IGTRCN's Peer-to-Peer Training Report

Adopting high-throughput and non-destructive RNAi technology in soybean aphid

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Background

RNA interference (RNAi) not only serves as a tool for functional gene analysis but also holds promise for management of aphid pests [1-3]. Nevertheless, aphid RNAi is marked by a considerable degree of inconsistency and poor silencing efficiency [4]. Moreover in aphid species like soybean aphid (*Aphis glycines*), a major soybean pest, RNAi success has remained elusive mainly due to the lack of an efficient delivery method. Microinjections for dsRNA (or siRNA) delivery have proved too stressful for *A. glycines* to survive due to its small (1.5 mm maximum length, 0.6 mm maximum width) and soft body. Furthermore, due to *A. glycines'* extreme host plant specificity on soybean, standardized artificial diet for *A. glycines* is not available, thus delivering dsRNA (or siRNA) through ingestion is not feasible.

The *A. glycines* is a pest of considerable economic importance throughout most of the soybean-growing regions of the United States (US) and Canada [5,6] as it can cause up to 58% yield losses in soybean [7]. In north-central states, which account for 80% of total soybean production in the US, annual losses due to *A. glycines* damage can be up to $2.4 billion [8]. The use of insecticides, a quick solution for managing *A. glycines*, has led to a dramatic rise in input costs for soybean production in the region [5,9]. Consequently, the cultivation of soybean that is naturally resistant to *A. glycines* offers an attractive opportunity because it is economical and environmentally safe [10]. But, the durability of aphid-resistant soybean is questionable since this insect has evolved virulent biotypes which overcome natural resistance [11]. Several candidate genes which are involved in biotype evolution as well as in mediating the aphid-plant interactions have been identified [12]. However, the lack of a successful RNAi

methodology in *A. glycines* has proved to be a big impediment for functional analysis of candidate genes and in turn, the understanding of molecular mechanisms in this insect.

Of late, there has been significant improvement in RNAi technology available for insect genetics research. One such technique involves a unique method of delivering small interfering RNAs (siRNAs), coupled with nanoparticles, through aerosolization with a nebulizer [13]. Specifically, siRNA-nanoparticle complexes are delivered into the insect in the form of an aerosol spray that penetrates the spiracles and travels through the tracheal respiratory system to silence targeted gene expression. This technology has been developed recently and was successfully demonstrated in the honey bee [13] and, if successful, can vastly improve functional genetic research in pest species. Recent development of the above-mentioned RNAi technology presents an ideal opportunity to test a unique way of siRNA delivery in *A. glycines*. Successful execution of this non-destructive delivery method in *A. glycines* is not only expected to result in significant RNAi silencing but also allow for a rapid evaluation of a large number of genes. Thus, through the IGTRCN's peer-to-peer training program, my overall goal was to learn and adapt this novel RNAi technology for use in the *A. glycines*, an insect where gene silencing has not been documented so far.

Methodology

Insect and plant source

Prior to arrival of trainee, an *A. glycines* colony was established in the host laboratory. *A. glycines* were obtained from a biotype 1 laboratory colony maintained at the Ohio Agricultural Research and Development Center (OARDC) Wooster, OH. This colony originated from insects collected at Urbana, IL (40° 06′ N, 88° 12′ W) in 2000

[14]. The laboratory population of these insects were maintained on susceptible soybean seedlings (Williams82) in a growth chamber at 24℃ and a photoperiod of 14:10 h (Light:Dark).

A. glycines BAT cDNA identification and siRNA design

For RNAi exploration in *A. glycines*, we aimed to target a gene that encodes for an enzyme called branched chain-amino acid transaminase (BCAT) (hereafter referred in the soybean aphid as *AyBcat*). The enzyme BCAT is generally involved in the degradation of the branch-chain amino acids, leucine, isoleucine, and valine. In aphids and other sap-feeding insects, BCAT is hypothesized to be involved also in the reverse reaction, i.e. the biosynthesis of the branch-chain amino acids [15-17]. The majority of sap-feeding obligate nutritional symbionts, including the aphid symbiont *Buchnera* encodes the majority of genes within the branch-chain amino acid biosynthesis pathway for the production of leucine, isoleucine, and valine, but has lost the homolog for BCAT [18]. In turn, it is hypothesized that aphid specialized cells that harbor *Buchnera* up-regulate BCAT to complement the symbiont's missing BCAT homolog enzyme for the production of branch-chain amino acids [15-17]. Sap-feeding insects including aphids require these branch-chain amino acids for survival, because they are not available at sufficient quantities in the aphid's sap diet. Moreover, they cannot be made *de novo* by the aphid, similar to other animals [18]. In turn, following the RNAi-mediated reduction in *AyBcat* expression, we predict a reduced body weight because of starvation of essential amino acids, and ultimately an overall negative impact on *A. glycines* fitness.

To retrieve the cDNA sequence for the *A. glycines* gene that encodes for branched chain-amino acid transaminase (*AyBcat*), the protein sequence of BCAT in

the pea aphid (*Acyrthosiphon pisum*, XP_008186504, LOC100167587) was used as a query in tblastn against the *A. glycines* transcriptome database [12]. The search yielded a single cDNA transcript showing a significant blast match [E-value 0.0; Identities 88% (381/430); Positives 93% (401/430)]. The putative *AyBcat* cDNA was 2,247 nucleotides long and contained an open reading frame (1,293 nucleotides) that encodes for an amino acid protein that consists of 430 amino acids. The identity of *AyBcat* cDNA was further confirmed through a blastx search at GenBank.

The *AyBcat* siRNAs were designed using an online tool available at https://rnaidesigner.lifetechnologies.com/rnaiexpress/ (Life Technologies, Carlsbad, CA). To track the penetration of siRNA within *A. glycines* body, the sense strand (CCUAUGAGGUUAGGUCUAAUGUAUA[dT][dT]) of the siRNA mixture (containing both sense and antisense strands) was labeled with Cyanine5 at the 5' end (Sigma-Aldrich, St. Louis, MO). The randomized siRNA sequence for the *ApTor* (encoding for enzyme carotene dehydrogenase in *A. pisum*), was employed as a control (sense strand: GGACGACTTAAGCGAGCGTTATGTT) (Hansen lab *in prep*).

A. glycines age synchronization and siRNA treatment

To obtain newborn nymphs, *A. glycines* adults (apterate females) were transferred (using a camel hair brush) and allowed to feed on first trifoliate soybean leaves (Williams82). Following the transfer, infested leaves were isolated with a small snap cage to restrict the insect movement. After ~20 h, the *A. glycines* adults were delicately removed and the leaves containing the newly hatched nymphs were again covered with snap cages. Snap cages contained holes covered with wire mesh to allow

for proper ventilation and maintenance of optimum growth conditions for *A. glycines* nymphs.

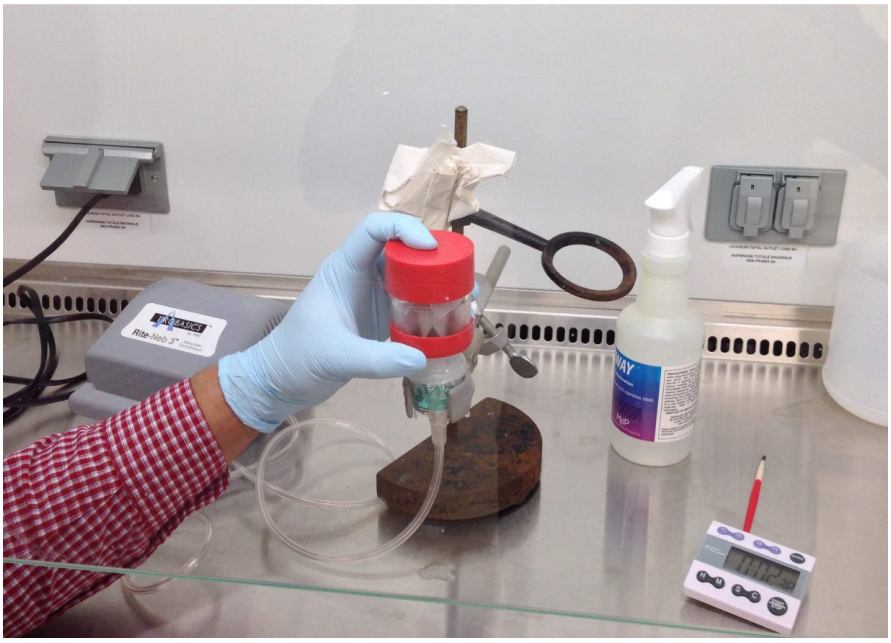

Figure 1. Experimental set up for RNAi delivery in *Aphis glycines*. Inside the enclosed plastic chamber (red edges), *A. glycines* individuals are exposed to nebulized siRNA-nanoparticle mixture.

The target or control siRNAs (@100nM and @200nM) were mixed with cationic CE perfluorocarbon nanoparticles (PFC-NP, 200pM: 65mol% DOTAP; 25mol% DOPE; 10mol% cholesterol) (Wickline laboratory, Washington University, St. Louis, MO). Before treatment, siRNAs and PFC-NP mixture was kept at room temperature for at least 40 min. For exposure to siRNA-nanoparticle mist, the *A. glycines* individuals (5-day old) were transferred on a wired mesh (sieve size 1mm x 1mm) placed horizontally at the center of a homemade plastic chamber. Subsequently, the siRNAs and PFC-NP mixture was administered to aphids using a nebulizer/compressor (Probasics, PMI, Marlboro, NJ), which aerosolizes the solution into the enclosed chamber containing insects (Figure 1). A volume of 3 mL for siRNAs and PFC-NP mixture was used for each spray treatment. All spray treatments were administered to 25-35 *A. glycines*

individuals at a time for ~5 min per chamber. Besides siRNA control (randomized *A. pisum Tor* siRNA+ PFC-NP), *AyBcat* siRNA alone (without PFC-NP) was also employed.

qRT-PCR analysis and sample processing for imaging

To measure *AyBcat* expression, *A. glycines* samples were collected at 3 and 5 day intervals post-treatment and were immediately frozen at -80℃ on dry ice, and were preserved in RNA *later*-ICE (Life Technologies, Grand Island, NY, US) prior to RNA isolation. Total RNA was extracted from individual aphid samples using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, US), following the manufacturer's protocol. To remove DNA contamination, samples were on-column treated with the Qiagen RNase-Free DNase treatment (Qiagen, Valencia, CA, US). RNA quality was checked using a Nanodrop 2000c (Thermo Scientific, Hudson, NH, US). Using iScript^{TM} cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), first strand cDNA was prepared with 100 ng RNA from each sample. The real-time reverse transcription-PCR (qRT-PCR) reactions were performed with iQ SYBR green super mix on a CFX-96 thermocycler system (Bio-Rad, Hercules, CA, USA). Specific primers for *AyBcat* (Sense: CTCTCGTACAACGGGTTTGAT; antisense: TGGTACAGCAGCAGTCATTAG) were designed using PrimerQuest tool (Integrated DNA Technologies, Inc, Coralville, IA, US). Due to its consistent expression, *RPL9* was used for normalizing the qRT-PCR data [19]. Each qRT-PCR reaction was performed with 2 µl (200 ng) of cDNA, 0.5 µl (100 µM) of each primer and 5 µl of iQ SYBR green super mix in 10 µl total volume. Each reaction was done in duplicate in a 96-well optical-grade PCR plates, sealed with optical sealing tape (Bio-Rad Laboratories, Hercules, CA). Relative expression level and fold

change were determined using comparative Ct method ($2^{-\Delta\Delta Ct}$) [20]. Statistical analysis was performed using unpaired, one-tailed t-test.

For imaging, the *A. glycines* samples were serially dehydrated in 25%, 50%, 75%, and 100% methanol solutions for 20 min each, and were kept in 100% methyl salicylate until imaging. The images were captured by a Zeiss LSM 700 confocal microscope. Data on body weight was recorded at 5 days post-treatment. Only apterate individuals were considered for body weight measurements. Data on aphid fecundity and survival was recorded daily up to 5 days after treatment. Daily average fecundity per adult was calculated by dividing the total number of nymphs laid with median adults surviving on that particular day.

Results

A. glycines stability in spray chambers

To begin with, trials were conducted to ascertain the stability of *A. glycines* individuals during aerosolized sprays in plastic chambers. The wired mesh of two sieve sizes 0.5mm x 0.5mm and 1.0mm x 1.0mm were tested individually and in combination. Twenty *A. glycines* individuals were transferred on the wired mesh(s) before being mock sprayed with water mist for 5 min. Following the spray, the counts of *A. glycines* individuals on the wired mesh or on the upper walls of chamber were similar (>80%) in all treatments and do not seem to be affected by the sieve size and the mesh number. Eventually, the wired mesh with sieve size 1.0mm x 1.0mm which is being used routinely for *A. pisum* treatments in Hansen lab was selected for subsequent *A. glycines* spray treatments.

siRNA penetration into *A. glycines* tissues

The whole-body image taken 24 h after the treatment indicated that the aerosolized siRNA-PFC-NP mixture penetrated into the *A. glycines* body and traveled to various tissues inside (Figure 2). Most likely, the nebulized siRNA-PFC-NP particles entered through spiracles on *A. glycines*' body surface and traveled through the tracheal respiratory system to reach various tissues as shown earlier [13].

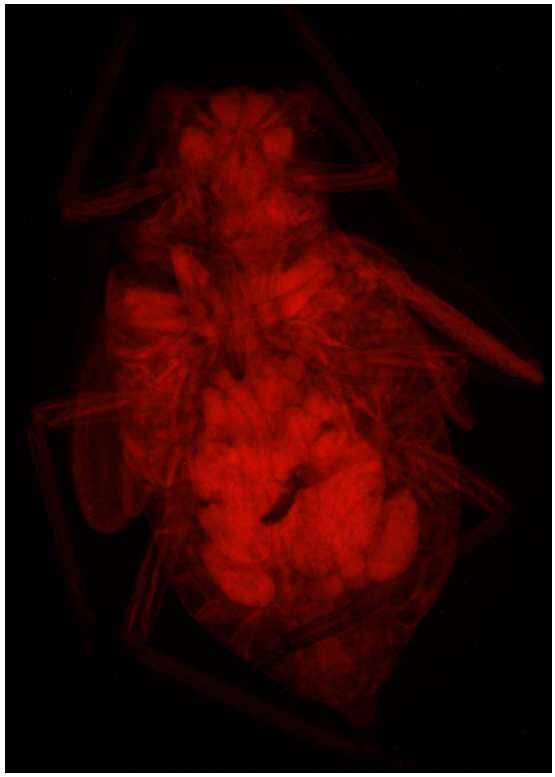

Figure 2. Nanoparticle mediated RNAi delivery in *Aphis glycines*. Whole body *A. glycines* as seen under confocal microscope 24 h after exposure to nebulized siRNA-nanoparticle mixture. The sense strand of siRNAs probe was labeled with cyanine5 at the 5'end.

Reduction in *AyBcat* expression

To determine whether aerosolized siRNA treatment alone or in combination with nanoparticles resulted in targeted gene silencing response in the *A. glycines*, we

measured *AyBcat* expression using qRT-PCR. At 3-day after treatment, we found no significant difference in *AyBcat* expression in both siRNA(*AyBcat)* treated ($P = 0.07$) and siRNA(*AyBcat*)-PFC-NP treated ($P = 0.12$) *A. glycines* compared to those treated with control siRNA-PFC-NP (Figure 3). At 5-day after treatment, *AyBcat* expression was not significantly different between siRNA(*AyBcat)* treated and control siRNA-PFC-NP treated *A. glycines* ($P = 0.48$). Further at 5DAI, *AyBcat* expression was significantly lower in siRNA(*AyBcat*)-PFC-NP treated *A. glycines* compared to those treated with control siRNA-PFC-NP ($P < 0.05$) (Figure 3). The relative transcript level of *AyBcat* were reduced by 52.13% in siRNA(*AyBcat*)-PFC-NP treated *A. glycines* compared to those in control.

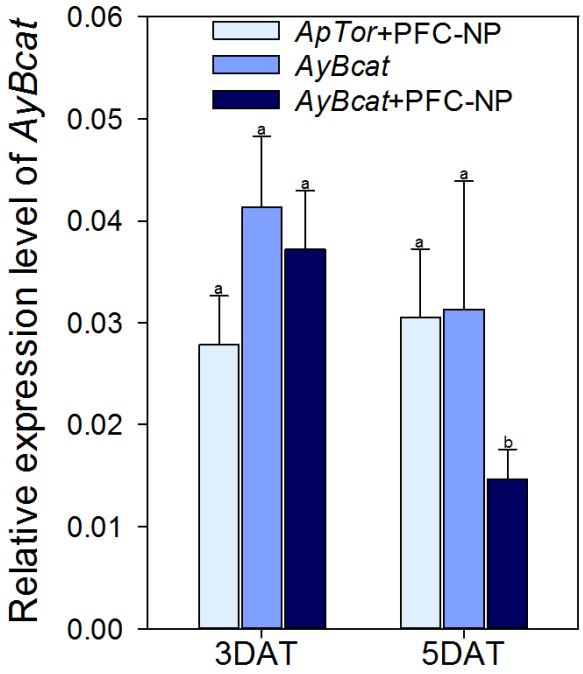

Figure 3. Effects of administering nebulized mixture of siRNAs and nanoparticles on *AyBcat* expression in *Aphis glycines*. The average expression (±standard error) at 3- and 5-day after treatment (DAT) was calculated after measuring *AyBcat* expression for six aphids individually in each treatment. The *RPL9* expression values were used for normalizing the qRT-PCR data [19]. The treatments marked with different letter indicate a significant difference ($P < 0.05$; one tail t-test) when compared to control (randomized *Acyrthosiphon pisum Tor* siRNA+ PFC-NP).

AyBcat silencing reduces aphid body weight

To determine the potential impact of reduced *AyBcat* expression on *A. glycines* biology, data on body weight were recorded. Following the spray administration at two different siRNA concentrations, the *A. glycines* body weight in different spray treatments is summarized in Table 1. At both siRNA concentrations, siRNA(*AyBcat*)-PFC-NP treated *A. glycines* had significantly lower body weight compared to those treated with the control treatment [siRNA(*ApTor*)+PFC-NP].

Table 1. *Aphis glycines* body weight statistics

siRNA concentration (nM)	Spray treatment[a]	Average body weight±S.E. (µg)[b]	T-test: *P* value[c]
100	siRNA(*AyBcat)*	124.51±6.05	0.07
	siRNA(*AyBcat*)+PFC-NP	88.10±7.57	<0.05
	siRNA(*ApTor*)+PFC-NP	110.65±6.16	-
200	siRNA(*AyBcat)*	90.58±10.05	0.19
	siRNA(*AyBcat*)+PFC-NP	69.81±8.25	<0.01
	siRNA(*ApTor*)+PFC-NP	102.84±9.01	-

[a]The PFC-NP concentration in all spray treatments was 200pM.
[b]Average weight of eight individuals in each trial at 5-day post-treatment is indicated; S.E. refers to the standard error of mean.
[c]Probability value for t-test when compared to control [randomized *Acyrthosiphon pisum Tor*, siRNA(*ApTor*)+PFC-NP] is indicated.

AyBcat silencing negatively affects survival and fecundity

Following the spray administration at two different siRNA concentrations, the percent *A. glycines* survival was comparable at different time points in both control and treated populations; though initially there was substantial decrease in survival when

siRNA(*AyBcat*)+PFC-NP was administered at 200nM (Figure 4A). The data on daily fecundity per surviving adult indicated no differences up to day 4 between control and treated populations (Figure 4B). However, there was substantial decrease in fecundity on day 5 at both concentrations used for siRNA(*AyBcat*)+PFC-NP.

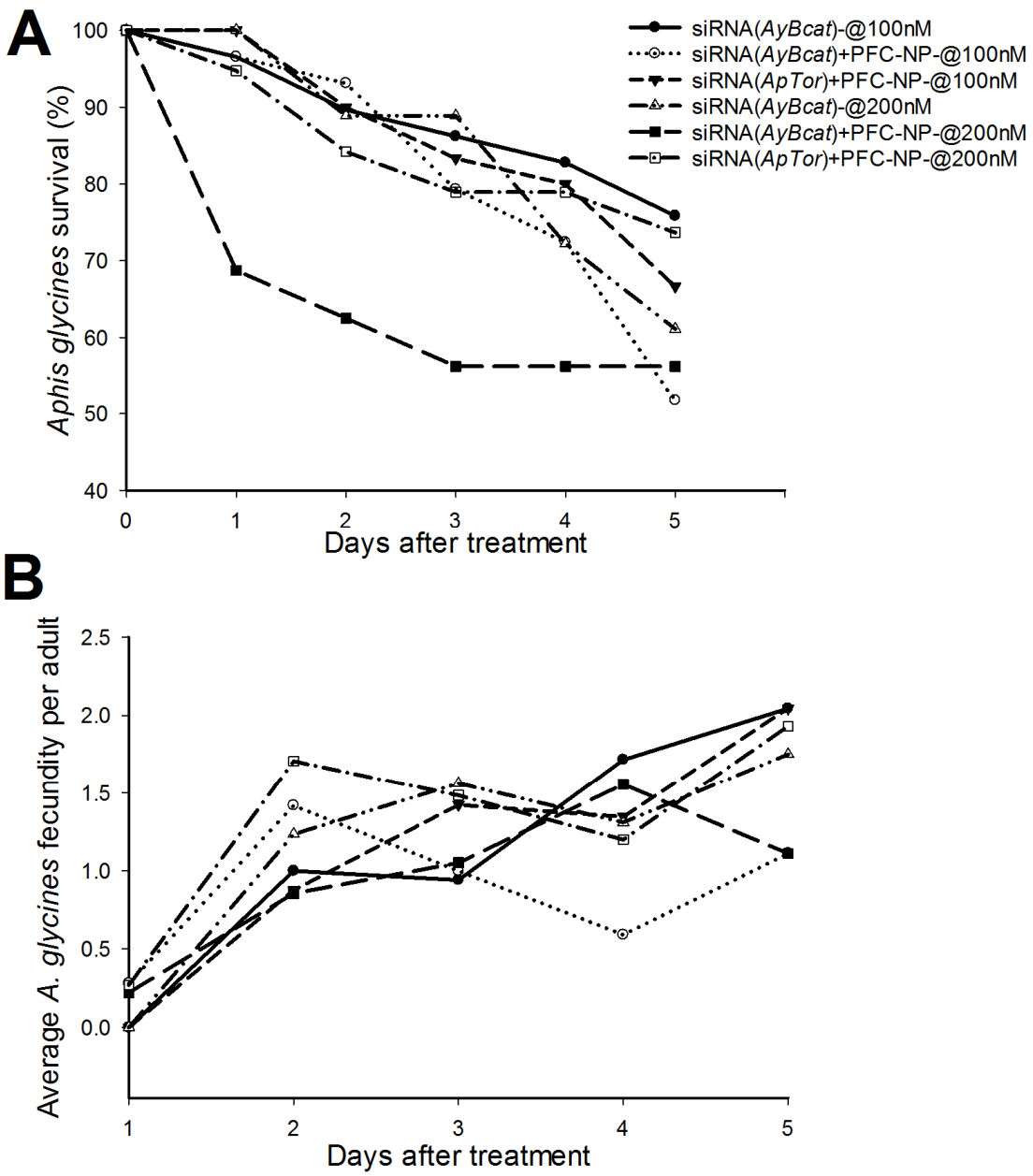

Figure 4. *AyBcat* silencing effect on *Aphis glycines* survival and fecundity. A. Daily percent survival is indicated following various spray trials. **B.** Daily average fecundity per adult is indicated following various spray trials. Two siRNA(*AyBcat*) concentrations [@100 nM: n=20; @200 nM: n=30] are indicated on the right in trial details. The nanoparticle (PFC-NP) concentration in all spray treatments was 200pM.

Conclusions

Here, we tested a novel mode of RNAi delivery using a mixture of aerosolized siRNA-nanoparticles in the soybean aphid, *A. glycines*. The imaging analysis indicated that the siRNA-nanoparticles penetrated into the *A. glycines* body and penetrated into a diversity of tissue and cell types. The qRT-PCR revealed that the aerosolized delivery of siRNAs significantly reduced the expression of target gene (*AyBcat*), when siRNA(*AyBcat*) was used in combination with nanoparticles, compared to control siRNA-nanoparticles and siRNA(*AyBcat*) alone (without nanoparticles). Due to silencing of *AyBcat*, *A. glycines* individuals exhibited a significantly reduction in body weight. Overall, our results suggest that the intended execution and implementation of this novel RNAi technology was successful in *A. glycines*, an insect system where RNAi has not been documented so far. In the long term, this technology carries potential to revolutionize the basic as well as applied research on *A. glycines* which is a serious pest in soybean growing regions of US and Canada.

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