretically provide functional compensation (Csorba et al., 2024b; Liu et al., 2023; Zhao et al., 2025). Given the fact that endosymbiotic bacteria can be leveraged to continuously produce dsRNA within the host (Whitten et al., 2016), and engineered entomopathogenic fungi can be used to express miRNAs targeting host genes, thereby effectively suppressing pest insects (Cui et al., 2022). The RNAi effect caused by insect endosymbiotic system may be more species-specific, directive and effective in plant RNAi-mediated aphid control in crops.

DsRNA delivery is one of the most important factors influencing RNAi efficacy in insects. Several innovative strategies for delivering dsRNA into insects have been

explored. For instance, combining RNAi with nanotechnology presents an alternative innovative methodology (Mathur et al., 2025; Xing et al., 2025). Viral-like particles (VLP) can serve as nano-vehicles for dsRNA delivery (Jiang et al., 2024; Xue et al., 2024; Xue et al., 2023). Bacteria-expressed and nanocarrier-delivered RNA pesticides were used for pest control in a greenhouse trial (Ma et al., 2023). Polymeric systems such as polycations and guanylated polymers (GNPs) have shown potential in protecting dsRNA from degradation, increasing cellular uptake, and ensuring controlled and precise release (Jain et al., 2022; Lin et al., 2025; Quilez Molina et al., 2024). These strategies present promising alternatives to enhance RNAi efficiency for pest management applications. In particular, dsRNA as biopesticide for direct field applications is emerging as a promising strategy for pest control. However, the cost-effective production of dsRNA-based pesticides continues to pose a significant challenge. Microbial expression platforms, such as the L4440-HT115 (DE3) strain, provide a scalable and sustainable solution for dsRNA synthesis (Abbasi et al., 2020). In addition, cell-free expression systems offer a promising alternative by eliminating the requirement for cellular viability, thereby streamlining the production process (Carlson et al., 2012; Garamella et al., 2016). While our study focuses on plant-mediated RNAi, direct application of dsRNA as a biopesticide is an emerging complementary strategy. Given the effectiveness of *Smrri1* in aphid control demonstrated in this study (Figure 3-1, 3-2, 3-3) and its potential as an RNAi target across multiple aphid species (Figure 3-S3), we propose that the development of *Smrri1* as RNAi-based biopesticide could serve as an alternative and effective strategy for broad-spectrum aphid control in different agriculturally important crop species.

In summary, we here identify a novel RNAi target from endosymbiotic *B. aphidicola* in grain aphid, and demonstrate the important roles of *Smrri1* in regulating grain aphid's growth, feeding behavior and fitness cost. Manipulating this mutualism by disrupting endosymbiont functionality through plant-mediated RNAi of *Smrri1* represents a potential for wheat aphid management. To further advance this strategy, future research should prioritize the identification of additional effective target genes in the endosymbionts, improving the stability and delivery efficacy of dsRNA when used as biopesticide, as well as implementing large-scale field trials to evaluate its durability, ecological impact, and practical application under diverse environmental conditions. By overcoming these challenges, we believe that this strategy holds great potential for integrated aphid management in crops for sustainable agricultural development.

5 Limitations and prospects

1. **Laboratory-to-field extrapolation.** The artificial diet assay represents a simplified, controlled system. The dsRNA concentrations required for efficacy and the resulting phenotypic effects observed in the laboratory cannot be directly extrapolated to predict outcomes under complex field conditions. Future studies involving field or semi-field trials are essential to evaluate the efficacy and durability of this strategy in agricultural settings.

2. **Mechanistic study.** The precise intracellular journey of plant-derived siRNAs to *Buchnera* and their final mode of action on the bacterial ribosome remain to be fully elucidated. Additionally, our study does not distinguish the relative contributions of

direct functional impairment of *Buchnera* versus potential secondary effects on its population titer within the host. Quantifying symbiont abundance and metabolic output in future work will be key to dissecting this mechanistic interplay.

3. Cross-species and non-target efficacy. The potential for broad-spectrum activity or impacts on non-target organisms remains predictive. The biological efficacy in other species depends on factors beyond sequence homology, such as RNAi machinery efficiency, which remain untested. Furthermore, effects on non-target *Buchnera* strains, other symbionts, or wider microbiomes are unknown. Therefore, empirical validation of efficacy in other aphid species and comprehensive environmental risk assessment constitute mandatory next steps before any broader application can be considered.

4. Resistance risk and management. The actual risk of resistance evolution to this strategy, and the efficacy of proposed management approaches such as gene stacking, were not evaluated. Future research should include long-term selection experiments and modeling to assess resistance risk and develop sustainable deployment strategies.

5. Mechanism of transgenerational persistence. While a transgenerational phenotypic effect was observed, our study did not measure persistent RNAi effectors in offspring or distinguish between the contributions of parental stress and heritable symbiont impairment. Elucidating this mechanism requires future studies designed to track dsRNA/siRNA across generations and analyze heritable changes in the host-symbiont unit.

6. Statistical Considerations. Our study used Student's *t*-tests for pre-planned pairwise comparisons. This approach does not include corrections for multiple comparisons, and we did not perform a formal power analysis; sample sizes were based on established practices in the field. Future studies should consider applying more robust statistical frameworks such as ANOVA with post-hoc corrections.

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Supporting Information

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Figure 3-S1. Nucleotide sequence of *Smrrl* and the 249 bp fragment used for RNAi.

Figure 3-S2. Phylogenetic trees of *Smrrl* gene in *Sitobion miscanthi* and other aphid species.

Figure 3-S3. Multiple sequence alignment of the selected 249 bp target sequence from 11 aphid species.

Figure 3-S4. Relative expression levels of *dsSmrrl* in different transgenic wheat lines.

Figure 3-S5. Effect of *Smrrl* silencing on the E1/(E1+E2) ratio.

Figure 3-S6. The longevity of different stages, adult preoviposition period (APOP), and total preoviposition period (TPOP) of aphids fed on RNAi lines and control lines.

Table 3-S1. Primers used in this study.

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AAGGTTAAGCCTCTCGGGTCATTAGTACTAGTTAGCTCAACACATTGCTATGCTTACACA 60
TCTAGCCTATCAACGTCGTAGTCTTCAACGTCCCTTCAGTAAACATTTCTGTTTCAGGGA 120
AGATTAATCTTGAGGCAAGTTTCGTGCTTATATGCGTTTCAGCGCTTATCTTTCCGTATT 180
TAGTACCGGGCAATGCCATTGGCATGACAACCCGAACACCAAGTATACGTCCACTTCGG 240
TCCTCTCGTACTAGAAGCAGCCCCCTCTCAATCTTCCAGCGCCACGGCAGATAGGGACCG 300
AACTGTCTCAGCAGTTCTAAACCCAGCTCGCGTACCCTTTAAATGGCGAACAGCCATA 360
CCCTTGGGACCTGCTTCAGCCCCAGGATGTGATGAGCCGACATCGAGGTGCCAAACACCG 420
CCGTCGATATGAACCTTTGGGCGGTATCAGCCTGTTATCCCCGGAGTACCTTTTATCCGT 480
TGAGCGATGGCCATCTCCATTGAGAACCACCGGATCACTAAGACCTGCTTTCGCACCTGCT 540
CGCGTTATCAGCTCAGAGTCAAACCTGGCTTATGCCTTTGCACTAACCTCAGATGTCCG 600
ACCGTGATTAGCCAATCTTCGTACTCCTCCGTTACTCTTTGGGAGGAGACCGCCCCAGTC 660
AAACTACCCACCAGACACTGTCTCTATGCCGGATTACGGCACTAGGTTAGAATACCGAAT 720
TTTAAAGGGTGGTATTTCAAGTGTGGCTCCATTAACCTAGCGTTTTAATTTTATAACCT 780
CCCACCTATCTACACATTAACATTCAGAATTCAGTGTCAAGCTATAGTAAAGGTTTCAG 840
GGGTCTTTCCGTCTTGCCGCGGATATACTGCATCTTCACAGCAAATTCATTTCACTGAG 900
TCCCAGGTGGAGACAGCCTGGCCATCATTACGCCATTCTGTCAGGTGCGAACTTACCCGA 960
CAAGGAATTCGCTACCTTAGGACCGTTATAGTTACGGCCGCCGTTTACCGGGGCTTCAG 1020
TCTAGAGCTTTAAGTTTCCTTTGACTCCTTCGATTAACCTTCCGGCACCGGGCAGGCGTC 1080
ACACCGTATACTTCCACTTTCGTGTTTGACAGTGTCTGTTTAAATAAACAGTTGCAG 1140
CCAGCTGATCTTCGATAGCTTCGGCTACAGGAGTAAATCCTTACACTTAAGTCTAGC 1200
GTGCTTCTCCGGAAGTTACGGCACTATTTTGCTAGTTTCTTACCTGGGTTCTCTCAA 1260
GCGCTTAGTATTTCTACTTAACTACCTGTGTGCGTTTAGGGTACGATTTAATTTTACC 1320
TGATGCTTAGAGGCTTTTCTTGGAAGCGTGGTATTAGTTACTTCATCACCGTAGTAATTC 1380
GTCATCATGCCTCAGATTAAAGATAATCGGATTTGCCTAATTATCATACCTACACATTTA 1440
AACCAGGATAACCGTCACCTGGATAACCTAACCTTCTTCGTCCTTCCAGTAAATAT 1500
TAAGCACAGGAATATTAACCTGTTGTCCATCGACTACGCTTTGACAGCTCGCCTTAGGGA 1560
TCGGCTTACCCTGCCCGATTAAACGTTGGACAGGAAACCTTAGTTTTCGGCGAGCAGGT 1620
TTTTACCTGCTTATCGTTACTCATGTACAGCATTGCACTTCTGATACCTCCAATATAT 1680
TTTACAATATATCTTCTTTGGCTTACAGAACGCTCCCTTACCAGTAAAAAATATATTT 1740
TACTGCCGAGCTTCGGTGCATAGTTTGAGCCCCGTATATCTTCCGCGCAGGCGCACTT 1800
GACCACTGAGCTATTACGCTTTCTTTAAATGATGGCTGCTTCAAGCCAACATCCTGGCT 1860
GTTTATGCCTTCCACATCGTTTCCCACTTAAGTATGACTTTGGGACCTTAGCTGGCGGT 1920
CTGGGTTGTTTCCCTTTCCACAACGAACGTTAGCACCCGCTGTGTGTCTCCCGTGATAAC 1980
ATTCTACGGIATTCGGAGTTTGATCGGTTTGGAAGCCGGGATGGCCCCCTAGCCGAAA 2040
CAGTGTCTACCCCCGAGATGAATTCACGAGGCGCTACCTAAATAGCTTTCGGGGAGAA 2100
CCAGCTATCTCCCGGTTTGATTGGCCTTACCCCTAGCCATAGGTCATCCGCTGATTTT 2160
TCAACATCAGTCGGTTCCGCTCCTCAGTTGGTTTACCCTAACCTTCAACCTGCCGTTGGC 2220
TAGATCACCGGGTTTCGGGTCTGTACCCTGAACTTAACGCCTGTTTAGGACTCGGTTTC 2280
CCTTCGGCTCCCCATTTGGTTAACCTTGCTACAGAGTACAAGTCGCTGACCCATTATAC 2340
AAAAGGTACGCAGTCACAAATTTATTAATAAAAAATGCTTCCACTGCTTGTACGTACACGGT 2400
TTCAGGTTCTATTTCACTCCCTCGCCGGGGTCTTTTCGCCCTTCCCTTACGGTACTAG 2460
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GGATTTCTCGTGTCCCGCTCTACTTTTGAACCTCACAATATAAGTTATTTTATATACAGG 2580
GCTATCACCTTGATCGCTGAATTTCCAAATCATTCTACTATAAATTATATTGATTATA 2640
GTTCTGGGCTGTTCCCTTTTCGCTCGCCACTACTAAGGGAATCTCAGTTGATTTCTTTC 2700
CTCAAGGTACTTAGATGTTTCAGTTCCTTGGTTTGCCTTATTAACCTATTTAATTCAGT 2760
TAATAATGATGCAAAAAAATTGCATCGGGTTTCCCATTCGGAATCGACGGTTATTGCG 2820
CTTCATATCAGCTTACCAGCGCTTTTCGAGATTAGCACGTCCTTCTTCGCTCTGACTG 2880
CCAAGGCATTCACCATGTACGCTTATTGCTTAACC 2916

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Figure 3-S1. Nucleotide sequence of *Smrrl* and the 249 bp fragment used for RNAi.

The sequences selected for construction of the RNAi vector are highlighted in yellow.



Figure 3-S2. Phylogenetic trees of *SmrriI* gene in *Sitobion miscanthi* and other aphid species

Insect phylogenetic trees showed the evolutionary relationships of the species addressed in this study. The deduced nucleotide sequences from 10 aphid species include wheat aphid (*Sitobion miscanthi*, CP084934), pea aphid (*Acyrtosiphon pisum*, AP036055.1), bird cherry-oat aphid (*Rhopalosiphum padi*, CP034858.1), cotton aphid (*Aphis gossypii*, CP042426.1), soybean aphid (*Aphis glycines*, CP009253.1), sugarcane aphid (*Melanaphis sacchari*, CP029161.1), corn aphid (*Rhopalosiphum maidis*, CP032759.1), peach aphid (*Myzus persicae*, CP002697.1), groundnut aphid (*Aphis craccivora*, CP034897.1) and turnip aphid (*Lipaphis pseudobrassicae*, CP034870.1). The joint enrooted tree was generated using MEGA 11 by the maximum likelihood method. Bootstrap values were indicated at each branch.

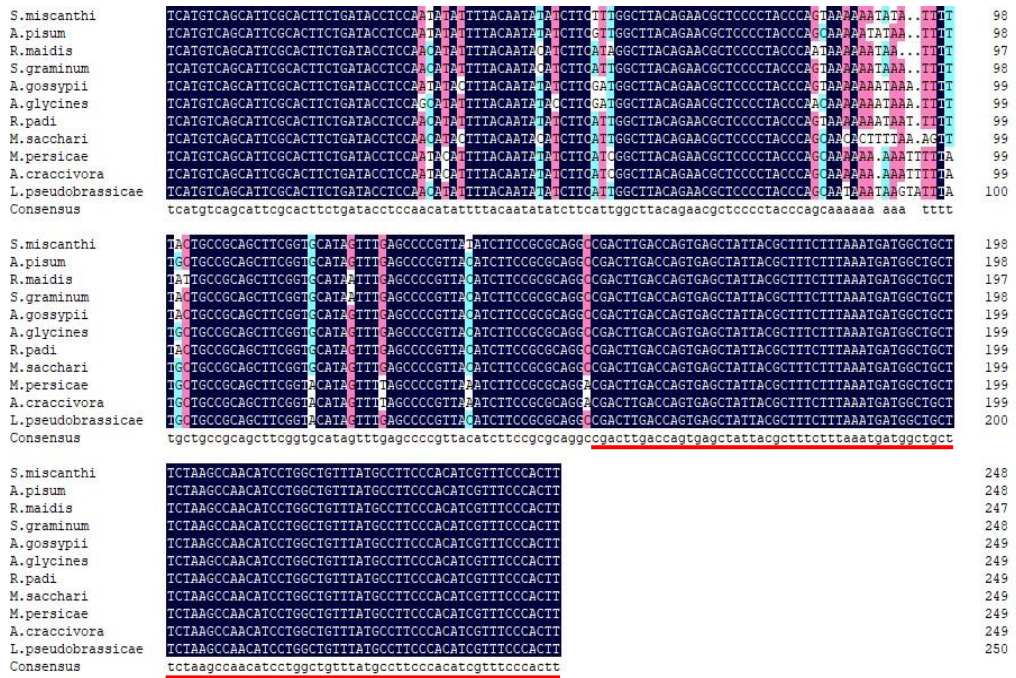


Figure 3-S3. Multiple sequence alignment of the selected 249 bp target sequence from 11 aphid species.

Black shades indicate identical sequences. Pink shades indicate similar sequences, and blue shades include the identical and similar sequences. More than three consecutive 21 bp sequences are highlighted with red lines.

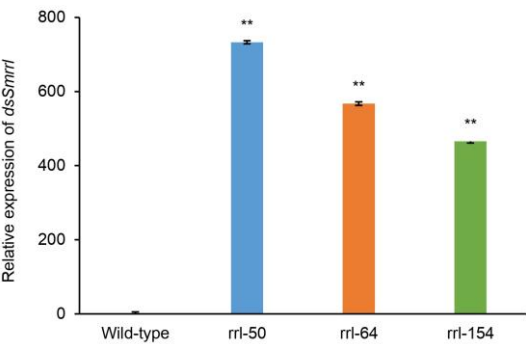


Figure 3-S4. Relative expression levels of *dsSmrri* in different transgenic wheat lines.

Values represent the mean $\pm$ SEM of three independent biological replicates (Student's *t*-test, **P*<0.05, ***P*<0.01).

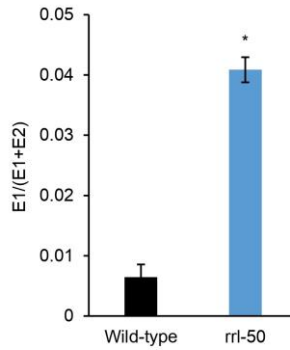


Figure 3-S5. Effect of *Smrrl* silencing on the E1/(E1+E2) ratio.

E1: phloem salivation, E2: phloem ingestion. Data shown are mean $\pm$ SEM. Asterisks above bars indicate significant differences between controls and treatments (Student's t-test, * $P < 0.05$, ** $P < 0.01$).

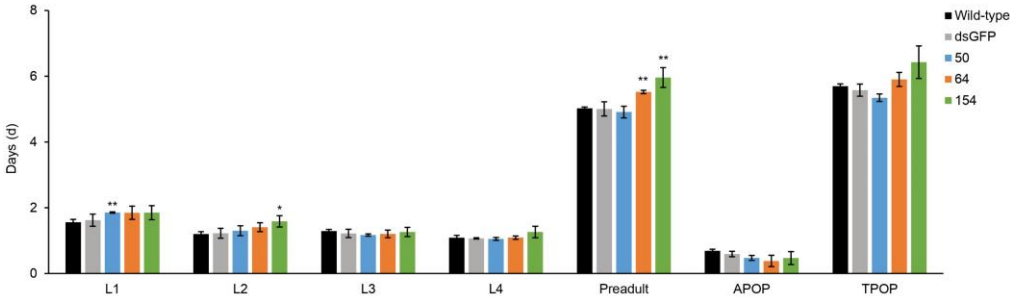


Figure 3-S6. The longevity of different stages, adult preoviposition period (APOP) and total preoviposition period (TPOP) of aphids fed on RNAi lines and control lines.

1st-4th: first-instar to fourth-instar aphids. Values represent the mean $\pm$ SEM (Student's *t*-test, * $P<0.05$, ** $P<0.01$).

Table 3-S1. Primers used in this study

Primers	Sequence (5' to 3')	Use of PCR products
qrrl-F	AGAACGCTCCCCTACCCAGT	Detect <i>Smrrl</i> in aphids
qrrl-R	GTGGGAAGGCATAAACAGCCAG	Detect <i>Smrrl</i> in aphids
rrlS-F	ATCCCCGGGGGATCCACTAGTTAAGTGGGA AACGATGTGGG	Construct vector for RNAi
rrlE-R	GCCTCCCAGATCGATTTCGATATCTCATGTCA GCATTTCGCACTT	Construct vector for RNAi
rrlH-F	GGGGCAGACTCCCGTTTGTAACTCATGTC AGCATTCGCACTT	Construct vector for RNAi
rrlS-R	CGATCGGGGAAATTCGAGCTCTAAGTGGGA AACGATGTGGG	Construct vector for RNAi
qactin-F	CGGTTCAAAAACCCAAACCAG	Internal control of qRT-PCR for aphid
qactin-R	TGGTGATGATTCCCGTGTTTC	Internal control of qRT-PCR for aphid
Ubi-F	TTTAGCCCTGCCTTCATACG	Detect <i>Smrrl</i> dsRNA transgenic wheat
Adh-R	CAGATAAGCCGCCAAGAAGG	Detect <i>Smrrl</i> dsRNA transgenic wheat
Adh-F	CCAAGGTATCTAATCAGCCATCC	Detect <i>Smrrl</i> dsRNA transgenic wheat
Nos-R	TATAATTGCGGGACTCTAATC	Detect <i>Smrrl</i> dsRNA transgenic wheat
T7-gfp-F	TAATACGACTCACTATAGGGGACGACGGCA ACTACAAGAC	DsRNA synthesis of <i>gfp</i>
T7-gfp-R	TAATACGACTCACTATAGGGCTCAGGTAGT GGTTGTCGG	DsRNA synthesis of <i>gfp</i>
T7-rrl-F	TAATACGACTCACTATAGGGGTGGGAAACG ATGTGGGAAG	DsRNA synthesis of <i>Smrrl</i>
T7-rrl-R	TAATACGACTCACTATAGGGCATGTCAGCA TTCGCACTTC	DsRNA synthesis of <i>Smrrl</i>

Chapter IV

**Host-induced gene silencing of *SmDSR32*
enhances wheat defense against
*Sitobion miscanthi***

Abstract

The grain aphid, *Sitobion miscanthi*, poses a serious threat to cereal crops worldwide, leading to considerable yield losses and demanding annual insecticide applications during the grain-filling stage. As a sustainable alternative, we explored host-induced gene silencing (HIGS) targeting an aphid-specific gene. In this study, we identified *SmDSR32*, a novel gene encoding a salivary peptide in *S. miscanthi*, and validated its suitability for RNAi. Transgenic wheat lines expressing *SmDSR32-dsRNA* were generated. Aphids feeding on these lines showed a 20-fold reduction in *SmDSR32* transcript levels compared with controls. This silencing disrupted normal feeding behavior in electropenetrography (EPG) analyses, characterized by a 1.94-fold prolongation of intercellular probing and a 61% shortening of phloem ingestion. Consequently, aphid performance was severely compromised, with at least a 56.7% decrease in survival, 5 days shortening in lifespan, and a reduction of 9-10 individuals in aphid progeny production. Impressively, upon transferred to wild-type plants, both the surviving aphids and their progeny sustained fitness deficits, with a 30% reduction in survival still observed in the first generation. These findings validate *SmDSR32* as a potent RNAi target and establish HIGS targeting essential salivary genes as a promising strategy for sustainable aphid management in wheat.

Keywords: wheat (*Triticum aestivum* L.), grain aphid (*Sitobion miscanthi* F.), RNA interference (RNAi); salivary peptide; aphid control

1 Introduction

Aphids (Hemiptera: Aphididae) rank among the most economically detrimental agricultural pests globally (Mou et al., 2023). Their capacity for rapid population growth, diverse reproductive modes, and broad environmental tolerance collectively amplifies their destructive potential on cultivated crops (Adhikari et al., 2019; Dampc et al., 2020; Gebretsadik et al., 2025). As obligate phloem-feeding insects, high population densities of aphids result in substantial nutrient depletion from sieve tube elements, while their feeding activity facilitates the transmission of numerous plant-pathogenic viruses (Khan and Naveed, 2022; Twayana et al., 2022). Despite documented ecological consequences and biodiversity threats associated with chemical control, its application persists as the prevailing approach, primarily due to its rapid efficacy in suppressing aphid outbreaks (Luo et al., 2021; Tang et al., 2021). Consequently, developing alternative control strategies represents a critical priority to mitigate dependence on chemical pesticides. RNA interference (RNAi)-mediated gene silencing represents a preferred strategy for enhancing crop resistance against diverse pests. This approach utilizes the host plant's capacity to produce and deliver silencing RNAs (sRNAs) into invading pests, thereby targeting essential pest genes (Lim et al., 2020; Zhang et al., 2013). One prominent application of this RNAi principle is known as host-induced gene silencing (HIGS) (Nowara et al., 2010). Within the dsRNA-mediated RNAi pathway, host-derived dsRNAs are generated by transcribing complementary sequences of pest genes. These molecules subsequently target corresponding mRNA transcripts in pests, thereby decreasing functional protein synthesis (Verma and Modgil, 2024). Transgenic plant-mediated RNAi offers a protective and environmentally compatible approach for aphid management, with demonstrated efficacy in recent research (Sun et al., 2019; Tian et al., 2025; Zhang et al., 2022a; Zhang et al., 2024).

Aphids utilize specialized piercing-sucking mouthparts, known as stylets, to extract phloem sap from host plants. This feeding process facilitates bidirectional substance exchange between the insect and its host (Shih et al., 2023). Through stylet penetration, aphids acquire essential nutrients including carbohydrates, proteins, and nucleic acids from the phloem while simultaneously injecting salivary secretions into plant tissues (Cherqui and Tjallingii, 2000; Twayana et al., 2022). The aphid saliva contains various bioactive components, with salivary proteins representing an important group of molecules. These proteins comprise diverse functional types, including digestive enzymes, immunomodulatory factors, and other bioactive components (Pavithran et al., 2024b). In piercing-sucking insects, salivary proteins are delivered into plants along with the gel and watery saliva secreted by salivary glands (Chen and Mao, 2020; Walling, 2008). These proteins play crucial roles in facilitating digestion, metabolizing secondary compounds, detoxifying, and modulating plant defense responses (Furch et al., 2015; Guo et al., 2020; Huang et al., 2024). Salivary effectors can also enhance the post-transmission infections of arboviruses, including plasmodesmata-mediated cell-to-cell movement and phloem-mediated long-distance transport (Jones et al., 2021; Wang, 2021). A study has found that aphid-excreted carbonic anhydrase facilitates intercellular movement of plant viruses during post-transmission infection (Kloth and Kormelink, 2020). The salivary protein glucose dehydrogenase (GLD) of *Myzus persicae* accelerates systemic movement of

cucumber mosaic virus (CMV) via oxidizing plant sieve element occlusion (SEO) proteins, and the GLD can be selected as a candidate target to impair the viral post-transmission infection process (Guo et al., 2025).

Notably, salivary protein composition exhibits both interspecific variation among aphid species and intraspecific plasticity depending on host plant species (Cooper et al., 2011; Pavithran et al., 2024a; Vandermoten et al., 2014), reflecting their crucial role in the ongoing co-evolutionary arms race between aphids and their host plants. Studies have implied that the genes encoding salivary proteins in aphids are potential candidates for aphid control in plants through plant-mediated RNAi. For instance, RNAi-mediated knockdown of the effector protein Armet transcript significantly impaired the feeding behavior of pea aphids (*Acyrtosiphon pisum*) (Wang et al., 2015b). The dsRNA targeting salivary effector *Sm9723* significantly reduced aphid survival rates and fecundity while impairing feeding behavior (Zhang et al., 2022c). Similarly, silencing salivary effector *Sg2204* triggered a robust wheat defense response, further decreasing aphid survival, reproduction, and feeding efficiency. Notably, RNAi-mediated knockdown of *Sg2204* homologs across four additional aphid species consistently compromised their performance on host plants (Zhang et al., 2022b). Furthermore, transgenic wheat expressing dsRNA targeting the salivary protein gene *SmDSR33* led to increased mortality and reduced fecundity of aphids (Zhang et al., 2022a).

In this study, we identified a novel putative salivary peptide gene, *SmDSR32*, in the grain aphid (*Sitobion miscanthi*) based on our previous transcriptomic analysis and dsRNA artificial diet assay (Wang et al., 2015a). Then we constructed RNAi vectors and generated stable transgenic wheat lines expressing *SmDSR32*-dsRNA. The effects on aphids were experimentally evaluated through bioassays, with a specific focus on alterations in feeding behavior, lifespan, population growth rate, and mortality. Surviving aphids showed effective gene silencing, and their offspring exhibited reduced fitness when subsequently reared on wild-type plants, suggesting a carry-over effect across generations.

2 Materials and methods

2.1 Plants and insects

In this study, the hexaploid wheat variety *Triticum aestivum* L. cv Zhengmai 7698 (ZM7698) was utilized. A total of 30 to 35 seeds from both wild-type and transgenic lines (32-64, 32-188 and 32-494) were sown in pots and cultivated in a climate chamber set at $21 \pm 1^\circ\text{C}$ with a 16-hour light photoperiod and $50 \pm 10\%$ relative humidity.

Grain aphids (*S. miscanthi*) were reared on two-leaf stage aphid-sensitive wheat seedlings in a controlled environment similar to growth conditions of plants. Aphids from a single clonal line were continuously reared on wheat seedlings for 24 hours to produce synchronized nymphs. The adults were then removed, and their offspring were used for subsequent experiments. All experiments were conducted in a climate chamber under the conditions described above.

2.2 RNA extraction and characterization of *SmDSR32*

Total RNA was isolated from pooled aphid adults using TransZol Up reagent (TransGen Biotech, Beijing, China), and first-strand cDNA was synthesized with the

FastKing RT Kit (Tiangen, Beijing, China). The full-length *SmDSR32* coding sequence was amplified using TransStart® FastPfu DNA Polymerase (TransGen Biotech, Beijing, China) following the instructions. PCR products were sequenced by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (Beijing, China). The theoretical isoelectric point (pI) and molecular weight (MW) of *SmDSR32* were predicted using ExPASy (https://web.expasy.org/compute_pi/). Transmembrane domains and signal peptide regions were analyzed using TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), respectively. Homologous sequences of *SmDSR32* in other aphid species were retrieved via the Basic Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>) database. A phylogenetic analysis of *SmDSR32* nucleotide sequences from twelve aphid species was performed using MEGA X software (www.megasoftware.net). The phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replicates to assess branch support.

2.3 Vector construction and wheat transformation

To construct the RNAi vector targeting *SmDSR32*, a 411 bp gene-specific fragment was amplified using designed primers (Table 4-S1). A 320 bp fragment of the *GFP* gene was used as a negative control in the aphid bioassay. Before vector construction, the targeted *SmDSR32* fragment was analyzed using BLAST to evaluate its specificity and potential off-target effects. The analysis compared the fragment's sequences with the genomes of aphid non-target organisms, including wheat (taxid: 4565), human (taxid: 9606), and key aphid natural enemies. These natural enemies comprised *Chrysopa* (taxid: 76806), *Asaphes vulgaris* (taxid: 338020), Coccinellidae (taxid: 7080), Reduviidae (taxid: 27479), flower flies (taxid: 34680), Araneae (taxid: 6893), and Mantodea (taxid: 7504).

The PCR products were purified and inserted in an inverted repeat orientation into the *Spe* I/*Eco* RV and *Sac* I/*Hpa* I restriction sites of the pEasy-Blunt-Zero-*Adh1* vector, resulting in the hairpin construct Bzero-*SmDSR32-adh1-SmDSR32*. The resulting plasmid was digested with *Ssp* I and *Bsr* GI to release the expression cassette, which was subsequently recovered for transformation. The RNAi construct was driven by the maize *ubiquitin* (*Ubi*) promoter. Transformation was carried out via particle bombardment using immature embryos derived from the wheat cultivar ZM7698. Immature embryos (14-16 days post anthesis) were isolated and placed with scutellum side up on callus induction medium. Cultures were maintained in the dark at $25 \pm 1^\circ\text{C}$ for 4 weeks to induce somatic embryogenesis. The induced somatic embryos were then transferred to Murashige and Skoog (MS) regeneration medium and incubated under a 16-h light/8-h dark photoperiod at $25 \pm 1^\circ\text{C}$ for 2-3 weeks. Somatic embryos were induced on medium, and regenerated plants were grown and selected. Healthy transgenic seedlings were transferred to soil and cultivated to maturity for further analysis (Green and Sambrook, 2012).

2.4 Quantitative real-time PCR analysis

To assess the expression level of *SmDSR32* at various developmental stages, total RNA was extracted from grain aphid nymphs (four instars) and adults reared on

susceptible wheat. For expression analysis in response to different host plants, adult aphids fed on transgenic and wild-type wheat were collected for RNA isolation. To investigate the relative expression level of *SmDSR32-dsRNA* in different transgenic lines, total RNAs were isolated from one-week-old leaves of wild type and different transgenic wheat plants.

First-strand cDNA synthesis was performed using standard protocols. Quantitative real-time PCR (qRT-PCR) was conducted with SYBR® Green Real-time PCR Master Mix (Tiangen, Beijing, China) on an ABI 7300 Real-Time PCR System. The wheat *actin* gene, the aphid *actin* gene, and *ribosomal protein S27 A (Rps27A)* gene were used as internal reference genes, and *SmDSR32*-specific primers were employed for expression quantification (Table 4-S1). All treatments had three biological replicates, and each replicate consisted of three technical replicates. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with normalization to the geometric mean of the two reference genes (Livak and Schmittgen, 2002).

2.5 Southern blot analysis

Genomic DNA was isolated from young T3 plant leaf tissues using the CTAB method, following the protocol of Russell and Sambrook (Russell and Sambrook, 2001). A total of 35 µg of genomic DNA was subjected to digestion with restriction enzyme *Hind* III overnight. The resulting DNA fragments were separated by electrophoresis on a 0.8% agarose gel prepared in 1× TBE buffer, run at 60 V for approximately 12-16 hours. Following electrophoresis, DNA was transferred onto Hybond-N⁺ membranes (Amersham, UK). For probe labeling and hybridization, the digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) was employed according to the manufacturer's instructions. Prehybridization, hybridization, washing, and signal detection were all performed using the DIG system. DNA probes were generated using the primers SmDSR32S-F/R to amplify the target sequence for labeling (Table 4-S1).

2.6 Aphid bioassays

A single apterous adult grain aphid clonal lineage was reared on wheat seedlings in enclosed cages. To obtain synchronized offspring, the aphids were allowed to reproduce for a 24-hour period, after which the newly produced nymphs were collected and transferred onto fresh transgenic wheat plants.

T3 generation homozygous wheat lines expressing *SmDSR32*-dsRNA were used to assess their effects on aphid survival and reproductive performance. At the 3- to 4-leaf stage, each wheat line was infested with 20 newly hatched first-instar nymphs. Aphid mortality was monitored daily throughout the experiment. For each wheat line, 10 individual plants were used as a biological replicate. The entire experiment was independently repeated three times to ensure reproducibility.

Life history traits of the aphids were calculated based on standard demographic parameters: the net reproductive rate, $R_0 = \sum l_x \cdot m_x$, the mean generation time, $T = \frac{\sum x l_x m_x}{\sum l_x m_x}$, the intrinsic rate of increase, $r_m = (\ln R_0)/T$, and the finite rate of increase, $\lambda = e^{r_m}$. In the equations, l_x is the surviving rate to a specific age x , and m_x is the number of newborn nymphs produced by per live adult for a specific age x (Biondi et al., 2013).

2.7 Electrical penetration graph technique analysis

The probing and feeding behavior of apterous adult aphids on wheat was monitored using a Giga-8 DC EPG amplifier (EPG-Systems, Wageningen, Netherlands) within a Faraday cage. Synchronously aged adult aphids were first allowed to feed on either 32-188 transgenic wheat or control plants for 48 hours. Following this preconditioning, aphids were starved for 2 hours. Each aphid was then individually tethered via the dorsal thorax using water-soluble silver conductive paint to a flexible gold wire (18 μm in diameter, 2 cm in length). The aphids were subsequently placed on the adaxial surface of wheat leaves at the three-leaf stage. The opposite end of the gold wire (2 mm in diameter, 3 cm in length) was affixed to a copper wire using conductive silver glue, which was connected to the DC amplifier. A plant electrode was inserted into the soil to complete the electrical circuit. Each aphid's EPG signal was continuously recorded for 8 hours under illuminated conditions. A total of 8 individual recordings were obtained per treatment group. Signal analysis was performed using Stylet+ software (EPG-Systems). Feeding behavior was interpreted based on waveform patterns, following the methodology established by (Tjallingii, 1985; Tjallingii, 1994). The non-probing (np) waveform indicates that the stylet remains outside the leaf tissue. The pathway phase includes two distinct waveforms, waveform C, representing the intercellular movement of the stylet, and potential drops (pd), which correspond to brief intracellular punctures during this phase. Waveform G is associated exclusively with active sap uptake from xylem vessels. The phloem phase consists of two sequential components, E1, marking the initial secretion of saliva into the sieve elements, and E2, indicative of passive phloem sap ingestion. EPG recordings were further analyzed using the EPG-Excel data workbook developed by Sarria et al. (Sarria et al., 2009).

2.8 Statistical analysis

A two-tailed Student's *t*-test was employed to assess statistical differences between wild-type and transgenic wheat lines. Significance levels were set at 1% or 5%. The standard error of the mean (SEM) was derived from three independent biological replicates for each treatment. In the EPG assays, mean values and corresponding SEMs were calculated based on individual aphid recordings, and statistical comparisons were performed using the Student's *t*-test. All results are expressed as mean $\pm$ SEM.

3 Results

3.1 Characterization of *SmDSR32* gene in grain aphids

Based on transcriptomic profiling and a dsRNA feeding assay, a candidate gene designated *SmDSR32*, which encodes a salivary peptide in the grain aphid, was identified (Wang et al., 2015a). The full-length sequence of this gene spans 261 bp and is predicted to encode an 86-amino-acid putative salivary peptide (Figure 4-1A). Bioinformatic analysis revealed that the *SmDSR32* peptide has a predicted molecular weight (Mw) of 8254.84 Da and an isoelectric point (pI) of 5.74. Furthermore, the peptide is predicted to contain a signal peptide with a cleavage site between residues 22 and 23, alongside three transmembrane helices (Figure 4-1B, Figure 4-S1).

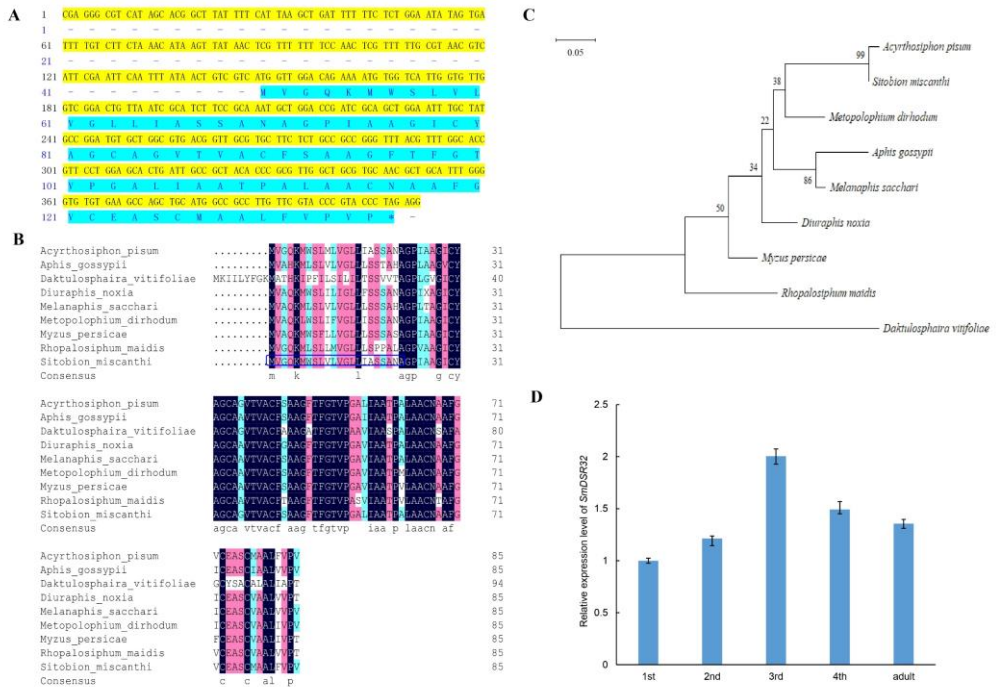


Figure 4-1. Characterization of *SmDSR32* and generation of wheat RNAi lines.

(A) The encoding sequence of *SmDSR32* and its deduced amino acid sequence. The sequences selected for construction of the RNAi vector are highlighted in yellow. (B) Multiple sequence alignment of *SmDSR32* protein and orthologs from other aphid species. The deduced amino acid sequences from eight aphid species include *Acyrtosiphon pisum* (XM_029487741.1), *Aphis gossypii* (XM_027982395.2), *Daktulosphaira vitifoliae* (XM_050677559.1), *Diuraphis noxia* (XM_015508058.1), *Melanaphis sacchari* (XM_025344765.1), *Metopolophium dirhodum* (XM_061021966.1), *Myzus persicae* (XM_022323304.1), and *Rhopalosiphum maidis* (XM_026966956.1). Black shades indicate identical amino acids. Pink shades indicate similar amino acid, and blue shades include the sequences with identical and similar residues. Signal peptide of *SmDSR32* is highlighted with blue box. (C) Phylogenetic tree of *SmDSR32* and its homologs from other aphid species constructed with the maximum likelihood method. Bootstrap supporting values (1000 replicates) are shown at the branch nodes. (D) The expression profile of *SmDSR32* in grain aphid at different development stages. The expression profiles of *SmDSR32* at different developmental stages of grain aphids fed on wheat. Values and error bars represent the mean and SEM of three independent biological replicates, each with a pool of 15 individual aphids.

To clarify the evolutionary relationships of this gene in different insect species, sequences of *SmDSR32* counterparts in pea aphid (*A. pisum*), cotton aphid (*Aphis gossypii*), grape phylloxera (*Daktulosphaira vitifoliae*), Russian wheat aphid (*Diuraphis noxia*), sugarcane aphid (*Melanaphis sacchari*), rose-grain aphid (*Metopolophium dirhodum*), peach aphid (*M. persicae*), and corn aphid (*Rhopalosiphum maidis*) were obtained by BLAST search against NCBI database.

The phylogenetic tree of SmDSR32 peptide was constructed using MEGA X software, with the resulting analysis revealing its closest relationship to orthologs from the pea aphid (*A. pisum*) (Figure 4-1C). Furthermore, the BLAST analysis showed that the 411 bp fragment of SmDSR32 did not share any contiguous 21-nucleotide sequences with known genetic sequences of aphid non-target organisms, including wheat, human, and key aphid natural enemies, indicating a low potential for off-target RNAi effects based on sequence homology (data not shown) (Bachman et al., 2013a).

The expression levels of *SmDSR32* across various developmental stages of the grain aphid were investigated using qRT-PCR, revealing that its transcript abundance accumulated to varying degrees throughout development (Figure 4-1D). The expression of *SmDSR32* reached its maximum in third-instar aphids, representing an approximate two-fold increase relative to the level observed in first-instar nymphs (Figure 4-1D).

3.2. In planta RNAi of *SmDSR32* in *S. miscanthi* via transgenic wheat expressing dsRNA

To assess the potential of *SmDSR32* as a target for dsRNA-mediated RNAi in aphid control, a 411 bp fragment of the gene was selected for hairpin RNAi vector construction (Figure 4-1A). A RNAi vector carrying *SmDSR32*-hairpin DNA was successfully engineered (Figure 4-2A). Following the transformation of immature wheat embryos (cultivar ZM7698), three independent transgenic lines (32-64, 32-188 and 32-494) were regenerated. Southern blot analysis confirmed the stable genomic integration of the transgene, with copy numbers varying from one to four (Figure 4-2B). The transcription levels and the relative expression levels of *SmDSR32*-dsRNA in different transgenic lines were investigated using RT-PCR and qRT-PCR. The result indicated that the transgenic line 32-494 had a higher dsRNA expression level than other two lines (Figure 4-2C, 2D). To evaluate whether the expression of *SmDSR32* in aphids was suppressed during feeding on transgenic wheat plants, one-day-old synchronized nymphs were individually transferred onto wild-type and transgenic wheat plants. Bioassay results revealed a significant suppression of *SmDSR32* expression ($P < 0.01$) in third-instar grain aphids fed on these transgenic lines compared to those on wild-type plants, confirming the successful induction of RNAi (Figure 4-2E).

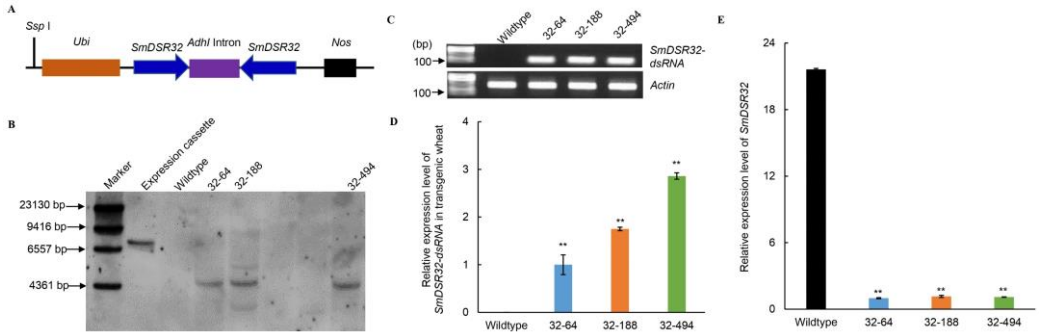


Figure 4-2. Generation and identification of wheat RNAi lines.

(A) A schematic diagram of the *SmDSR32* expression cassette and the position of *Ssp* I restriction enzyme. (B) Southern blot analysis of the transgenic wheat lines. Genomic DNA was digested with *Ssp* I and hybridized with a *SmDSR32* gene fragment with the expression cassette digested with *Ssp* I as a positive control. (C) RT-PCR analysis of *SmDSR32-dsRNA* expression in different transgenic lines. *Actin* served as a reference transcript. (D) Relative expression levels of *SmDSR32-dsRNA* in different transgenic lines. (E) Relative expression levels of *SmDSR32* of grain aphid fed on wild-type and transgenic wheat lines. The expression level of *SmDSR32* in the adult aphids fed on wild-type and different transgenic wheat lines after inoculation of one-day-old newborn nymphs, respectively. Values and error bars represent the mean and SEM of three independent biological replicates, each with a pool of 15 surviving individual aphids (Student's *t*-test, ** $P < 0.01$).

3.3. The impact of *SmDSR32* silencing on feeding behavior of *S. miscanthi*

To assess aphid feeding behavior, the transgenic wheat line 32-188 was selected for electropenetrography (EPG) assays. As illustrated in Figure 3A-F, *SmDSR32* silencing significantly impeded the feeding process. Compared to the control, aphids fed on transgenic plants exhibited a prolonged latency to first probe and spent significantly more time in non-probing (np) and C phases, indicating greater difficulty in locating suitable feeding sites (Figure 3-3A, 3B, 3C, 3D). Crucially, the duration of the sustained phloem ingestion (E2) phase was markedly shorter, demonstrating a compromised ability to acquire nutrients from the phloem (Figure 3-3F). The shortened E2 phase further reflects a decline in nutrient uptake efficiency. These findings collectively demonstrate that *SmDSR32* silencing disrupts key aspects of feeding behavior in grain aphids.

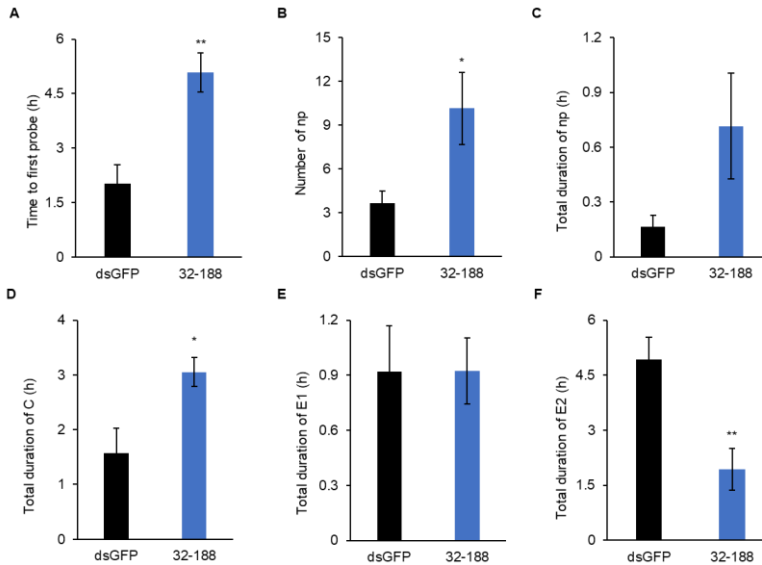


Figure 4-3. Feeding behavior of aphids fed on transgenic plants.

(A–F) Representative parameters of aphid feeding behavior. Non-probing (np), stylet probing (C), intracellular stylet puncture (pd), phloem salivation (E1), and phloem ingestion (E2). Data shown are mean $\pm$ SEM. Asterisks above bars indicate significant differences between controls and treatments (Student's *t*-test, * $P < 0.05$; ** $P < 0.01$).

3.4. Silencing *SmDSR32* via transgenic wheat impairs fitness of *S. miscanthi*

To evaluate the fitness consequences of *SmDSR32* silencing, key fitness parameters were compared between aphids reared on transgenic and wild-type wheat lines. Bioassays revealed a pronounced increase in mortality, exceeding 70% by 18 days after feeding (DAF) on transgenic plants (Figure 4-4A). While the adult preoviposition period (APOP) and total preoviposition period (TPOP) remained unaffected (Figure 4-S2), aphids on transgenic wheat exhibited significantly reduced longevity, along with shorter adult lifespan and reproductive duration ($P < 0.01$) (Figure 4-4B). Consequently, a significant decline in total fecundity was observed across all three transgenic lines compared to the wild-type control ($P < 0.01$) (Figure 4-4C).

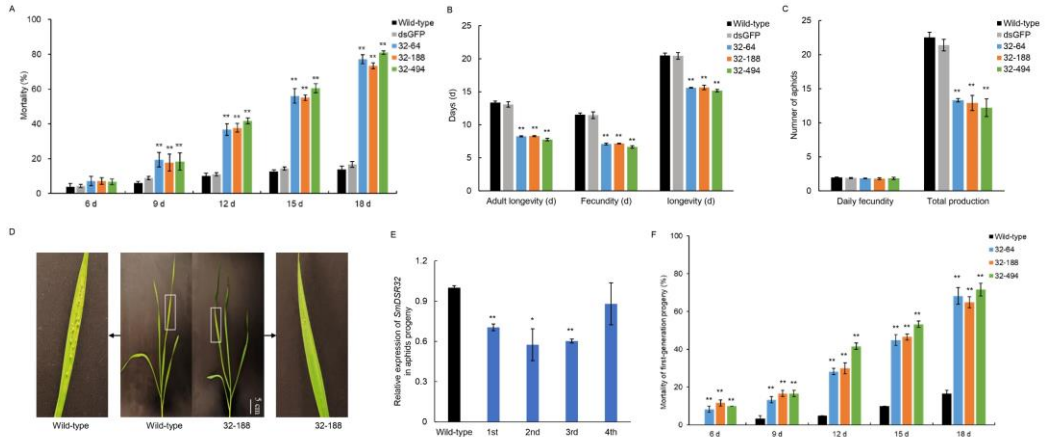


Figure 4-4. Fitness analysis of aphids fed on transgenic plants.

(A) Mortality of aphids fed on wild-type and transgenic wheat lines. The mortality of aphids fed on wild-type and *dsSmDSR32* expression transgenic wheat lines. Twenty synchronous one-day-old nymphs were put into clip cages individually on transgenic and wild-type wheat plants. All experiments were repeated three times. Values and bars represent the mean $\pm$ SEM (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$). (B) The adult longevity, fecundity and the total longevity of aphids fed on transgenic wheat lines and wild-type control. (C) The reproduction of aphids fed on transgenic wheat lines and the wild-type control. All experiments were repeated three times, each with 20 synchronous one-day-old nymphs. Values and bars represent the mean $\pm$ SEM (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$). (D) Phenotype of aphid resistance for wild-type (control) and 32-188 transgenic wheat plants. 10 synchronized one-day-old nymphs were placed on the leaf of plants. Photos were taken 12 days later. (E) The *SmDSR32* transcript levels of adult aphids were determined in fourth successive aphid generations. (F) The mortality of the first generation of the offspring of aphids fed on transgenic lines at different time points after being switched to wild-type plants. Values and bars represent the mean $\pm$ SEM (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$).

Collective analysis of key population parameters, including the net reproductive rate (R_0), mean generation time (T), the intrinsic rates of increase (r_m), the finite rate of increase (λ), and doubling time of the population (DT), revealed significant disparities between aphids reared on transgenic versus wild-type wheat lines (Table 4-1). Notably, R_0 , r_m , and λ were all significantly suppressed in aphids feeding on the transgenic plants ($P < 0.01$) (Table 4-1).

Table 4-1. Life table parameters of aphids fed on wild-type and different transgenic wheat lines.

Parameters	Wild-type	32-64	32-188	32-494
R_0	21.27±0.52	10.21±0.36**	9.50±0.41**	8.99±0.56**
T	14.49±0.30	13.51±0.21	13.23±0.28*	13.10±0.24*
r_m	0.21±0.01	0.17±0.00**	0.17±0.01**	0.17±0.01*
λ	1.24±0.01	1.19±0.01**	1.19±0.01**	1.18±0.01**
DT	3.29±0.08	4.04±0.11**	4.10±0.16*	4.17±0.20*

All data are expressed as means ± SEM based on 3 repeated experiments. R_0 , net reproductive rate; r_m , the intrinsic rate of increase; λ , the finite rate of increase; T, the mean generation time; DT, Doubling time (day). Student's *t*-test, *n*=3, **P*<0.05, ***P*<0.01.

3.5. Dietary RNAi induces persistent fitness effects across generations in *S. miscanthi*

To investigate whether the effects of *SmDSR32* silencing persist across generations, newborn nymphs from a parallel experiment were analyzed. Offspring derived from aphids fed on transgenic wheat exhibited constitutively suppressed *SmDSR32* expression and high mortality (Figure 4E, 4F), even when reared exclusively on wild-type plants. This suppression persisted from the first to the fourth generation (Figure 4E).

4 Discussion

Plant-mediated RNAi is widely regarded as a promising approach for engineering insect-resistant crops, notably for controlling wheat aphids, with important implications for global food security, human health, and agroecosystem sustainability (Saakre et al., 2024; Yu et al., 2016; Zhang et al., 2025). HIGS, also known as host-mediated RNAi, involves the production of dsRNA molecules inside transgenic plants that target essential genes of insect pests. In our study, we expressed a hairpin RNA (hpRNA) complementary to the *SmDSR32* transcript in wheat. The plant's intrinsic RNAi machinery processes this hpRNA into small interfering RNAs (siRNAs) or microRNAs (miRNAs), depending on the secondary structure of the dsRNA transcript. When aphids feed on the transgenic phloem, they ingest these processed siRNAs together with some unprocessed precursor dsRNAs (Koch and Wassenegger, 2021). Upon entry into the aphid gut cells, these RNA molecules trigger the insect's own RNAi pathway which results in the silencing of the targeted gene (Saakre et al., 2024). Here, we successfully validated the salivary peptide gene *SmDSR32* of *S. miscanthi* as an effective RNAi target, and demonstrated that RNAi wheat based on HIGS strategy could significantly impact aphid feeding behavior and fitness. Selecting an insect-specific salivary peptide gene *SmDSR32* as the target minimizes potential risks to humans and other non-target organisms, because the target fragment did not share any contiguous three 21-nucleotide sequences with the known genetic sequences of these organisms (Bachman et al., 2013b). Our results confirmed that feeding on RNAi wheat effectively silenced the target gene in aphids (Figure 4-2E). Compared to aphids on wild-type plants, those on RNAi lines showed

significantly reduced *SmDSR32* expression levels, which directly led to a sharp decline in their survival rate, fecundity, and reproductive capacity (Figure 4-4A, 4B, 4C). This finding is consistent with numerous previous studies concluding that silencing key essential genes in insects, particularly those involved in feeding and digestion, can effectively impair pest survival and reproduction (Zhang et al., 2022a; Zhang et al., 2022b; Zhang et al., 2022c).

EPG analysis provided direct behavioral evidence for the physiological mechanism underlying *SmDSR32* silencing. Aphids feeding on RNAi plants exhibited prolonged durations of non-probing (np) and intercellular probing (C-wave), and substantially shortened durations of passive phloem sap ingestion (E2-wave) (Figure 4-3). The E2 reduction is a direct measure of impaired sap uptake from sieve elements. This indicates that silencing *SmDSR32* severely impairs the aphid's ability to locate and establish a rewarding feeding site. The aphids required more time for probing to find a suitable feeding location but struggled to maintain sustained sap ingestion. Since *SmDSR32* is a putative salivary peptide, we hypothesize that it is secreted into sieve elements during the E1 (salivation) phase and normally facilitates sustained sap flow—perhaps by suppressing sieve tube occlusion or by modulating plant defense responses such as callose deposition (Furch et al., 2015; Walker, 2022). These results provide direct *in vivo* behavioral evidence linking a specific salivary gene to phloem ingestion efficiency. This alteration in feeding behavior closely correlates with the observed reduction in aphid fitness, further underscoring the critical role of salivary components in aphid-plant interactions (Adhikari et al., 2019; Åhman et al., 2019; Liu et al., 2025; Thompson and Goggin, 2006).

Furthermore, not only did the aphids directly feeding on transgenic plants incur fitness costs, but their offspring also continued to exhibit reduced fitness, even when reared on wild-type plants. By the fourth generation, *SmDSR32* expression returned to normal (Figure 4E-F). This transgenerational RNAi effect has been reported in aphids and likely results from persistent siRNAs in the germline or early embryos (Coleman et al., 2015). The absence of an RNA-dependent RNA polymerase (RdRP) in insect limits amplification, which explains why the effect fades without continuous dsRNA exposure. This multiplicative effect significantly enhances the practical application potential of HIGS technology, as even short-term or partial feeding could exert long-term, profound inhibitory impacts on pest populations (Abdellatef et al., 2015; Zand Karimi and Innes, 2022).

Selecting an insect-specific salivary peptide gene *SmDSR32* as the target minimizes potential risks to humans and other non-target organisms, because the target fragment did not share any contiguous three 21-nucleotide sequences with the known genetic sequences of these organisms (Bachman et al., 2013a). Moreover, the world's first sprayable RNA-based biopesticide "Calantha" marks a milestone breakthrough in the field of crop protection. Field trials have demonstrated that Calantha not only effectively controls pest populations but also exhibits rapid degradation and high environmental safety, with no significant impact on non-target organisms (Pallis et al., 2023). Our transgenic HIGS approach provides an alternative delivery platform that could be integrated with sprayable dsRNA or stacked with other RNAi targets.

While our results are promising, the exact molecular function of *SmDSR32*—whether it binds to specific plant receptors, modifies sieve tube proteins, or inhibits callose

deposition-requires direct biochemical investigation. Besides, translating this technology into practical applications requires consideration of potential challenges. For instance, continuous monitoring is needed to assess whether aphid populations could develop RNAi resistance through target gene mutations (Yoon et al., 2020). Furthermore, stacking *SmDSR32* dsRNA with dsRNAs targeting other genes could build a more robust and durable multi-gene pest resistance system (Yang et al., 2023). Additionally, the combination of entomopathogenic fungus *Metarhizium anisopliae* and virus-induced gene silencing-RNAi (VIGS-RNAi) is also an efficient strategy for managing herbivorous pests (Shi et al., 2025). Finally, evaluating the performance of our transgenic wheat lines under diverse field conditions across different wheat genetic backgrounds will be essential before commercial deployment.

5 Conclusions

In this study, we generated transgenic wheat lines expressing *SmDSR32*-dsRNA using a HIGS approach. EPG analysis revealed that aphids fed on transgenic plants exhibited prolonged non-probing and pathway phases (np, C) and drastically shortened phloem ingestion (E2). Silencing *SmDSR32* significantly reduced aphid survival, fecundity, and population growth. Furthermore, the suppressed gene expression and high mortality persisted in offspring even without continuous exposure to transgenic plants.

In conclusion, our study demonstrates that *SmDSR32* is a highly effective RNAi target for controlling the grain aphid, *S. miscanthi*. Silencing this gene via HIGS technology impairs aphid fitness by disrupting their feeding behavior and induces transgenerational effects. This lays a solid theoretical foundation and provides technical support for developing novel and sustainable wheat aphid management strategies. Future work should focus on elucidating the molecular interaction of *SmDSR32* with host defense regulators, developing multi-target RNAi stacks, testing field performance across different wheat cultivars, and integrating HIGS with sprayable RNAi biopesticides.

6 References

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Supporting Information

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Figure 4-S1. The predicted signal peptide and transmembrane helix of SmDSR32.

Figure 4-S2. The longevity of different stages, adult preoviposition period (APOP) and total preoviposition period (TPOP) of aphids fed on transgenic lines and wild-type control.

Table 4-S1. Primers used in this study.

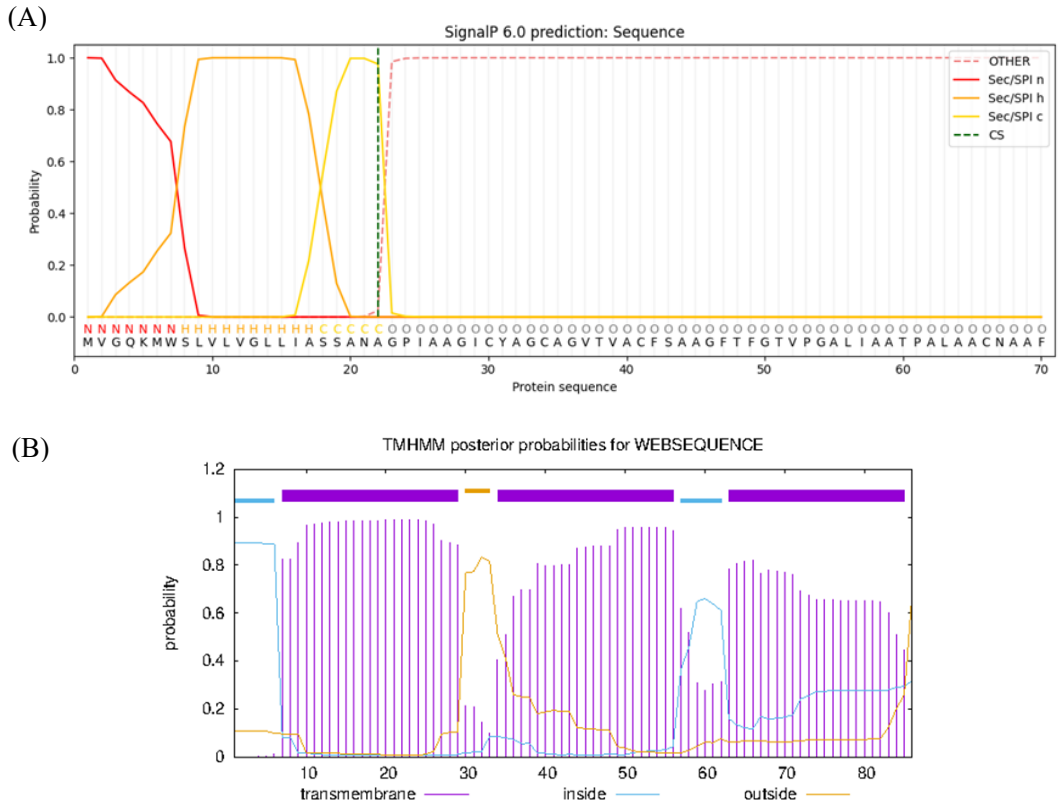


Figure 4-S1. The predicted signal peptide and transmembrane helices of *SmDSR32*.

(A) The predicted signal peptide of *SmDSR32*. The cleavage site is between residues A20N21A22-G23P24.

(B) The predicted transmembrane helices of *SmDSR32*.

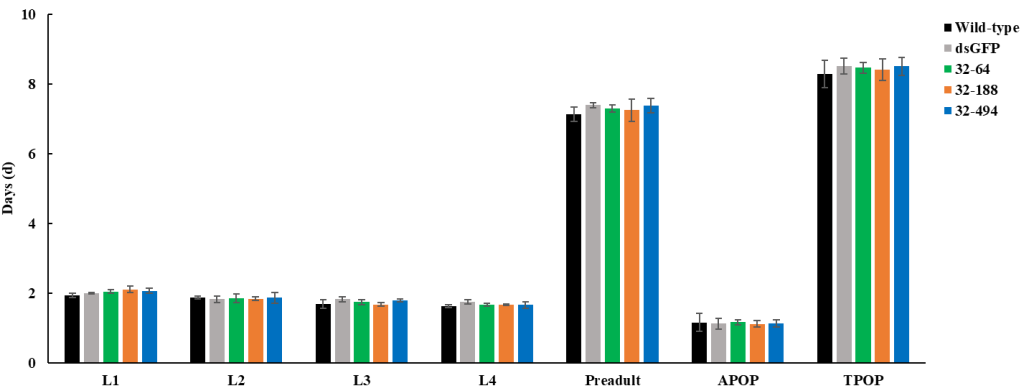


Figure 4-S2. The longevity of different stages, adult preoviposition period (APOP) and total preoviposition period (TPOP) of aphids fed on transgenic lines and wild-type control.

L1-L4: first-instar to fourth-instar aphids.

Table 4-S1. Primers used in this study

Primer	Sequence (5' to 3')	Use of PCR products
SmDSR32c-F	ATGGTTGGACAGAAAATGTGGTC	Full length cDNA cloning
SmDSR32c-R	CTAGGGTACGGGTACGAACA	
SmDSR32S-F	AGGTCGACTCTAGAGGATCCCCGGGGGATC CACTAGTCGAGGGCGTCATAGCACGG	Construct vector for RNAi
SmDSR32E-R	GGCCTCCCAGATCGATTGATATCCCTCTAG GGTACGGGTACGAAC	
SmDSR32H-F	AGGGGCAGACTCCCGTTTGTTAACCCTCTAG GGTACGGGTACGAAC	
SmDSR32S-R	TGAACGATCGGGGAAATTCGAGCTCCGAGG GCGTCATAGCACGG	Construct vector for RNAi
WactinF	TCAGTGGAGGTTCTACCATGTTTC	Internal control of qRT-PCR for wheat
WactinR	CATTATTTTCATACAGCAGGCAAGC	
qactin-F	CGGTTCAAAAACCCAAACCAG	Internal control of qRT-PCR for aphid
qactin-R	TGGTGATGATTCCCGTGTTTC	
Rps27-F	TGTGAAGACGTTGACTGGGAAA	Internal control of qRT-PCR for aphid
Rps27-R	CGTTGCTGATCCGGAGGAATAC	
SmDSR32S-F	CGACGAGTCTAACGGACACC	Southern blot
SmDSR32S-R	CCCTAACCATGGACCGGAAC	
SmDSR32q-F	GTGGTCATTGGTGTTGGTCG	Detect <i>SmDSR32</i> in aphids
SmDSR32q-R	AACGGTGCCAAACGTAAACC	
Ubi-F	GCCTTCATACGCTATTTATTTGCTTGGTAC	Detect <i>SmDSR32</i> dsRNA in transgenic wheat
ADH1-R	GGAGTCTGCCCCTAAGACAGATAAGC	
ADH1-F	CTGGGAGGCCAAGGTATCTAATCAGC	Detect <i>SmDSR32</i> dsRNA in transgenic wheat
Nos-R	CGCAAGACCGGCAACAGGATTC	

Chapter V

General discussion, conclusions and perspectives

1 Discussion, conclusions and perspectives

1.1 Discussion and conclusions

This thesis is based on two highly complementary studies that employed host-induced gene silencing (HIGS) to manage the grain aphid *S. miscanthi* on wheat. The first study targeted *Smrrl*, a gene up-regulated in the obligate endosymbiont *B. aphidicola* during aphid feeding. The second one focused on *SmDSR32*, a novel aphid-specific gene encoding a salivary peptide. Although the two targets are fundamentally different in biological function-*Smrrl* participates in nutrient synthesis and supply via mutualistic symbiotic bacteria, and *SmDSR32* functions as an aphid effector to suppress plant defense responses-both approaches produced consistent and significant anti-aphid phenotypes. Together, these two independent lines provide strong evidence that plant-mediated RNAi is a viable and sustainable strategy for aphid control in wheat.

Silencing either *Smrrl* or *SmDSR32* significantly suppressed multiple key population fitness parameters of grain aphids, including reduced survival, decreased fecundity, shortened lifespan and lower progeny production. Targeting the symbiont gene *Smrrl* caused relatively rapid mortality, presumably due to an abrupt interruption of essential amino acid supply. In contrast, targeting the salivary peptide *SmDSR32* appeared to progressively impair aphid fitness through disrupting normal feeding behaviors. This differential inhibitory pattern, pending verification under standardized controlled conditions, provided targeted guidance for the practical deployment of HIGS transgenic materials: symbiont-targeted lines might be preferable for rapidly suppressing outbreak populations, whereas lines targeting aphid salivary effectors might provide longer-lasting, more subtle population suppression with potentially lower selective pressure on non-target organisms.

Electrical penetration graph (EPG) analysis demonstrated consistent alterations in aphid feeding behaviors in both treatments: silenced aphids showed prolonged intercellular probing (non-feeding phases) and drastically shortened phloem ingestion. This convergent phenotypic change indicated that regardless of the distinct molecular regulatory pathways, both strategies ultimately interfered the aphid's ability to recognize, access or continuously feed from phloem sap. From a physiological perspective, *Smrrl* silencing inhibited the nutrient synthesis function of *Buchnera*, thereby indirectly impairing aphid salivary gland activity and energy metabolism, which in turn prevented effective suppression of plant sieve-tube defenses, such as callose deposition or phloem protein plugging (Wang et al., 2020). *SmDSR32* silencing directly deprived aphids of a core effector that counteracts plant phloem defenses (Guo et al., 2020; Ma et al., 2024). In both cases, wheat phloem tissues maintain a defensive, non-permissive state, forcing aphids to engage in frequent but ineffective probing attempts. This hypothesis can be tested in future studies by comparing callose deposition, phloem protein profiles and salivary proteomes between the two transgenic lines and wild-type controls.

Both studies reported that the silencing effect persisted beyond the directly exposed generation. From an applied perspective, such transgenerational suppression is highly attractive because it would extend the effective protection window even after aphids migrate away from transgenic plants. However, it is essential to emphasize that the

current data do not prove that the observed transgenerational fitness defects are caused by heritable RNAi. Alternatively, non-genetic explanations are equally plausible: (1) maternal physiological debilitation-aphids that have fed on transgenic plants may produce fewer or lower-quality eggs simply because they were starved or stressed (Trotta et al., 2024); (2) irreversible somatic damage-prolonged silencing of *Smrrl* might cause lasting damage to the symbiont population, which is vertically transmitted, so the offspring inherit a reduced symbiont load rather than a specific RNA signal (Kashkouli and Khajehali, 2025); (3) residual dsRNA/siRNA carryover-small amounts of dsRNA might be present on the egg surface or inside the egg but do not represent genuine germline inheritance. Therefore, the mechanism of transgenerational suppression remains an open question. Only after multi-generational rearing experiments on dsRNA-free plants with systematic detection of target gene expression, small RNA profiles, and epigenetic modifications can we clarify the underlying regulatory pathway.

No obvious negative effects of RNAi lines on wheat growth, development, or yield were observed in our studies. However, the potential indirect effects of HIGS on wheat physiological defense and ecological interactions cannot be ignored. For *Smrrl*-targeted HIGS, although intracellular *Buchnera* within aphids cannot directly trigger plant immune responses, the prolonged probing and repeated stylet punctures caused by the silencing may physically induce local defense responses, such as reactive oxygen species bursts and callose deposition, which could enhance basal resistance (Nalam et al., 2019). For *SmDSR32*-targeted HIGS, silencing eliminates the key effector that suppresses wheat phloem defense, thereby fully activating the plant's intrinsic occlusion response to aphid feeding (Walker, 2022). Future studies should quantify phloem-specific defense markers (defense gene expression, salicylic acid and jasmonic acid contents) in transgenic wheat lines before and after aphid infestation. Additionally, the metabolic cost of constitutive dsRNA expression should be assessed under field conditions, especially when high-copy transgenes or strong constitutive promoters are used, as excessive transgene expression may cause unnecessary plant fitness consumption and affect field yield.

Compared with currently dominant aphid management strategies, HIGS technology exhibits unique advantages and inherent limitations in practical agricultural applications. In China, the grain aphid is mainly controlled by neonicotinoids and pyrethroids. While these chemicals are fast-acting, they suffer from resistance development, non-target toxicity to natural enemies and environmental persistence (Gong et al., 2021; Liao et al., 2024; Xu et al., 2022). HIGS offers high sequence specificity: *Smrrl* has no homologues in free-living bacteria, and *SmDSR32* appears to be aphid-specific, making the risk to pollinators, predators and soil microbiota theoretically low. HIGS is more flexible because the target can be switched or multiple targets can be combined (Dong et al., 2024). Also, HIGS has clear drawbacks: (1) the long development and regulatory timeline for transgenic crops, (2) public acceptance issues in many regions, (3) variability in dsRNA expression levels among independent transformation events due to position effects and epigenetic silencing, and (4) a relatively slow onset of action compared to chemical sprays. In summary, HIGS is best positioned as a long-term, preventive component of integrated pest management (IPM) rather than a direct replacement for fast-acting chemical controls.

Several limitations of the study should be acknowledged. Firstly, all experiments were conducted under controlled greenhouse or growth-chamber conditions; field validation is missing. Factors such as UV radiation, temperature fluctuations, rainfall and microbial degradation could affect dsRNA stability and HIGS efficacy in the field. Secondly, non-target safety assessments have not been performed. Although sequence analyses suggest low off-target risk, empirical data on soil microbiomes, rhizosphere nematodes and natural enemies are required. Thirdly, the constitutive Ubi promoter used in both studies drives dsRNA expression in all tissues, which may be unnecessary and could increase metabolic load. Phloem-specific or aphid-inducible promoters might achieve equivalent or better control with lower fitness costs. Fourthly, no long-term selection experiments have been conducted to examine whether aphids or their symbionts can evolve resistance to HIGS. Resistance mechanisms could include target-site mutations, enhanced dsRNA degradation by gut nucleases, or reduced uptake of dsRNA. Proactive research on HIGS resistance mechanisms and supporting management strategies is essential for the sustainable application of this technology.

Taken together, the two studies provide the systematic proof-of-concept that HIGS targeting either a symbiont gene or an aphid salivary effector gene can effectively control the grain aphid on wheat. Both target genes mediate aphid population suppression by disrupting phloem feeding behavior, and both reveal transgenerational fitness defects (though the mechanism remains to be elucidated). By comparing two independent functional nodes in the wheat-aphid-symbiont tripartite interaction system, this study confirms multiple vulnerable targets for RNAi-mediated aphid control. These findings lay a solid foundation for developing environmentally friendly, sustainable aphid management strategies and provide valuable genetic resources for future breeding efforts.

1.2 Perspectives

1.2.1 Multi-target stacking for synergistic control

Given that *Smrrl* and *SmDSR32* act through complementary mechanisms (nutritional symbiosis disruption and feeding defense inhibition), stacking both dsRNAs in a single transgenic wheat line represents a promising strategy. A multi-target cassette, for example, a fused fragment containing parts of both genes driven by a bidirectional promoter, could simultaneously impair symbiont function and effector secretion. This would not only enhance overall efficacy but also delay the evolution of resistance, because aphids would need to overcome two independent silencing effects simultaneously. Additional targets could be added, such as chitin synthase genes, ecdysone receptor genes or dsRNA uptake genes. The principle of pyramiding different RNAi targets has already shown promise in insect-crop systems (Salim et al., 2020).

1.2.2 Elucidating the mechanism of transgenerational effects

The transgenerational fitness defects observed in this study are intriguing but mechanistically unresolved. To distinguish genuine heritable RNAi effects from speculative non-genetic maternal carryover effects, a standardized multi-generational verification experiment needs to be implemented. The core experimental design is to rear aphids on HIGS plants for one generation, then transfer them to wild-type plants for three or more consecutive generations. Dynamic detection of target gene expression, small RNA accumulation profiles, and genomic epigenetic modifications

across generations will contribute to clarify the inheritance pattern of silencing effects (Burton and Greer, 2022; Duempelmann et al., 2020; Rieger et al., 2023). Moreover, because *Buchnera* is vertically transmitted, silencing *Smrrl* might reduce symbiont titer or function in the maternal body, and this reduced symbiont load could be passed directly to progeny through the egg, independent of the aphid's own RNAi machinery (Koga et al., 2012; Xin Yee Tan and Shigenobu, 2025). This possibility can be tested by quantifying symbiont density (by qPCR of *Buchnera* genes) in progeny from silenced versus control mothers.

1.2.3 Comprehensive environmental safety and non-target assessment

Non-target ecological risk is a key prerequisite for the field promotion and application of HIGS technology, which requires systematic and empirical verification rather than speculative inference. Before field release, thorough environmental risk assessment is necessary. Firstly, soil microbial safety assessment: high-throughput sequencing (16S rRNA for bacteria, ITS for fungi) of rhizosphere soils from transgenic versus non-transgenic wheat, with special attention to bacteria that harbor sequences homologous to *Smrrl* or *SmDSR32*. Secondly, non-target arthropods: standard laboratory toxicity tests on common natural enemies (*Harmonia axyridis*, *Chrysoperla sinica*, *Episyrphus balteatus*) and pollinators (*Apis mellifera*) using pollen, phloem sap or whole aphid prey reared on transgenic plants. Thirdly, trophic transfer: feed HIGS-reared aphids to predatory insects and assess survival, development and reproduction of the predator. Although sequence specificity argues for low risk, empirical validation remains essential.

1.2.4 Resistance management strategies for HIGS

Resistance evolution is an inevitable challenge for all pest control technologies, and HIGS has multiple potential resistance mechanisms that require targeted preventive management. Potential resistance mechanisms in aphids include: point mutations in the target sequence that abolish siRNA binding, down-regulation or mutation of dsRNA uptake pathways, up-regulation of dsRNA-degrading nucleases in the gut or hemolymph, and compensatory gene duplication or mutation in *Buchnera* (Bass and Nauen, 2023; Chong and Moran, 2018; Farhan et al., 2025; Han et al., 2025; Mishra and Jurat-Fuentes, 2025; Munkhbayar et al., 2024; Silva et al., 2012). To delay resistance, three targeted management strategies are proposed: (1) Pyramiding and rotation: deploy multi-target HIGS lines, or rotate different single-target lines across seasons (Fuchs, 2017; Rimbaud et al., 2018). (2) High-dose/refuge strategy: ensure that dsRNA expression is sufficiently high to kill most heterozygous resistant individuals and plant a non-transgenic refuge to maintain susceptible alleles (Brewer and Bonsall, 2021; Campagne et al., 2016; Zhou et al., 2018). (3) IPM integration: combine HIGS with biological control (releases of natural enemies), cultural practices (crop rotation, trap crops) and, as a last resort, selective chemical insecticides that are compatible with the overall programme (Castellanos et al., 2019; Ni et al., 2017; Zhang et al., 2020).

1.2.5 Optimizing dsRNA expression and delivery systems

To address the limitations of transgenic technology in popularization, non-transgenic RNAi delivery systems and tissue-specific expression optimization are effective improvement strategies. Nanocarriers such as layered double hydroxide (LDH) clay nanosheets or chitosan nanoparticles can be used to deliver *in vitro*

synthesized dsRNA via foliar spray or root irrigation. This approach would provide temporary, season-long control without releasing transgenic organisms into the environment (Cheng et al., 2024; Tang et al., 2024). For the transgenic route, tissue-specific or inducible promoters should replace constitutive ones. Phloem-specific promoters (*AtPP2*, *AtSUC2*) or jasmonate-inducible promoters would restrict dsRNA production to the exact site and time of aphid feeding, reducing plant metabolic costs and off-target risks (Zheng and Baum, 2008). Additionally, aphid-specific viral vectors can be modified to deliver RNAi constructs in a more efficient and specific manner (Hao et al., 2025; Huang et al., 2024). In natural environments, where pests are exposed to fluctuating conditions, the longevity of RNAi effects may vary (Burc et al., 2025; Guan et al., 2025; Rokosh et al., 2025). Therefore, future research should focus on developing more stable RNAi constructs that sustain bioactivity throughout the growing season. This could involve engineering plants that express dsRNA in response to specific environmental cues or using inducible systems that allow for temporal control of gene silencing (Christiaens et al., 2020a; Christiaens et al., 2020b; Duanmu et al., 2025).

1.2.6 Cross-species conservation and broader applications

The RNAi strategy targeting symbiont and salivary effector genes has broad cross-crop and cross-pest application potential. Beyond wheat and *S. miscanthi*, this technical framework can be extended to major aphid pests including *Aphis gossypii* (cotton aphid), *Myzus persicae* (green peach aphid), and *Schizaphis graminum* (greenbug) and other major aphid pests that cause significant damage to various crops worldwide. Given the shared genetic makeup of aphid species, it is plausible that similar RNAi strategies could be applied to other economically important crops such as cotton, tobacco, and vegetables (Huang et al., 2025; Shigenobu and Yorimoto, 2022). Expanding RNAi-based pest control to multiple crops would further reduce the reliance on chemical pesticides, contributing to more sustainable agricultural practices on a global scale (Mahanta et al., 2025; Willow and Smagge, 2025). Moreover, the strategy of targeting obligate symbionts or salivary effectors can be extended to other piercing-sucking pests, including whiteflies (*Bemisia tabaci*), planthoppers (*Nilaparvata lugens*) and psyllids (*Diaphorina citri*), all of which rely on symbionts and effectors for successful feeding.

1.2.7 Field validation, scale-up application and regulatory system improvement

While laboratory and greenhouse experiments show promising results, large-scale field trials are essential to confirm the viability of RNAi as a practical pest management tool in commercial agriculture (Koch, 2023; Mead-Briggs, 1998). In addition to technical optimization, the industrial promotion of HIGS requires improved supporting regulatory frameworks. Formulating transparent and unified safety evaluation standards for RNAi transgenic crops will standardize the risk assessment of food safety and environmental ecology, eliminating policy bottlenecks for large-scale commercial application (Gunasekara et al., 2025; Koch, 2023; Rinaldi et al., 2025; Zhao et al., 2024). This requires collaboration between researchers, agricultural practitioners, and policymakers to realize the industrialization and popularization of green pest control technology.

1.2.8 Ethical and socio-economic considerations for equitable adoption

Finally, it is important to consider the ethical and socio-economic implications of

using RNAi for pest control. Technically, HIGS reduces chemical pesticide consumption, lowering planting costs and pesticide exposure risks for smallholder farmers (Brunelle et al., 2024). Conversely, the patent monopoly of RNAi constructs and transgenic materials may lead to uneven technological access, restricting the equitable popularization of green pest control technology (Mat Jalaluddin et al., 2019). Future research and policy formulation need to balance technological innovation and social equity, optimize intellectual property and promotion mechanisms, and ensure that sustainable RNAi pest control technology benefits all agricultural producers.

In conclusion, this study provides strong evidence for the potential of HIGS strategy targeting aphid genes *Smrrl* and *SmDSR32*. The results demonstrate that HIGS cause significant alterations in aphid feeding behavior, while also effectively reduce aphid survival, fecundity, and population growth. Moreover, the transgenerational effects observed in this study suggest that HIGS can have long-lasting impacts on aphid populations. As a novel, precise, and sustainable pest management strategy, HIGS technology overcomes the limitations of traditional chemical prevention and control, providing a new solution for green wheat production and food security guarantee. With continuous technical optimization, field verification, and standardized industrial regulation, HIGS strategy will become a core component of modern sustainable agricultural IPM systems.

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Appendices

List of publications

Accepted publications

1. **Xue Zhong**[#], Xiudao Yu[#], Jiahui Zhang[#], Jiajing Xu, Mengchao Qin, Mingxin Cao and Frédéric Francis and Lanqin Xia, RNAi technologies for insect control in crop protection. *The Crop Journal*, 2025. Doi: <https://doi.org/10.1016/j.cj.2025.05.006>.
2. Jiahui Zhang[#], **Xue Zhong**[#], Mingxin Cao, Jiajing Xu, Mengchao Qin, and Frédéric Francis and Lanqin Xia, Host-induced gene silencing of *SmDSR32* enhances wheat defense against *Sitobion miscanthi*. *Current Issues in Molecular Biology*, 2026. Doi: <https://doi.org/10.3390/cimb48050523>.
3. Chen Zhang, **Xue Zhong**, Shaoya Li, Lei Yan, Jingying Li, Yubing He, Yong Lin, Yangjun Zhang and Lanqin Xia, Artificial evolution of *OsEPSPS* through an improved dual cytosine and adenine base editor generated a novel allele conferring rice glyphosate tolerance. *Journal of Integrative Plant Biology*, 2023. Doi : [10.1111/jipb.13543](https://doi.org/10.1111/jipb.13543).

Under review

4. **Xue Zhong**[#], Yongwei Sun[#], Jiajing Xu, Mengchao Qin, Mingxin Cao, and Frédéric Francis and Lanqin Xia, Manipulation of a novel endosymbiont gene for wheat aphid control through plant-mediated RNA interference.