Research Article

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Target gene selection for sprayable dsRNA-based biopesticide against *Tetranychus urticae* Koch (Acari: Tetranychidae)

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Abstract

BACKGROUND: Because of the excessive use of synthetic chemicals, the two-spotted spider mite, *Tetranychus urticae* Koch, a highly polyphagous pest, has developed comprehensive resistance to a broad spectrum of pesticides with diverse modes of action, raising severe concerns over agroecosystems and human health. To resolve this emerging issue, we initiated a project to develop double-stranded RNA (dsRNA)-based biopesticides against *T. urticae*, aiming for a species-specific and sustainable pest management alternative.

RESULTS: To examine the uptake of dsRNAs using the egg-soaking delivery method, we fluorescently labeled extraneous dsRNAs, and later showed that *T. urticae* dsRNAs can permeate through eggshell in a time-dependent manner within the first 24 h. For target gene screening, silencing of *Prosbeta-1* and *-5* resulted in the highest mortality (>90%) and a dark body phenotype in *T. urticae*. Notably, each target gene was effective in both avermectin laboratory susceptible and field resistant populations. As such, *Prosbeta-5* was selected as the candidate target gene for subsequent spray-induced gene silencing (SIGS). After two rounds of spray at day 5 and day 12, SIGS led to a substantial suppression of *T. urticae* populations (>90%).

CONCLUSION: Our combined results suggest viable molecular targets, confirm the feasibility of SIGS against *T. urticae*, and lay the foundation for the development of dsRNA-based biopesticides to control this devastating pest.

Supporting information may be found in the online version of this article.

Keywords: *Tetranychus urticae*; molecular target; egg-soaking method; dsRNA-based biopesticides; spray-induced gene silencing

1 INTRODUCTION

Two-spotted spider mite, *Tetranychus urticae* Koch, is known as one of the most destructive polyphagous pests worldwide, infesting more than 1100 plant species, including vegetables, fruits, maize, and cotton, as well as various agricultural systems such as orchards, greenhouses, and gardens.[1–3] *T. urticae* exhibits various physiological and biological traits, including high fecundity, haplo-diploid sex determination, a very short life cycle resulting in many generations per year, and the ability to produce silk-like webs. These webs facilitate the mite's movement from one plant to another, contributing to its rapid dispersal and colony establishment. All these traits contribute to the devasting damage the mite causes to plants and global agriculture.[1,4] Currently, *T. urticae* management depends heavily on chemical acaricides.[2,5,6] Excessive use of synthetic chemicals, however, has led to the development of resistance in *T. urticae*.[7,8] Challenging issues of resistance coupled with negative impacts on agroecosystems have led to the exploration of alternative pest management strategies against *T. urticae*.

RNA interference (RNAi) is a gene-regulatory mechanism that utilizes exogenous RNA molecules, such as double-stranded RNA (dsRNA), to induce specific gene suppression in eukaryotic organisms.[8,9] In arthropods, RNAi can achieve high species selectivity and efficacy in pest control, making it a promising tool for

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agricultural pest management.[10,11] Recently, dsRNA-based biopesticides have been verified and commercialized in North America.[12–14] RNAi in Coleopterans, Hemipterans, and other insects has shown long-lasting, strong responses with minimal doses.[15,16] Most recently, a Massachusetts biotech company, GreenLight Biosciences, has petitioned for regulatory approval for a dsRNA-based biopesticide targeting the varroa mite, *Varroa destructor*, an external parasitic mite and one of the most destructive pests of honey bees.[17,18] Similar to *T. urticae*, *V. destructor* has developed resistance to broadly used commercially available pesticides.[19] However, RNAi biopesticides in spider mites are still in the relatively early stages of investigation.

Multiple approaches, including injection, ingestion, and immersion, have been used to deliver dsRNA in mites.[18] In *T. urticae*, the initial successful RNAi experiment involved direct injection of a dsRNA solution into the abdomen of adult females via microinjection. It simultaneously demonstrated parental effects between adults and subsequent generation embryos following RNAi treatment.[20] However, because of the mite's soft cuticle and ~0.5 mm body length, the injection procedure requires high-precision instruments and a complex operating procedure.[21] In addition, injection is impractical for use in integrated pest management. In general, leaf disc-mediated dietary or soaking methods have become the most widely implemented approaches for spider mite RNAi.[22] However, the high concentration requirements and vast usage of dsRNA have raised concerns about cost-effectiveness.[23] Recently, we developed an egg-soaking method to deliver dsRNA into spider mites, successfully knocking down multiple target genes in hawthorn spider mite *Amphitetranychus viennensis*, resulting in significant phenotypic changes and mortality.[24] The applicability of the egg-soaking method in *T. urticae* remains to be demonstrated.

Different species exhibit varying sensitivities to RNAi, and even within the same species, distinct target genes demonstrate differing efficiencies of RNAi.[25] The selection of target genes is crucial in the RNAi process, determining the efficacy of gene knockdown and the effectiveness of pest management. Genes essential for insect growth and development can serve as targets for dsRNA-based biopesticides, disrupting physiological processes in pests and ultimately leading to death.[26] DEAD-box (DDX) RNA helicases, broadly conserved across prokaryotes and eukaryotes, play a pivotal role in RNA metabolism and represent a key family of RNA-binding proteins vital for genome protection.[27–29] The 20S proteasome, a core component of the proteasome complex, is responsible for the processive degradation of proteins, oligopeptides generation, and specific stress responses.[30–32] Signal recognition particle 54k is a key component of the ribonucleoprotein complex that mediates the co-translational targeting of secretory and membrane proteins to the endoplasmic reticulum.[33,34] Three essential DEAD box genes, namely *ATPHel-31B* (*ATP-dependent RNA helicase me31b*), *Belle* (*ATP-dependent RNA Helicase DDX3Y*) and *eIF-4A-1* (*Eukaryotic initiation factor 4A-I*), as well as two 20S proteasome genes, *Prosbeta-1* and *Prosbeta-5* (*Proteasomes β1* and *β5 subunits*) and *Srp54k* (*Signal recognition particle 54k*), were selected as candidate genes in this study.

Because of the effectiveness of spray-induced gene silencing (SIGS) as a system for controlling pest populations in field conditions, SIGS biopesticides like Ledprona have been registered by the United States Environmental Protection Agency (US EPA) and officially applied in the field, and BioDirect is under commercial evaluation by the United States Department of Agriculture and other agricultural departments in various countries.[12,13] Although the SIGS system has demonstrated its potential as a powerful tool against Lepidopterans and Hemipterans,[35,36] more work needs to be done to assess the viability of this method in spider mites.

To explore the feasibility of managing *T. urticae* using dsRNA-based biopesticides, we carried out the following objectives: (i) evaluated the applicability of the egg-soaking method in delivering dsRNAs in *T. urticae*, (ii) screened gene targets for SIGS, and finally (iii) assessed the efficacy of the selected target gene and the feasibility of SIGS in managing *T. urticae*.

2 MATERIALS AND METHODS

2.1 *Tetranychus urticae* collection and rearing conditions

2.1.1 *Laboratory population*

Adults and larvae of *T. urticae* were collected from apple trees in August 2021 at Linyi Orchard, Yuncheng, Shanxi Province (E110° 72′, N35°20′). The population was maintained in a climate-controlled growth chamber at a constant air temperature of 26 ± 0.5 °C, 50% relative humidity, and a 16:8 h light/dark photoperiod. Mites were reared with Chinese wild peach, *Prunus davidiana*.

2.1.2 *Field population*

A *T. urticae* population was collected from green bean, *Phaseolus vulgaris*, in September 2024 at the greenhouse located in Yuci District, Jinzhong City, Shanxi Province (E112°45′, N37°36′). The population was then transferred to *P. davidiana*, for indoor rearing. The population was acclimated for two generations under the conditions detailed in Section 2.1.1.

2.2 Avermectin bioassay

To evaluate the susceptibility of *T. urticae* populations to avermectin, bioassays were conducted on the laboratory population and field population. Fifty adult mites from each population were placed on 17-mm diameter leaf discs prepared from fresh host plant leaves. Avermectin (97% purity) (Shanghai Yuanye Bio-Technology Co., Shanghai, China) was dissolved in acetone and then diluted with 0.2% Tween 80. The final concentrations were 1.0 and 1220.7 mg L^{-1}; each concentration was further diluted using a 2.5× gradient to obtain six and eight concentrations, respectively. The leaf discs were submerged in 25 mL of the prepared avermectin solutions for 5 s. Excessive solution was carefully removed using filter paper. Mortality was recorded 24 h after treatment. Mites that did not respond to gentle probing with a fine brush were considered dead. Each treatment group included at least three biological replicates.

2.3 dsRNA synthesis and delivery

2.3.1 *Total RNA extraction and complementary DNA synthesis*

Approximately 200 adult *T. urticae* were collected into a 1.5-mL centrifuge tube containing multiple 2-mm sterilized steel balls and then submerged in liquid nitrogen. *T. urticae* were thoroughly fractured before adding TriZol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The total extracted RNA was subsequently dissolved in 15 μL of nuclease-free water and quantified using NanoDrop NC2000 (Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China), following the manufacturer's instructions.

2.3.2 Gene amplification

Target gene amplification was conducted using cDNA as a template mixed with 2× SevenBasis Taq PCR Mix (Dye+) (Seven Biotech, Beijing, China) on the C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). DNA fragments were purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. The quality and quantity of the gel extraction products were established by NanoDrop NC2000 (Thermo Fisher Scientific). The cloning vector pVMG was used to amplify the control gene *GUS*, a 561-bp fragment of *β*-glucuronidase from *Escherichia coli*. Primers used in this study are listed in Supporting Information, Table S1.

2.3.3 *dsRNA* in vitro *synthesis*

Synthesis and purification of dsRNA was conducted following the manufacturer's guidelines detailed below and using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). For dsRNA synthesis, combine the following components in a reaction tube: 10 μL of RiboMAX™ Express T7 2X Buffer, 1–8 μL of linear DNA template [the purified polymerase chain reaction (PCR) products served as templates, volume of DNA solution are calculated according to the concentration of each target gene], and 2 μL of T7 Express Enzyme Mix. Adjust the final volume to 20 μL with nuclease-free water. Mix gently and incubate at 42 °C for 8 h. Anneal RNA strands by mixing complementary RNAs at 70 °C and cooling to room temperature. Add 1 μL of both diluted RNase and DNase Solution. Incubate for 30 min at 37 °C, then precipitate RNA with sodium acetate and isopropanol alcohol. Mix and place on ice for 5 min, resulting in a cloudy appearance. Spin at 14 000 rpm in a microcentrifuge for 10 min to form a visible white pellet. Carefully pour off the supernatant, wash the pellet with 1 mL of cold 70% ethanol, and remove all ethanol. Air-dry the pellet for 15 min at room temperature. Following the synthesis, all dsRNAs were resuspended in 40 μL of nuclease-free water and preserved at −80 °C. The quality and quantity of dsRNAs were established by NanoDrop NC2000 (Thermo Fisher Scientific) and 1.5% agarose gel electrophoresis.

2.3.4 *Egg-soaking method to deliver dsRNA in* Tetranychus urticae

The egg-soaking method was performed according to the protocol outlined in our previous publication.[24] dsGUS and nuclease-free water mixed with Tween 80 served as the negative control and vehicle control. Approximately 50 newly laid *T. urticae* eggs were placed on a leaf disc treated with 20 μL of H_2O, 250 ng μL^{-1} dsGUS, and 250 ng μL^{-1} dsRNA solution. All experiments were conducted with five biological replicates. Hatching rate and mortality were calculated on day 6 and day 12, respectively. After the mites had developed into adults and become capable of producing eggs at day 14, 20 female mites from each treatment were transferred to new leaf discs individually. Eggs produced by each female were assessed at day 23.

2.4 dsRNA intake

To assess the feasibility of the egg-soaking method in *T. urticae* and track the temporal dynamics of dsRNAs post soaking, dsGUS was synthesized using the T7 RNAi Transcription Kit (Zhishengyougu Co., Shanghai, China) and labeled with a fluorescein RNA-labeling mix (Merck KGaA Co., Darmstadt, Germany) following established protocols.[37] Newly laid eggs were treated with a final concentration of 250 ng μL^{-1} fluorescently labeled dsGUS solution. The solution was left to naturally air-dry at a constant air temperature of 26 ± 0.5 °C, and eggs were then collected continuously after 0, 6, 12 and 24 h. Each egg was washed thoroughly five times using ddH_2O to remove any remaining fluorescein residues on the surface. Unwashed eggs at 0 h served as the positive control, whereas eggs treated with ddH_2O served as the negative control. Images were captured using an epifluorescence Leica DM2500 microscope (Leica, Wetzlar, Germany) and quantified using ImageJ software (NIH, Bethesda, MD, USA). This assessment was conducted using a total of five biological replicates, each comprising four technical replicates.

2.5 Candidate target genes

The dsRNA templates for target genes included a 500-bp fragment of *Prosbeta-1*, a 495-bp fragment of *Prosbeta-5*, a 479-bp fragment of *eIF-4A-1*, a 439-bp fragment of *ATPHel-31B*, a 333-bp fragment of *Belle*, and a 459-bp fragment of *Srp54k*.

2.6 Sample collection and target gene expression

To evaluate the RNAi efficiency of the candidate genes in *T. urticae*, mites at the deutochrysalis life stage were collected for each dsRNA treatment and control. Approximately 150 individual *T. urticae* were collected for the total RNA extraction. Total RNA extraction and cDNA synthesis were performed as described in Section 2.3.1.

Quantitative PCR (qPCR) analysis was used for quantification of target gene expression. *CycA* was used as the internal reference gene.[38] Standard curves were established for each candidate gene qPCR primers using cDNA with five concentrations, starting at 1000 ng and diluted fivefold serially. One hundred nanograms of total RNA served as the template for qPCR analysis. Both processes used the CFX96 Real-Time PCR Detection System (Bio-Rad). Each reaction mix (20 μL) comprised 7.76 μL of ddH_2O, 10 μL of 2× TB Green Premix Taq II (Tli RNaseH Plus; Takara), 0.32 μL of forward qPCR primer, 0.32 μL of reverse qPCR primer, and 1.6 μL of cDNA template. The PCR cycle consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s concluding with a melting curve analysis. The qPCR analysis protocol consisted of an initial 95 °C for 30 min, followed by 40 cycles of 95 °C for 5 s and annealing at 60 °C for 30 s.

qPCR analysis was conducted with at least three biological replicates, each consisting of three technical replicates. Primers used for qPCR analysis are listed in Supporting Information, Table S2. The standard curves for qPCR primers are listed in Supporting Information, Table S3.

2.7 *Prosbeta-1/5* dose–response analysis

A 20-μL volume of either nuclease-free water or dsRNAs solution at varying concentrations (600, 500, 400, 300, 150, 75, 37.50, 18.75, 9.38, 4.69, 2.34, 1.17, 0.59, and 0.29 ng μL^{-1}) was applied to the eggs using egg-soaking method. Each treatment had three biological replications. The mortality of mites was recorded at day 14. SPSS 27.0 (IBM, Armonk, NY, USA) and GraphPad Prism 9.5 (GraphPad Software Inc, Boston, MA, USA) software was used to calculate median (LC_{50}) and 90% (LC_{90}) lethal concentrations with 95% confidence intervals, R^2 degrees of freedom, standard deviation and generate the dose–response curve.

2.8 Spray-induced gene silencing of dsTuProsbeta-5

The experimental peach plants were grown in a climate-controlled growth chamber for 1 month under the following conditions: constant air temperature of 26 ± 1 °C, 60% relative

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humidity, 30 000 lx illuminance and a 16:8 h light/dark photoperiod. Subsequently, ten adult *T. urticae* were evenly transferred onto each peach plant. The entire plant was covered with 400-mesh gauze to prevent the dispersal of mites. According to the result of the *Prosbeta-5* dose–response analysis, the plant was then treated with H_2O, 432.10 ng μL^{-1} dsGUS and dsTuProsbeta-5 (concentration value is used in the LC_{90} calculation in SPSS 27.0). After mite transfer, the plants were divided into three groups and sprayed with 700 µL of H_2O, dsGUS, and dsTuProsbeta-5 solution using a handheld water oxygen airbrush. The first group was sprayed once on day 5, the second group was sprayed once on day 12, and the third group was sprayed twice on day 5 and day 12. Each treatment in every group has three biological replications. The number of eggs, larvae and adults from 12 leaf samples were counted at day 19 and day 26. Leaf samples were randomly collected from four orientations and varied in size (large, medium, small) (Fig. 1). The suppression rate was calculated by comparing the number of eggs, Larvae, and adult mites in the dsTuProsbeta-5 treatment group with the average of both control groups (H_2O and dsGUS treatments).

2.9 Imaging

Mite images were captured using a SZ680 light microscope (Optec, Chongqing, China) with an UCMOS03100 assorted industrial digital camera (Optec) and SMZ18 research stereo microscope (Nikon, Tokyo, Japan). Plant and leaf images were captured using $\alpha 7$ mark iv mirrorless camera (Sony, Tokyo, Japan) with a 50-mm F1.4 DG HSM camera lens (Sigma, Tokyo, Japan).

2.10 Statistical analysis

Statistical analyses were conducted using SPSS 27.0 software (IBM). Means and standard errors (SEM) were analyzed via analysis of variance followed by Tukey's honestly significant difference test ($P < 0.05$). Bioassay and dose–response results were analyzed using the probit regression method in SPSS 27.0 (IBM), with concentrations converted to $\log_{10}$.

3 RESULTS

3.1 Avermectin bioassay

The LC_{50} values for avermectin were 0.18 mg L^{-1} for the laboratory population and 61.02 mg L^{-1} for the field population; the toxicity regression equations were $y = 1.15x + 0.06$ and $y = 1.06x - 1.90$, respectively. The field population exhibited a 340-fold resistance to avermectin in comparison with the laboratory population (Supporting Information, Table S4).

3.2 dsRNA uptake in *Tetranychus urticae* via the egg-soaking method

Previously, we found that the fluorescence intensity of dsRNA through the eggshell was time-dependent within the first 24 h in *A. viennensis*. In this study, we observed a consistent result in *T. urticae*. Specifically, after ~2 h of air drying, a significant fluorescence signal was observed on the eggshell (Fig. 2(F)), whereas no obvious signal was detected in the ddH_2O negative control group (Fig. 2(A)). After thorough washing with ddH_2O, the fluorescence intensity of dsGUS gradually accumulated through the eggshell in a time-dependent manner within the first 24 h after the egg-soaking process ($R^2 = 0.5923$, $P < 0.0001$; Fig. 2(B)–(G)). In addition, the fluorescence intensity in *T. urticae* (76.08 ± 8.02) was lower in compared with *A. viennensis* (96.32 ± 20.97).

3.3 Selection of molecular targets for SIGS

3.3.1 *Toxicity of target genes*

We previously developed an egg-soaking method delivering dsRNAs in *T. urticae*, and caused ~90% mortality by targeting an extensively used housekeeping gene, *Vacuolar-type H + ATPase subunit A* (*TuV-ATPase A*).[39] In this study, we used this method to screen for the potential molecular targets for SIGS biopesticide against *T. urticae*. There was no significant difference in the hatching rate of eggs among the different treatments (Fig. 3(A)). However, the mortality of *T. urticae* differed significantly. Specifically, the mortality of mites treated with dsTuProsbeta-5, dsTuProsbeta-1, dsTuSrp54k, dsTuelF-4A-1, dsTuATPHel-31B, and dsTuBelle exhibited 97.39%, 94.09%, 67.87%, 63.27%, 60.90%, and 37.90% mortality, respectively, significantly higher than controls: dsGUS (10.01%) and H_2O (10.91%) (Fig. 3(B)). For fecundity, because of the high mortality of dsTuProsbeta-1 and dsTuProsbeta-5 treatments, most adult mites died before they could produce eggs and were therefore excluded from the count. The average number of eggs produced by each female significantly decreased when treated with dsTuSrp54k (47.86%) and numerically decreased when treated with dsTuBelle (43.50%), dsTuATPHel-31B (42.67%), and dsTuelF-4A-1 (35.96%), compared with the control group treated with H_2O (Fig. 3(C)).

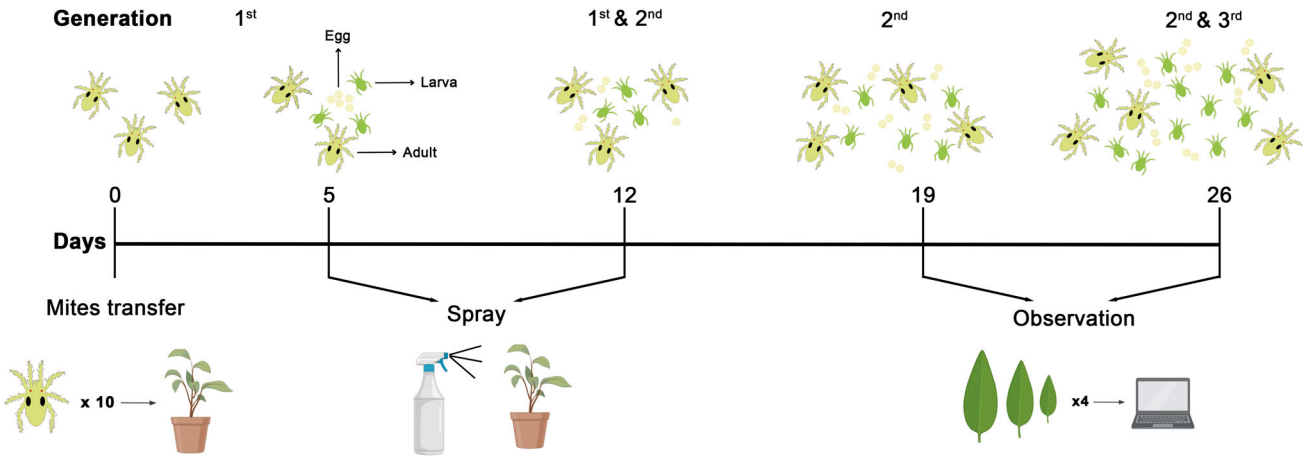

Figure 1. Spray-induced gene silencing in *Tetranychus urticae* experimental procedure overview.

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3.3.2 Phenotypical impact

In addition to the mortality effect observed after treating *T. urticae* with dsTuProsbeta-1, dsTuProsbeta-5, dsTuelF-4A-1, and dsTuSrp54K, a dark body color phenotype was observed. The whole body turned except the appendages turned black (Fig. 4 (C)–(E), (H)). The dsTuATPHel-31B- and dsTuBelle-treated phenotypes did not exhibit any changes (Fig. 4(F), (G)) compared with the controls (Fig. 4(A), (B)). Phenotypical changes in the community of *T. urticae* are shown in Supporting Information, Fig. S1.

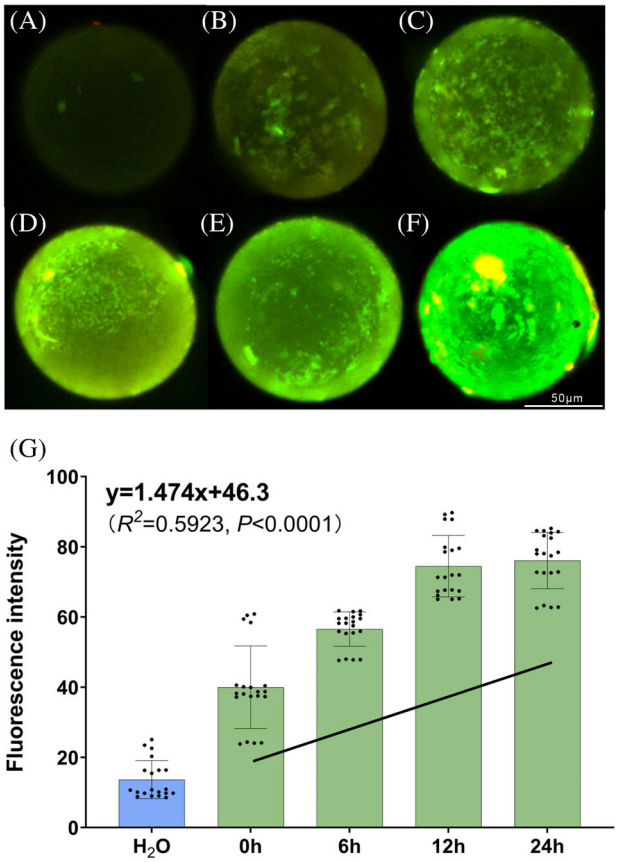

Figure 2. Temporal fluorescence profile of dsGUS during the egg-soaking process in *Tetranychus urticae*. Newly laid eggs were treated with either RNase-free water (A) or fluorescein-12-UTP labeled dsGUS (B–F). Eggs were thoroughly washed five times with ddH_2O (B–E) or left unwashed (F). Fluorescence signal was visualized at 0 h (B, F), 6 h (C), 12 h (D), and 24 h (E), using an epifluorescence Leica DM2500 microscope and the fluorescence intensity (G) was quantified using ImageJ software. Each treatment consisted of five biological replicates, and each biological replicate contained four technical replicates. The linear regression was carried out by GraphPad Prism 9. Scale bars, 50 μm.

3.3.3 Target genes suppression

The expression level of each target gene was significantly reduced compared with the controls after treatment with the corresponding dsRNAs. Specifically, the expression levels of *Prosbeta-1*, *Prosbeta-5*, *eIF-4A-1*, *ATPHel-31B*, *Belle*, and *Srp54k* were significantly decreased by 31.34%, 41.94%, 36.27%, 64.59%, 59.20%, and 74.10%, respectively, compared with control levels (Fig. 5).

3.4 Efficacy and feasibility of SIGS in controlling *Tetranychus urticae*

3.4.1 Prosbeta-1/5 *dose–response analysis*

Prosbeta-1 and *-5*, which showed significant mortality, were selected to assess the efficacy of this dsRNA-based biopesticide. In the laboratory population, with 0.29, 0.59, 1.17, 2.34, 4.69, 9.38, 18.75, 37.50, 75, 150, 300, 400, 500 ng μL^{-1} of dsTuProsbeta-1, the mortality of *T. urticae* was 13.37%, 15.54%, 22.23%, 30.96%, 44.76%, 61.40%, 70.10%, 75.82%, 82.36%, 88.78%, 94.37%, 96.27%, and 96.34%, respectively. The LC_{50} and LC_{90} values for dsTuProsbeta-1 were 4.86 and 177.61 ng μL^{-1}. With the same series of concentrations of dsTuProsbeta-5, the mortality of *T. urticae* was 12.97%, 13.64%, 14.46%, 28.37%, 30.12%, 42.08%, 60.94%, 66.90%, 78.56%, 83.07%, 89.50%, 90.61%, and 91.98%, respectively. The LC_{50} and LC_{90} values of dsTuProsbeta-5 were 9.93 and 432.10 ng μL^{-1}. The mortality of the control (H_2O treatment) was 11.59%. The response (% max) presented in the figures was calculated using corrected mortality (Supporting Information, Fig. S2). In the field population, with 0.29, 0.59, 1.17, 2.34, 4.69, 9.38, 18.75, 37.50, 75, 150, 300, 600 ng μL^{-1} of dsTuProsbeta-1, the mortality of *T. urticae* was 11.56%, 14.99%, 22.16%, 28.36%, 42.49%, 58.40%, 63.43%, 71.58%, 80.53%, 86.13%, 89.86%, and 94.57%, respectively. The LC_{50} and LC_{90} values of dsTuProsbeta-1 were 8.19 and 270.88 ng μL^{-1}. With the same series of concentrations of dsTuProsbeta-5, the mortality of *T. urticae* was 14.13%, 14.77%, 18.91%, 27.10%, 34.63%, 46.15%, 56.90%, 65.27%, 73.20%, 77.41%, 88.59%, and 91.97%, respectively. The LC_{50} and LC_{90} values of dsTuProsbeta-5 were 12.69 and 562.15 ng μL^{-1}. The mortality of the control (H_2O treatment) was 9.50%. The response (%max) presented in the figures was calculated using corrected mortality (Supporting Information, Fig. S3).

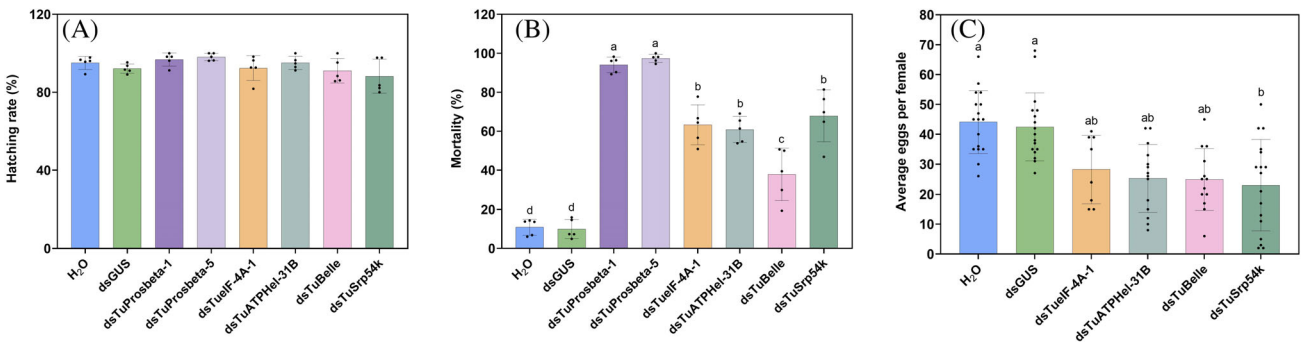

Figure 3. RNA interference responses following dsRNA treatment in *Tetranychus urticae* via the egg-soaking method. The hatching rate of eggs (A), mortality (B), and average number of eggs produced by one adult female (C) are shown. Different letters above the bars indicate a significant difference determined by analysis of variance with Tukey's honestly significant difference test ($P < 0.05$). Each treatment consisted of at least five biological replicates.

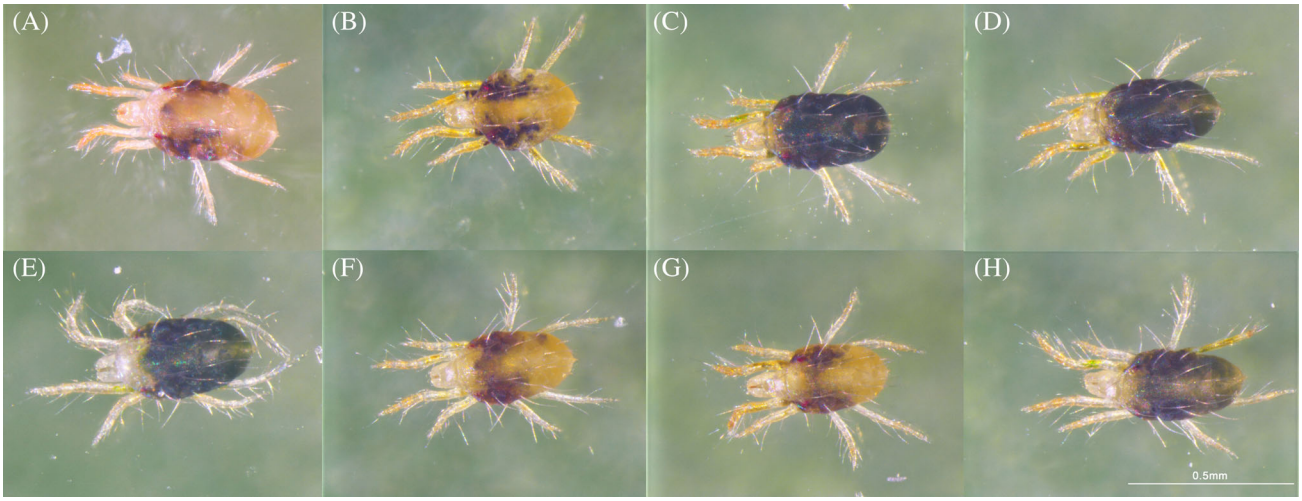

Figure 4. Phenotypic impact after dsRNA treatment in *Tetranychus urticae* via the egg-soaking method. Phenotype of a single adult female under treatment with H_2O (A), dsGUS (B), dsTuProsbeta-1 (C), dsTuProsbeta-5 (D), dsTuelF-4A-1 (E), dsTuATPHel-3B (F), dsTuBelle (G), and dsTuSrp54K (H). Scale bars, 0.5 mm.

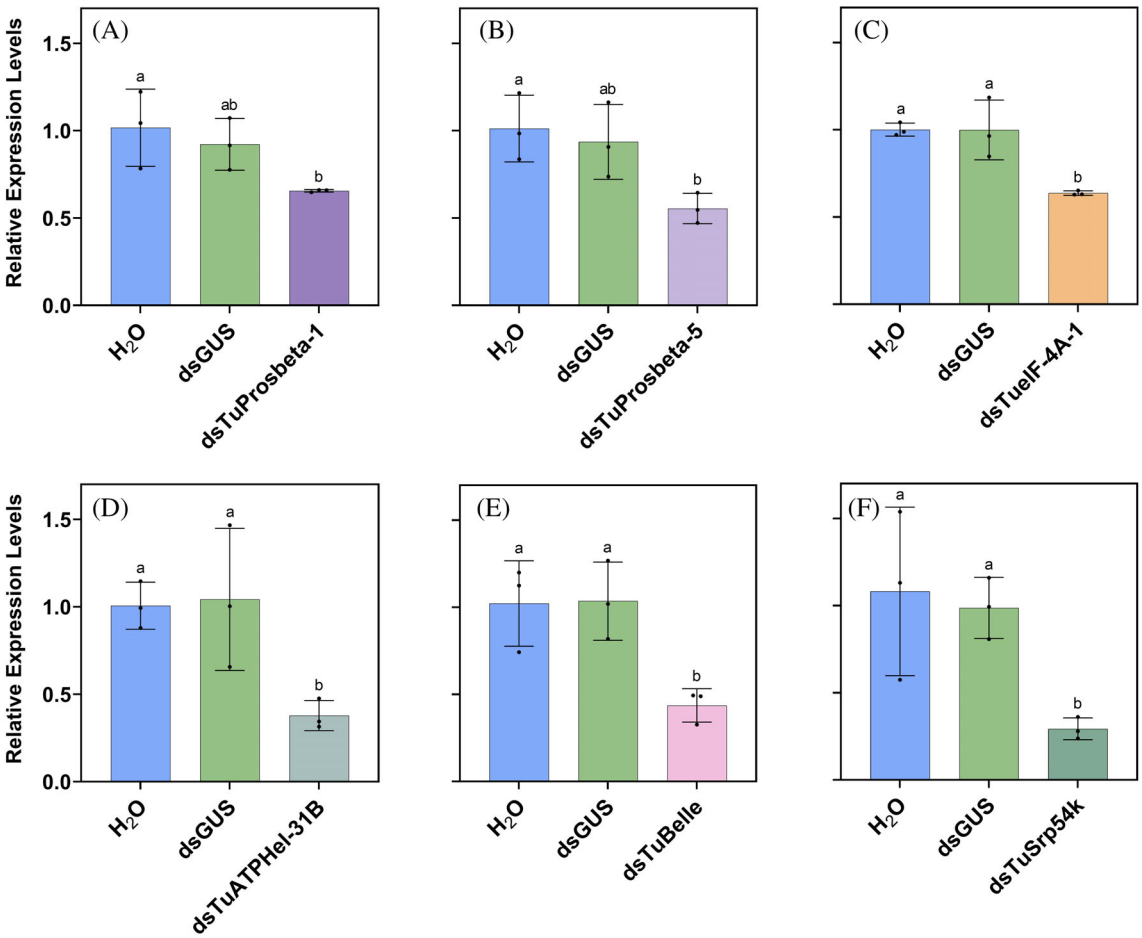

Figure 5. Target gene suppression under treatment with dsRNA via the egg-soaking method. The relative expression levels of *Prosbeta-1* (A), *Prosbeta-5* (B), *elF-4A-1* (C), *ATPHel-31B* (D), *Belle* (E), and *Srp54k* (F) are shown. Different letters indicate a significant difference determined by analysis of variance with Tukey's honestly significant difference test ($P < 0.05$). Each treatment consisted of three biological replicates, and each biological replicate contained three technical replicates.

3.4.2 Prosbeta-5 *SIGS experiments*

To assess the efficacy of the selected target gene in managing *T. urticae*, dsTuProsbeta-5 spraying experiments were conducted. On day 19, the H_2O- and dsGUS-treated plants exhibited severe feeding damage and leaf spotting (Fig. 6(A), (B)). By day 26, symptoms had worsened, including wilting, leaf drying, yellowing, and chlorosis (Fig. 6(F), (G)). By contrast, the 5-day dsTuProsbeta-5 sprayed group and 12-day dsTuProsbeta-5 sprayed group initially

showed less damage on day 19 (Fig. 6(C), (D)). However, by day 26, both groups had begun to exhibit wilting and leaf yellowing, along with significant leaf spotting damage (Fig. 6(H), (I)). Notably, the 5- and 12-day dsTuProsbeta-5 sprayed group maintained minimal overall damage, with the fewest feeding spots, and continued to grow healthily to day 26 (Fig. 6(E), (J)).

At day 19, the 5-day dsTuProsbeta-5 spray treatment group showed significant suppression of *T. urticae*. In this group, the total mite population was 356.33, compared with 750.67 in the H_2O control and 756.34 in the dsGUS control, resulting in a total suppression rate of 52.71%. In the 12-day dsTuProsbeta-5 spray treatment group, the total mite population was 581.33, compared with 874.00 in the H_2O control and 732.00 in the dsGUS control, yielding a suppression rate of 27.61%. In the combined 5- and 12-day dsTuProsbeta-5 spray treatment group, the total mite population was 52.33, compared with 749.00 in the H_2O control and 810.33 in the dsGUS control, resulting in the highest suppression rate of 93.29% (Supporting Information, Table S5 and Fig. 6(K)–(M)).

At day 26, the total mite population in the 5-day dsTuProsbeta-5 spray treatment group was 712.67, compared with 1232.99 in the H_2O control and 1161.33 in the dsGUS control, resulting in a suppression rate of 40.47%. In the 12-day dsTuProsbeta-5 spray treatment group, the total mite population was 736.34, compared with 1053.33 in the H_2O control and 1204.33 in the dsGUS control, resulting in a suppression rate of 34.77%. In the combined 5- and 12-day dsTuProsbeta-5 spray treatment group, the total mite population was significantly reduced to 140.67, compared with 1071.00 in the H_2O control and 1189.00 in the dsGUS control, leading to the highest suppression rate of 87.55% (Supporting Information, Table S5 and Fig. 6(K)–(M)).

4 DISCUSSION

4.1 Egg-soaking RNAi in *Tetranychus urticae*

Recently, it was confirmed that the egg-soaking method can deliver dsRNA into the eggshell of hawthorn spider mite *A. viennensis* using

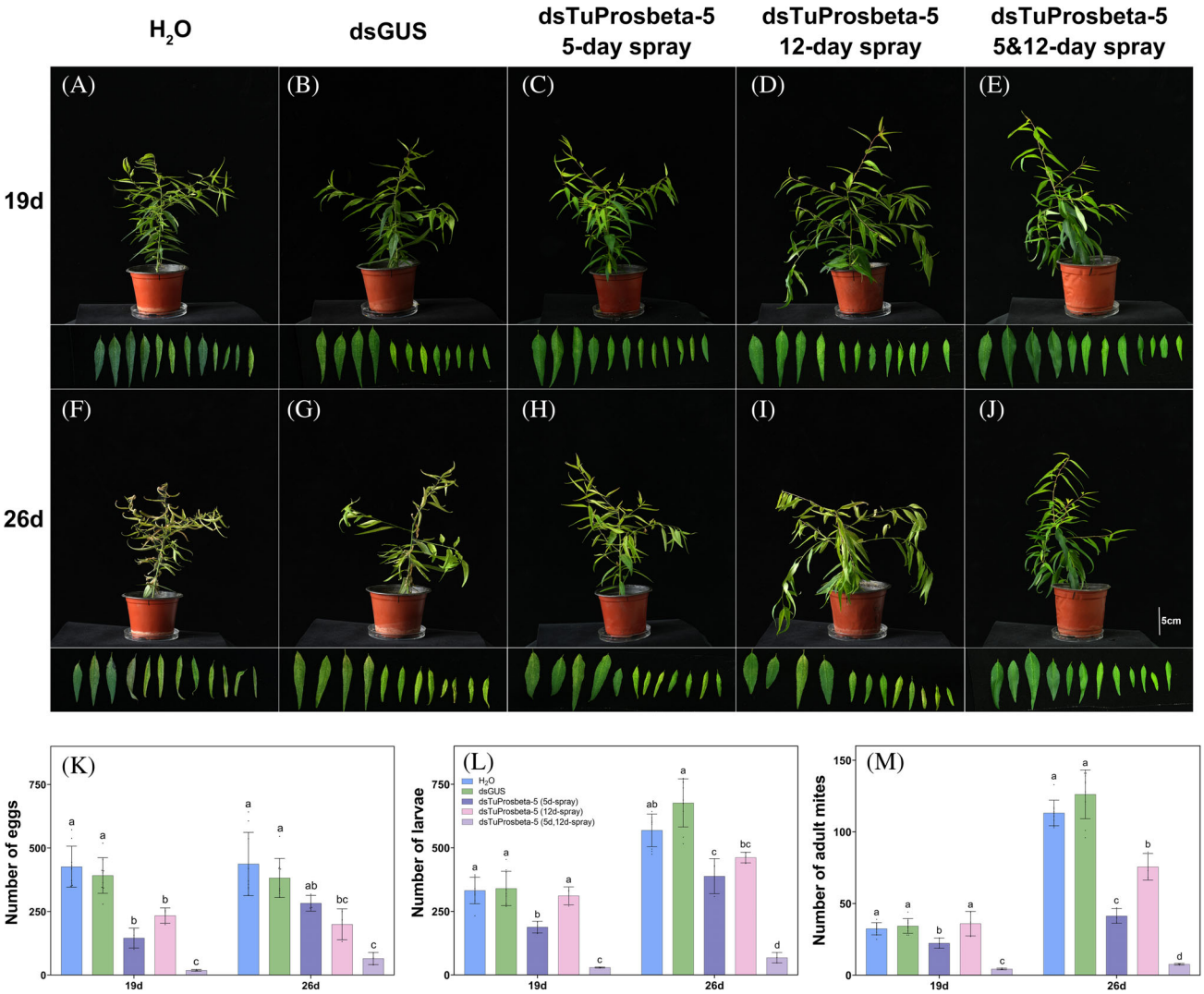

Figure 6. RNA interference effect by spray-induced gene silencing in *Tetranychus urticae*. Overall perspective of plants and 12 leaves selected for counting the mite population (A–J). Plant and leaves were treated with H_2O, dsGUS at day 19 (A, B) and day 26 (F, G). 5-day dsTuProsbeta-5 sprayed plant and leaves at day 19 (C) and day 26 (H). 12-day dsTuProsbeta-5 sprayed plant and leaves at day 19 (D) and day 26 (I). 5 - and 12-day dsTuProsbeta-5 sprayed plant and leaves at day 19 (E) and day 26 (J). The number of eggs (K), larvae (L), and adult mites (M) calculated on day 19 and day 26 on the selected leaves are shown. Different letters above the bars indicate the significant difference determined by analysis of variance with Tukey's honestly significant difference test ($P < 0.05$). Each treatment consisted of three biological replicates. Scale bars, 5 cm.

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fluorescein-12-UTP labeled dsRNA, revealing the time-dependent dynamics of dsRNA under environmental conditions.[24] In addition, the injection of fluorescein-labeled yellow fluorescent protein (YFP) dsRNA (dsYFP) or green fluorescent protein (GFP) siRNA (siGFP) into the abdomen of adult female *T. urticae* showed that the fluorescence signal was detectable in both adults and offspring embryos after 3 days, demonstrating that both dsRNA and siRNA can efficiently cross cell membranes in various spider mite tissues.[20] Studies have shown that water-soluble dsRNA can cross the eggshell and enter the egg in red spider mite, *Tetranychus cinnabarinus*, when combined with water-soluble pigment and fluorescence-labeled retinoid X receptor 1 dsRNA (dsTcRXR1).[40] In this study, we used fluorescein-12-UTP labeled dsGUS to trace the temporal distribution following egg-soaking in *T. urticae*. The temporal distribution of the fluorescence signal demonstrated a time-dependent increase in fluorescence intensity over the first 24 h, confirming the continuous accumulation of absorbed dsRNA in the eggshell in a time-dependent manner. Although RNAi in embryos still holds potential, the feasibility of egg soaking in *T. urticae* has been demonstrated.

In comparison with other dsRNA delivery methods, the leaf-coating method was the least efficient and required the highest minimum dosage (192 ng per mite, 30% effectiveness) targeting a highly lethal gene *VATPase A* in *T. urticae*.[21,38,39] The soaking method for newly emerged adult females faces obstacles in target gene selection and analysis because of post-soaking recovery, achieving ~75% mortality of the *VATPase A* gene with 160 ng per mite.[38] This is followed by the mesh method with less damage to adult females, which requires mandatory replicated procedures for mite transfer and high doses of dsVATPase A (1 μg μL^{-1}, ~40% mortality).[41] The egg-soaking method in *T. urticae* silencing *VATPase A* with a maximum dosage of ~100 ng/mite, achieved >90% mortality.[39] Recently, this method has been used in mites to investigate gene function. In the red spider mite, *T. cinnabarinus*, eggs continuously treated with dsTcRXR1 showed knockdown of the target gene *RXR1* and caused high egg mortality.[40] In conclusion, the egg-soaking method stands out as the most efficient, practical, and cost-effective technique for delivering dsRNAs into spider mites. This method minimizes the dsRNA dosage and does not require additional handling of mites, making it highly suitable for functional genomic research and target gene selection studies. It also showcases significant potential for incorporating sprayable RNAi biopesticides into spider mite management strategies.

4.2 Target gene RNAi response in *Tetranychus urticae*

Avermectins, one of the most important insecticides worldwide, have been registered and widely used for several decades to control major agricultural pests and insects of public health importance.[42] It primarily disrupts electrical signaling in invertebrate nerve and muscle cells by enhancing glutamate activity at glutamate-gated chloride channels, which are specific to protostome invertebrates.[43] Ultimately causing paralysis of their neuromuscular systems and lethal effects.[44] Excessive use of avermectins has caused *T. urticae* to rapidly develop high levels of resistance.[2]

With the development of RNAi technology, RNA biopesticides have emerged as environmentally friendly, highly accurate, and species-specific alternatives to chemical pesticides, offering different modes of action for pest management.[45] The first commercialized oral application of RNAi using transgenic maize and artificial diet has been implemented to manage the western corn rootworm (WCR), *Diabrotica virgifera virgifera*, with Bayer's SmartStax PRO maize (MON87411),[11] which had been deregulated in Canada (2016), the USA (2017), and China (2021).[12] This approach targets the *Snf7* gene, a housekeeping gene encoding the SNF7 subunit of the ESCRT-III complex. RNA interference-mediated silencing of this gene, which is involved in the transport of transmembrane proteins, causes lethality in WCR, ultimately leading to reduced root damage.[11] *Ledprona*, the first sprayable dsRNA biopesticide registered with the US EPA, targets the *proteasome subunit beta type-5* (*PSMB5*) gene in the Colorado potato beetle *Leptinotarsa decemlineata*. A concentration of 0.025 ng μL^{-1} dsRNA caused 90% mortality after 6 days of initial exposure.[46] In summary, the selection of highly effective target genes is crucial for the development of dsRNA-based biopesticides.

In this study, we assessed the toxicity of genes in the DEAD box gene family, proteasome subunits, and signal recognition particle. The DEAD box gene family is the largest subfamily of ATPase activity-dependent RNA helicase,[47] encoding RNA helicases genes that are highly conserved and widely expressed across prokaryotes and eukaryotes. They play essential roles in various aspects of RNA metabolism, including unwinding RNA and remodeling RNA–protein interactions through their ATP-dependent RNA helicase activity.[48] *ATPHel-31B* and *Belle* are members of the DEAD box ATP-dependent RNA helicase genes, which are known to modulate numerous biological processes, such as innate immunity and participation in the RNAi pathway.[29] Various approaches have shown that silencing DEAD box ATP-dependent RNA helicase genes can significantly compromise fitness and cause phenotypical changes in several arthropods, including migratory locust *Locusta migratoria*,[48] common fruit fly *Drosophila melanogaster*,[49] and *A. viennensis*.[39] In this study, knockdown of *ATPHel-31B* and *Belle* resulted in 60.9% and 37.9% mortality, respectively, with a >50% decrease in relative gene expression levels. No phenotypic changes were observed. Previous research been demonstrated that *Belle* is essential for both male and female fertility.[50,51] dsTuBelle treatment in *T. urticae* caused a numerical decrease in female fecundity. However, there were no significant differences compared with the controls, likely because of an insufficient concentration of the treatment. *Eukaryotic initiation factor 4A-I* (*eIF-4A-1*) belongs to the DEAD box superfamily 2 (*SF2*) and is critical for protein translation in eukaryotes.[28] It facilitates ribosome loading onto messenger RNA and is essential for cell growth and development.[52] *eIF4A* is regulated by the *DREF* pathway, which is involved in controlling protein synthesis in *Drosophila*.[53] Injecting dsTueIF4A in *L. migratoria* nymphs caused 100% mortality before molting, with appendages darkening and withering.[48] In this study, silencing *eIF4A* in *T. urticae* resulted in 63.3% mortality with a dark body phenotype.

Proteasomes, specifically the β1 and β5 subunits, are integral components of the 20S proteasome complex, which comprises four stacked rings (α1-7/β1-7/β1-7/α1-7). The proteasome exhibits chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1) activities, cleaving peptides after hydrophobic, basic, and acidic residues, respectively.[31,32,54] The β1 subunit plays crucial roles in cleaving peptide chains into smaller segments, enabling the proteasome to degrade a wide range of protein substrates.[30] The β5 subunit, part of the ubiquitin/proteasome machinery, is responsible for removing damaged proteins and preventing the accumulation of poly-ubiquitinated protein aggregates in cells.[31,55] In multiple studies, knocking down proteasome genes has been shown to significantly affect various of pests, leading to pronounced phenotypic changes and substantial increases in

mortality. For *T. urticae*, silencing the *Rpt7* and *Rpn3* genes from the 26S proteasome resulted in a dark body phenotype and more than 90% mortality.[38] In 28-spotted potato ladybird *Henosepilachna vigintioctopunctata*, targeting *proteasome β5* causes developmental stunting and more than 80% mortality.[56] For oriental fruit fly *Bactrocera dorsalis*, knockdown of *proteasome β3* effectively blocks ovarian development, preventing sexual maturation.[57] In cabbage stem flea beetle *Psylliodes chrysocephala*, dsRNA targeting *Prosβ6* leads to almost 100% mortality, demonstrating a dose-dependent effect.[58] In this study, we demonstrated that dsTuProsbeta-1 and dsTuProsbeta-5 effectively induced high mortality in *T. urticae*, achieving 94.09% and 97.39%, respectively. They also caused a dark body phenotype, highlighting their impact on mite physiology. Dose–response analysis showed that dsTuProsbeta-1 and dsTuProsbeta-5 led to increasing mortality at higher concentrations. Specifically, dsTuProsbeta-1 and dsTuProsbeta-5 had LC_{50} values of 4.86 and 9.93 ng μL^{-1}, respectively, indicating their high potency even at low concentrations. We also evaluated the efficacy of these two gene targets on avermectin-resistant populations. The differences in sensitivity to RNAi between the laboratory and field *T. urticae* populations were numerical in nature, suggesting that the effectiveness and bioactivity of dsRNA fragments are independent of the population's resistance status. This result is unsurprising given the fundamentally different modes of action between chemical pesticides and RNA biopesticides. In addition, both dsRNAs significantly reduced the expression levels of their target genes, confirming effective gene silencing. The effectiveness of two proteasome β subunit genes make them promising candidates for dsRNA-based pest control.

Signal recognition particle 54k (*Srp54k*) binds to the hydrophobic signal peptide as the nascent pre-protein emerges from the ribosome's N-terminal end, resulting in a slowing or pause in translation, a phenomenon termed 'elongation arrest'.[33] *Srp54k* gene knockdown in Coleoptera pests caused high mortality at low doses. The LC_{95} value in second-instar larvae of willow leaf beetle *Plagiodera versicolora* is 0.275 ng μL^{-1} and has dose-dependence effect.[59] Injecting 3 ng of dsSrp54k per individual in red flour beetle *Tribolium castaneum* caused almost 100% mortality, with higher doses causing earlier death.[60] In *T. urticae*, soaking female adults in 267 ng of dsRNA per individual for 24 h to silence the *Srp54k* gene resulted in >90% mortality by day 10, a dark body phenotype, and a 40% decrease in fecundity.[38] In this study, the egg-soaking method for *Srp54k* knockdown caused >60% mortality in day 2 adult females, resulting in a dark body phenotype and reducing egg production by 40% in female adults using 100 ng of dsRNA per individual. This demonstrates that the egg-soaking method can use lower doses to achieve higher mortality at earlier life stages.

Our combined results suggest that the *Prosbeta-1*, *Prosbeta-5*, *eIF-4A-1*, *ATPHel-31B*, and *Srp54k* are suitable candidate targets for dsRNA-based pest control against *T. urticae*.

4.3 SIGS in *Tetranychus urticae*

In recent years, excessive use of chemical pesticides has led to the contamination of agricultural land and water bodies.[61,62] The accumulation and magnification of pesticides pose significant harm to humans, wildlife, and even remote areas like the Arctic regions.[63] In addition to Ledprona and BioDirect, a SIGS-based platform developed by Bayer, has confirmed the potential of RNAi technology to control the Varroa mite *V. destructor* in honeybees.[12] The current implementation of SIGS has attracted attention because of its feasibility and effectiveness in crop protection.[64] Despite challenges related to the high costs and instability of dsRNA, it is emerging as a potential method to silence essential genes in target organisms, resulting in environmentally friendly and effective pest control.[65]

In Lepidoptera and Hemiptera, SIGS is widely used and has resulted in mortality and abnormal development. Among Lepidoptera, mortality of 60–100% and developmental stunting have been observed in the diamondback moth *Plutella xylostella* with *Acetylcholinesterase 2* (*AchE2*) siRNA spraying[66] and in Asian corn borer *Ostrinia furnalalis* with *chymotrypsin-like serine proteinase C3* and *carboxypeptidase 4* dsRNA spraying.[67] Abnormal ecdysis has occurred in larvae and pupae in the cotton bollworm *Helicoverpa armigera* with *methionine-rich storage protein* dsRNA spraying.[35] The application of SIGS on various Hemiptera species yielded notable results. For white-backed planthopper *Sogatella furcifera*, targeting *protein phosphatase I alpha at 96a* and *heat shock 70-kDa protein cognate 3* with star polycation dsRNA formulation (SPc-dsRNA) resulted in significant phenotypic changes including abdominal deformity, melanization, wing deformity, ecdysis failure, and an approximate mortality of 50–70%.[37] In green peach aphid *Myzus persicae*, the targeting of *ATP-binding cassette sub-family G member 4* and *vitellogenin receptor* using micro RNA caused significant mortality and abnormalities in body type.[68] For the black striped plant bug *Adelphocoris suturalis*, the use of star polycation dsRNA formulation (SPc-dsRNA) to silence juvenile hormone pathway genes led to abnormal ovarian development fertility.[19] The effectiveness of SIGS is varied among different pests, target genes and different molecular types. The application of SIGS in spider mites is still limited.

To explore effectiveness and targeted control methods, we evaluated the effectiveness of dsTuProsbeta-5 via SIGS. Day 5 spraying targets offspring (eggs and first-hatched larvae) and transferred adult mites. Day 12 spraying can cover all developmental stages of offspring and residual transferred adult mites. Our previous research showed that RNAi efficiency was substantially higher in the early stages of *A. viennensis*. In this study, a high percentage of immature *T. urticae* from day 5 to day 9 (the first generation of offsprings) and from day 12 to day 16 (the first and second generation of offsprings) was observed. Thus, the selected spraying days align with our previous results and observations.

The study demonstrates that dsTuProsbeta-5 effectively reduces *T. urticae* populations across all life stages, particularly with repeated application. The singly sprayed groups demonstrated mediocre population suppression, whereas the spraying twice strategy proved significantly more effective and sustained in controlling *T. urticae*. The data indicate that a single spraying operation, although initially effective, is insufficient to maintain control over time as mite populations rebound. This highlights the importance of applying dsRNA-based treatments more than once, especially during early developmental stages. This approach ensures comprehensive and sustained control, leading to significant mite population suppression and minimal detrimental effects on plant health.

To further establish the feasibility of RNA biopesticide target, assessing biosecurity for non-target organisms is crucial. High sequence similarity across species can significantly increase the risk of unintended gene silencing.[69] In addition, application method, dosage, timing, and frequency are key factors that may influence effects on non-target organisms.[70] For instance, 240-nucleotide dsRNA designed to target the *Snf7* gene in the

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WCR demonstrates a highly selective insecticidal effect. The dsRNA also effectively targets specific beetle species that are phylogenetically closely related to WCR, while having no adverse effects on non-target organisms.[19] In other research, testing on various non-target organisms, including Coleoptera, Hymenoptera, Neuroptera, and Hemiptera, showed that WCR Snf7 dsRNA had no statistically significant adverse effects.[71] The v-ATPase A dsRNA in WCR may trigger a sequence-unspecific response, leading to prolonged development time and increased mortality, probably because of a higher number of 21-nucleotide matches found in the two ladybird beetle species when exposed through dietary RNAi[72]; it also had no significant effects on larval and adult survival in honey bee *Apis mellifera* at higher doses.[73] For several gene targets in the 28-spotted potato ladybird *H. vigintioctopunctata*, including *Prosbeta5*, specific dsRNA molecules demonstrate high specificity for *H. vigintioctopunctata* management with no impact on its natural predator, *Propylaea japonica*.[56] Although safety testing on non-target organisms was not conducted in this research, the study did screen for gene targets and proved the feasibility of using SIGS to control *T. urticae*. Future research should explore the potential impacts on non-target organisms to ensure the ecological safety and sustainability of RNA biopesticides.

AUTHOR CONTRIBUTIONS

JY, YW, and MR designed the experiment. YG, ZL, PZ, LH, and RF collected samples from the field. YW, YD, ML and MR conducted the experiments and analysis. YW drafted the manuscript, and JY, XZ, and MR revised the manuscript and oversaw the entire project. All authors have read and approved its final version.

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CONFLICT OF INTEREST

The authors declare that they have no conflict interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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