A first greenhouse application of bacteria-expressed and nanocarrier-delivered RNA pesticide for *Myzus persicae* control

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Research Article

Keywords: hpRNA expression system, Nanocarrier, Nanocarrier/hpRNA pesticide, RNA interference, RNA pesticide

Posted Date: January 24th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-236095/v1

Version of Record: A version of this preprint was published at Journal of Pest Science on February 18th, 2022. See the published version at https://doi.org/10.1007/s10340-022-01485-5.

Abstract

There are two main limitations for sprayable RNA pesticide development: delivery efficiency and synthetic cost of double-stranded RNA (dsRNA). We previously constructed a nanocarrier-based transdermal dsRNA delivery system and a novel bacteria-based hairpin RNA (hpRNA) expression system to solve these challenges. Herein, as a subsequent exploration of RNA pesticide (sprayable ds/hpRNA for pest control), we performed a greenhouse application of bacteria-expressed and nanocarrier-delivered RNA pesticide on green peach aphid. The nanoparticle SPc could combine and deliver dsRNA across the aphid cuticle, and V-type proton ATPase subunits d (ATP-d) and G (ATP-G) were selected as the potential RNA interference (RNAi) targets. Our plasmid- Escherichia coli system simultaneously expressing ATP-d and ATP-G hairpin RNAs (hpRNAs) was constructed for mass production of hpRNA. The expressed hpRNA was mixed with SPc and detergent to form RNA formulation, which showed a certain insecticidal activity through the spray application in the greenhouse. Total control efficacy of our RNA pesticide could reach 61% on 3 d, and maintained at 50% until the sixth day. To our knowledge, our study is the first attempt to apply the bacteria-expressed and nanocarrier-delivered RNA pesticides for pest control in the greenhouse trial, which is beneficial for promoting the development of RNA pesticides.

Key Message

· SPc can protect and deliver dsRNA from degradation for efficient gene silencing

· hpRNA is expressed in *E. coli* system to develop a novel RNA insecticide

· Our RNA pesticide can be applied to control aphids in greenhouse

· Our study brings the concept of RNAi-based pest management to practical application

Introduction

RNA interference (RNAi) was first identified in the nematode *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), resulting in the sequence-specific suppression of gene expression (Fire et al., 1998). This technology offers new opportunities for the development of sustainable integrated pest management plans (Price and Gatehouse, 2008; Whangbo and Hunter, 2008; Bolognesi et al., 2012; Zotti and Smagghe, 2015). RNAi has shown a promising control effect against some plant chewing pests, especially the coleopteran pests (Baum et al., 2007; Zhang et al., 2019). Transgenic plants can be engineered to express hairpin RNAs (hpRNAs) against some functional genes in chewing and piercing-sucking pests (Pitino et al., 2011; Zha et al., 2011; Thakur et al., 2014; Zhang et al., 2017). However, the major obstacle may come from the potential ecological consequences and public concerns of genetically modified foods (Yan et al., 2015, 2018, 2020a). Meanwhile, there are also promising non-transformative RNAi applications/approaches available. RNAi via sprayable or soaking approaches has been recognized as a potential strategy for pest control, and thereafter, it has been well studied in a number of insect species (Avila et al., 2018; Petek et al., 2020). However, the RNAi efficiency can greatly vary among

different insect species. Instability of dsRNA, insufficient dsRNA internalization, deficient RNAi machinery, impaired systemic spreading of the RNAi signal, and refractory target genes have been proposed as factors constraining RNAi efficiency (Wang et al., 2016; Singh et al., 2017; Christiaens et al., 2018; Cooper et al., 2019).

In recent years, nanoparticle-mediated RNAi has been reported as a novel method for delivering dsRNA to improve RNAi efficiency (Shen et al., 2014; Xu et al., 2014; Christiaens et al., 2018). We previously constructed a nanocarrier-based dsRNA delivery system through oral feeding to control plant chewing pests (He et al., 2013; Xu et al., 2014). To broaden its application scope, we recently constructed a transdermal dsRNA delivery system with a fluorescent nanoparticle on soybean aphids (Zheng et al., 2019). However, the high production cost restrains the practical application of synthetic polycations, and there is a great need for highly efficient with low-cost gene vectors (Wightman et al., 2001; Rai and Ingle, 2012; Kumar et al., 2019). To this end, we synthesized a star polycation (SPc) with low cost and high efficiency, which could deliver dsRNA across the insect cuticle for efficient gene silencing and pest control (Li et al., 2019; Yan et al., 2020b). Although the technical bottleneck of dsRNA delivery was solved, the absence of cost-effective methods for dsRNA production should be another challenging factor. To solve this problem, we removed the *rnc* gene encoding endoribonuclease RNase from *Escherichia coli* BL21 (DE), and introduced an RNAi expression vector containing a single T7 promoter to construct a novel plasmid-*E. coli* system. Its hpRNA expression efficiency was three times that of L4440-HT115(DE3), a widely used hpRNA production system (Ma et al., 2020). The combination of these two technologies may overcome the current technical bottlenecks and promote the development of RNA pesticides.

Aphids are a group of sap-sucking insects that not only suck nutrients from plant phloem but are also plant virus vectors (Hogenhout et al., 2008). Currently, their control measure largely relies on the application of insecticides, which may lead to the emergence of severe resistance (Niu et al., 2019). Thus, there is an urgent need for alternatives to control aphids to avoid the side effect of chemical pesticides. The eukaryotic V-type ATPase (V-ATPase) is a membrane protein complex that consists of multiple subunits, such as A, B, C, D, E, F, G, H, a, c, c', c'', d and e, and its disruption induces the lethal effect in various insect species, suggesting that this molecule is a promising RNAi target for pest control (Baum et al., 2007; Li and Xia, 2012; Basnet and Kamble, 2018; Sato et al., 2018; Tariq et al., 2019). In the current study, we performed a greenhouse test of bacteria-expressed and nanocarrier-delivered RNA pesticide on an insecticide-resistant species green peach aphid. The *V-type proton ATPase subunit d* (*ATP-d*) and *ATP-G* were selected as the RNAi target genes, and the hpRNAs targeting these two genes were fused together to simultaneously expressed in *E. coli* BL21(DE3). The insecticidal activity of hpRNA/nanocarrier pesticide was assayed through the spray application in the greenhouse. Our RNA pesticide achieved a promising control effect on aphids, which was the first attempt to apply the bacteria-expressed and nanocarrier-delivered RNA formulation in the greenhouse for pest control.

Materials And Methods

Insect rearing

Myzus persicae were reared in an environmental chamber under a photoperiod of 16 h light: 8 h dark at 25°C. The aphids were fed on radish plants (*Raphanus sativus*).

Infiltration assay of transdermal dsRNA delivery system in *Myzus persicae*

A gel retardation test with various mass ratios of ds*eGFP*/SPc complexes was performed to determine whether the complexes were stable. The ds*eGFP* mass was 1 μg for each sample, and the ds*eGFP*/SPc mass ratios were 1:0, 2:1, 3:2, 1:1, 2:3, and 1:2. The fluorescent ds*eGFP* was synthesized using Fluorescein RNA labeling Mix (Roche Diagnostics, USA) according to the manufacturer's protocol. To test the transdermal dsRNA delivery system, the SPc (1 μg) was mixed with fluorescent ds*eGFP* (1 μg) at the recommended mass ratio of 1:1 at room temperature for 15 min, and the 0.5% volume of detergent (LIBY, China) was then added into the formulation to prepare the fluorescent dsRNA/SPc/detergent formulation (the final concentration for both SPc and dsRNA was 100 ng/μl). The corresponding fluorescent dsRNA/detergent and SPc/detergent formulations were prepared similarly, and the final concentration for both SPc and dsRNA was 100 ng/μl. The 0.2 μL fluorescent dsRNA/SPc/detergent, fluorescent dsRNA/detergent and SPc/detergent solution were applied on the notum of 4th instar *M. persicae* with an RN Needle (34/10mm/4) (Hamilton, USA), respectively. The fluorescence images of aphids were scanned on the fluorescence microscope EVOS FL (AMG, USA) at 6 h after the topical application. Then, the aphids were submerged in a drop of optimum cutting temperature (O.C.T) compound (SAKURA, USA), and placed in the cryostat chamber (− 30 °C) for 30 min to prepare cryosection. The aphids were crosscut into 10 μm sections and were scanned with the fluorescence microscope EVOS FL (AMG). Fluorescence intensities of aphids were calculated using ImageJ 1.48v (National Institutes of Health, USA) from six independent samples.

Stability test of nanocarrier-delivered dsRNA in aphid hemolymph

The aphid hemolymph was collected from 4th instar aphids according to the method previously described by Tassetto et al. (2017). Aphids were transferred onto a sheet of parafilm in a drop of cold PBS (200 μL per 25 aphids), and quickly wounded with an insect needle. Aphids were then transferred to a centrifuge tube previously punctured with an insect needle and placed into a 1.5 mL tube and spun at 3000 g for 3 min at 4℃. The collected supernatant in a 1.5 mL tube was transferred to a new tube and spun at 10000 g for 5 min to remove the tissue contaminants. For degradation assays, different dilutions of hemolymph were prepared and mixed with 1 μg ds*eGFP*. The mixture (10 μL) was incubated at room temperature for 1.5 h and then analyzed by 1% agarose gel electrophoresis.

To investigate the stability of nanocarrier-delivered dsRNA, the concentration of hemolymph capable of degrading 1 μg ds*eGFP* was used to treat ds*eGFP*/SPc complexes. The ds*eGFP*/SPc complexes were decomplexed by adding 1 μL of a 3% SDS solution, and then analyzed by 1% agarose gel electrophoresis. The relative band density was determined using the ImageJ 1.48v from 3 independent samples. Meanwhile, the 1 μg ds*eGFP* was mixed with SPc, and was treated with hemolymph under the same incubation condition. The resulting complexes were decomplexed by adding 1 μL of a 3% SDS solution,

and then purified using MEGAclear Kit (Thermo Fisher Scientific). The resulting dsRNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Three replications were prepared for determining the relative dsRNA concentration. Naked ds*eGFP* solution was used as the control.

Biochemical synthesis of dsRNA

Total RNA was extracted from ten aphids using RNA simple Total RNA Kit (TIANGEN, Beijing), and then prepared for cDNA synthesis using Prime-Script RT reagent Kit (TaKaRa, Japan). All primers were synthesized by TSINGKE (China), and the primers for the biochemical synthesis of dsRNA are listed in Table S1. The sequence fragments of the target genes (*ATP-A*: 413 bp, LOC111039523; *ATP-d*: 383 bp, LOC111041166; *ATP-G*: 301 bp, LOC111040044) were amplified using the 2×Taq PCR Master Mix kit (TIANGEN). The amplified sequences were cloned into pMD19T-Vector (TAKARA) and transformed into DH5α competent cells (TSINGKE). The plasmids extracted from *E. coli* were used as templates for dsRNA synthesis using a T7 RiboMAX Express RNAi System (Promega, USA) according to the manufacturer's protocol.

In vivo hpRNA synthesis and extraction

The *ATP-d* and *ATP-G* hpRNAs were selected to simultaneously express in plasmid-*E. coli* BL21(DE3) RNase III- system. Plasmid pMD19T-*ATP-d* and pMD19T-*ATP-G* were used as templates for the amplification of ATP-d-L (508 bp), ATP-d-R (413 bp), ATP-G-L (331 bp), and ATP-G-R (333 bp). ATP-d-L and ATP-G-L contained an overlap at their ends, and were fused by fusion PCR. The combined fragment was digested with EcoR□-Xba□ (NEB, USA) to generate ATP-L. ATP-d-R and ATP-G-R also contained to overlap at their ends, and their combined fragment was digested with Xba□-Xho□ (NEB) to generate ATP-R. ATP-d-L contained a 95 bp DNA sequence for the formation of a stem-loop structure. Two fragments, including ATP-L and ATP-R, were cloned into the EcoR□-Xho□ site of pET-28a (TIANDZ, China) together to generate pET28-*ATP-d+G*. pET28-*ATP-d+G* vector was identified by restriction enzyme digestion of EcoR□-Xho□ (Fig. 4A). Primers for plasmid construction are listed in Table S1.

Two hpRNAs were simultaneously expressed in the pET28-ATP-BL21(DE3) RNase III- system according to the method described by Ma et al. (2020). The only difference was that IPTG was added when the OD_{600nm} value reached 0.5. For sonication treatment, 40 mL of the bacterial cultures were sonicated on ice using an ultrasonic cell disruptor (XINCHEN, China) equipped with a microprobe. The sonication was performed at maximum power on ice for 10 s 90 times with 10 s interval between each sonication, and the cell culture was then centrifuged at 12,000 g for 10 min to remove the cell debris (Ahn et al., 2019). 5 μL supernatant was then evaluated by 1% agarose gel. The resulting RNA and DNA were extracted from the supernatant by MEGAclear Kit (Thermo Fisher Scientific) and TIANamp Bacteria DNA Kit (TIANGEN, China), respectively, from the same sample, and then quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The quantification of nucleic acids was repeated 3 times to determine the concentration.

Laboratory RNAi assays

The 9-day-old adults were transferred on radish plants, and then removed one day later, while nymphs were maintained on the plants to the 4th instar. The SPc was mixed with dsRNA at the mass ratio of 1:1 at room temperature for 15 min, and the 0.5% volume of detergent (LIBY, China) was then added into the formulation to prepare the dsRNA/SPc/detergent formulation (the final concentration for both SPc and dsRNA was 720 ng/μl). The corresponding dsRNA/detergent formulation was prepared similarly, and the final concentration for dsRNA was 720 ng/μl. The ds*ATP-d* and ds*ATP-G* were used for double-gene RNAi with the final concentration of 360 ng/μL for each target gene. Total nucleic acids (105.3±1.33 ng/μL) extracted from pET28-ATP-BL21(DE3) RNase III- cells contained 67.33±2.96 ng/μL RNA (hpRNA) and 38±1.73 ng/μL DNA. Nucleic acids extracted from BL21(DE3) RNase III- cells were mixed with SPc at the mass ratio of 1:1, and the 0.5% volume of detergent (LIBY) was added to the formulation to prepare hpRNA/SPc/detergent formulation (final concentration for hpRNA: 100 ng/μL). The 0.2 μL formulation was applied on the notum of 4th instar aphids. Aphids treated with ds*eGFP*-SPc and pET28-EV-SPc were used as the control. The aphids were transferred to a new plant after the topical application. Each treatment contained 15 aphids. Three replicates were prepared for determining RNAi efficiency, and 6 replicates for mortality bioassay. Dead aphid number was recorded on 3rd day after the application.

Samples for determining RNAi efficiency were collected at 24 h post the topical application. The 900 ng RNA was used for cDNA synthesis. The quantitative real-time PCR (qRT-PCR) was performed with Step One Plus Real-Time PCR system (Applied Biosystems, USA) using Power SYBR® Green Master Mix (Applied Biosystems). All qRT-PCR reactions were performed adopting the previously published method (Zheng et al., 2019). The metadata of qRT-PCR experiments is shown in Table S3. The linear ranges and amplification efficiencies were determined across five 10-fold serial dilutions of cDNA. The relative mRNA levels of target genes were normalized to the abundance of *EF-1α* (*elongation factor 1 alpha*) and *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) gene using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001). The primers for qRT-PCR are listed in Table S2.

Control efficacy analysis of RNA pesticide in greenhouse

Control efficacy was analyzed in an oilseed rape greenhouse (Beijing Liangzhiyuepin Agricultural Co., China) with a day/night temperature of 25/18°C. Rows were spaced 0.5 m apart, and plants within rows were spaced 0.15 m apart. The aphids were natural populations, and the population density was approximately 290 per plant. As shown in Fig. 1, the bacteria-expressed hpRNA/SPc formulation was performed as described in laboratory RNAi assays. Then, 0.5% volume of detergent was added to obtain RNA pesticide, which was sprayed directly on top of leaves of four-week-old oilseed rapes infested with *M. persicae*. The spraying experiment was carried out using a pneumatic water sprayer (JIALESHI, China) with an application amount of 100 mL/m^2. A chemical pesticide thiamethoxam (Syngenta, Switzerland) targeting aphids was diluted 10000 times with ddH_2O and served as a positive control, and ddH_2O served as a negative control. The area of each plot was approximately 20 m^2, and each plot contained 40-60 plants. Ten plants from each plot were selected as ten replicates to record the aphid number. Numbers of aphids on top of leaves and leaf backs were counted and recorded separately on 0, 1, 2, 3, 4, 5, and 6 d

after the treatment. Ten plants from each plot were weighted as ten replicates using an electronic analytical balance (METTLER TOLEDO, Switzerland) to estimate the potential side effects of RNA pesticides. The dropping rate of insects and the control efficacy were calculated as below.

Dropping rate of insect (%) = (number of pests before pesticide application − number of pests after pesticide application)/number of pests before pesticide application

Control efficacy (%) = (dropping rate of an insect in the treatment plot − dropping rate of an insect in the control plot)/(100 − dropping rate of an insect in the control plot)

Absorption detection of dsRNA/SPc complex by plants

To investigate whether SPc could promote the absorption of dsRNA by plants, the roots of ten-day-old oilseed rapes were immersed in 100 μL of fluorescent dsRNA/SPc, fluorescent dsRNA and SPc, respectively (the final concentration for both dsRNA and SPc was 200 ng/μL). The fluorescence images of roots were obtained on the fluorescence microscope EVOS FL (AMG) at 6 h after the treatment.

Furthermore, the method described by Wang et al. (2016) was adopted and modified to test the absorption. Serial dilutions of known ds*eGFP* amounts were reverse transcribed into cDNA, creating a five-point standard curve for ds*eGFP* quantification. The 100 μL of ds*eGFP*/SPc and ds*eGFP* were applied to treat plant roots similarly as above, and the 10 μL of ds*eGFP*/SPc/detergent and ds*eGFP*/detergent were applied on the top of leaves (the final concentration for both dsRNA and SPc was 200 ng/μL). Leaves from each treatment were washed 3 times with PBS, and added with 180 μL prepared reagent containing 2% SDS, 0.1mol/L Tris-HCl (PH 8.0), 0.5mol/L Nacl, 0.05mol/L EDTA and 0.05% DEPC bought from Solarbio (China). Then, 20 μL of KAC (PH 4.8) (Sigma-Aldrich, Germany) was added to reach a final concentration of 0.3 mol/L. Phenol water (Solarbio) was added into the solution, vortexed vigorously, and then centrifuged for 15 min. The supernatant was mixed with isopropanol (Sigma-Aldrich) of the same volume, stored at -20 °C for 30 min, and then centrifuged. The nucleic acid pellet was carefully washed using 70% ethanol and centrifugation. After removing residual ethanol, the pellet was air-dried in a sterile hood and resuspended in nuclease-free water. RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). All centrifugations were performed at 12,000 g and 4℃. Each treatment was repeated 6 times. The 900 ng RNA was used to synthesize cDNA, and qRT-PCR was performed with Step One Plus Real-Time PCR system described above, except for no housekeeping gene normalization. The cycle number performed from either ds*eGFP*/SPc or ds*eGFP* treatments could indirectly represent the dsRNA content.

Statistical analysis

A two-tailed Student's *t*-test was performed for statistical analyses of two columns with Prism 7.0 (GraphPad Software Inc., USA) at the $p = 0.05$ level of significance. Statistical analyses of multiple columns were conducted using SPSS 20.0 (SPSS Inc., USA). The ANOVA with Tukey HSD test was

conducted to determine statistical differences at the $p = 0.05$ level of significance. The descriptive statistics are shown as the mean value and standard errors of the mean.

Results

Infiltration assay of transdermal dsRNA delivery system in *Myzus persicae*

A gel retardation test was first performed to determine the optimal mass ratio for the combination of dsRNA with SPc, and the results showed that the mass ratio of 1:1 was recommended to be used in the following study (Fig. 2A). The fluorescent dsRNA/SPc/detergent droplet smoothly spread around the aphid integument, and high-intensity fluorescence was detected at 6 h post topical application. As expected, the fluorescence intensities of the two other treatments were much weaker (Fig. 2B-D). The results of frozen section assays were similar. The fluorescence signal was detected in sections of both dsRNA/SPc/detergent and dsRNA/detergent-treated aphids (Fig. 2E-J'). More specifically, the fluorescence intensity of dsRNA/SPc/detergent-treated aphids was over 5 times stronger than those treated with dsRNA/detergent (Fig. 2K).

Enhanced stability of SPc-delivered dsRNA and potential RNAi target gene screening

The dsRNA could be completely degraded upon incubation in 12.5% of aphid hemolymph (Fig. S1A). To investigate the protective effect of SPc on dsRNA, the ds*eGFP*/SPc complex was incubated in 12.5% of aphid hemolymph, which could not degrade SPc-delivered ds*eGFP* (Fig. S1B-D). The strong protective effect of SPc on dsRNA might be beneficial for improving RNAi efficiency. The *ATP-A*, *ATP-d* and *ATP-G* were selected as the potential RNAi target genes in the current study. As shown in Fig. 3A, the relative expression of *ATP-A*, *ATP-d* and *ATP-G* were significantly downregulated from 35% (*ATP-G*) to 20% (*ATP-A*) after treated with SPc-delivered dsRNA; however, the expression level of three target genes showed no significant change when treated with naked dsRNA. Silencing of *ATP-d* and *ATP-G* resulted in an obvious lethal effect with the percent survival of 46% and 59%, respectively (Fig. 3B). Co-silencing of these two target genes led to higher mortality of 70% (Fig. 3C-D).

Bacteria-based hpRNA expression and lethal effect evaluation in the laboratory

Two reverse complementary sequences of the *ATP-d+G* gene were introduced into pET-28a, one of them contained a loop structure. The constructed vector pET28-*ATP-d+G* was transformed into BL21(DE3) RNase III- to produce hp*ATP-d+G* (684 bp) (Fig. 4A-B). Plasmid pET28-*ATP-d+G* was digested with EcoR□-Xho□ to identify the recombination of the RNAi vector, generating a 1463 bp fragment (Fig. 4C). The expressed hpRNA could downregulate the expression of *ATP-d* and *ATP-G* simultaneously through the topical application (Fig. 5A), resulting in a moderate lethal effect (40% mortality rate) against aphids (Fig. 5B).

Spray application of bacteria-expressed and nanocarrier-delivered RNA pesticide in greenhouse

The ddH_2O, hpATP-*d+G*/SPc complex, and thiamethoxam were sprayed directly on top of leaves, and the numbers of aphids on top of leaves and leaf backs were recorded separately. The population density of aphids on top of leaves was significantly decreased after the application of RNA pesticide or thiamethoxam, and the body of dead aphids turned black and shriveled (Fig. 6A-C). Surprisingly, the population density of aphids on leaf backs showed a significant reduction (Fig. 6A-C). The dropping rate of aphids from top of leaves treated with RNA pesticides increased significantly, with the highest dropping rate (47%) occurred on 2 d, and its control efficacy varied from 39% to 63%, which was lower than that of thiamethoxam (Fig. 6D and 6F). The dropping rate of aphids on leaf backs treated with RNA pesticides showed a rapid reduction on 4 d (from -36% to -241%) (Fig. 6E). Meanwhile, its highest control efficacy (70%) occurred on 3 d post-application (Fig. 6G).

To further reveal how the hpATP-*d+G*/SPc complex showed a certain insecticidal activity against aphids on leaf backs, we tested the absorption of SPc-delivered dsRNA by plants (Fig. S2). Compared to dsRNA along, the fluorescence intensity of plant roots was much stronger after treating with dsRNA/SPc (Fig. S2A-C'). The calibration line for ds*eGFP* quantification was constructed by calibration experiments with different amounts of ds*eGFP* (Fig. S2D). The cycle number in ds*eGFP*/SPc-treated samples either from the spraying or soaking method was much smaller than ds*eGFP*-treated samples, indicating the enhanced absorption of SPc-delivered dsRNA by plants (Fig. S2E-F).

The total dropping rate and control efficacy of aphids are shown in Fig. 7. The dropping rate of aphids treated with RNA pesticide was consistently higher than the negative control, but lower compared to thiamethoxam. Although not as good as thiamethoxam, the control efficacy of RNA pesticide reached 61% on 3 d, and was maintained at about 50% until the sixth day. In addition, the weight of oilseed rapes treated with different formulations showed no significant difference (Fig. S3).

Discussion

RNA pesticides are suitable for suppressing pests on stems, foliage or fruits, and the dsRNA/siRNA formulation can be sprayed on pests or plants, which may penetrate the pest cuticle or feed pests to induce lethal effects (Dalakouras et al., 2016; Yan et al., 2020c, d). The application of RNA pesticides showed a promising control effect on an RNAi-sensitive species *Leptinotarsa decemlineata* (San Miguel and Scott, 2016). However, the dsRNAs are usually not stable in the environment, and the RNAi efficiency can vary greatly among different insect species. Efficient delivery methods such as liposomes, artificial extracellular vesicles or nanoparticles were applied to improve the RNAi efficiency (Numata et al., 2014; Shen et al., 2014; Huang et al., 2019). In the current study, high-intensity fluorescence was observed after the treatment of fluorescent dsRNA/SPc/detergent formulation, indicating the successful construction of nanocarrier-mediated transdermal dsRNA delivery system in green peach aphids. In addition, the SPc exhibited a strong protective effect on dsRNA, which was crucial for its stability in the environment.

The silencing of *ATPase* genes shows great potential for pest control, and the most inspiring breakthrough is the development of transgenic maize expressing *ATP-A* hpRNA to efficiently control the

western corn rootworm (Baum et al., 2007). In the current study, the *ATP-A*, *ATP-d,* and *ATP-G* were first tested as the potential RNAi target genes, and their relative expressions were downregulated through the nanocarrier-based transdermal dsRNA delivery system. The higher efficacy of the nanocarrier-mediated RNAi might be due to the high delivery efficiency and good stability of SPc-delivered dsRNA. Meanwhile, co-silencing of *ATP-d* and *ATP-G* in *M. persicae* led to a mortality rate of 70%, suggesting the suitable RNAi target genes for pest control.

The large-scale production of dsRNA has been regarded as a limitation of RNA pesticides for a long time (Bellés, 2010; Zotti and Smagghe, 2015). With the rapid development of RNA pesticides in recent years, companies such as GreenLight Biosciences achieved the massive production of dsRNA at a low cost of $0.5/g (Taning et al., 2019). Previously, we constructed a pET28-BL21(DE3) RNase Ⅲ- system for expressing hpRNA to decrease its production cost for field application (Ma et al., 2020). In the current study, the concentration of hpRNA produced by our system was approximately 67 ng/μL. However, the actual concentration of hpRNA could be higher due to the inevitable loss in the purification process. With the application of the sonication method and reduced cost of LB culture, our novel expression system could decrease the production cost of hpRNA to $0.47/g. In laboratory RNAi assays, the RNAi efficiency of bacteria-expressed hpRNA was lower than the biochemical-synthesized dsRNA, which was related to the different concentrations of hpRNA between dsRNA. However, the exposure dose of RNA pesticides in the greenhouse would be higher compared to the 0.2 μL dsRNA/SPc/detergent formulation through the topical application in the laboratory. The insecticidal activity of RNA pesticides through the spray application might be acceptable in the field.

The spraying experiment against green peach aphids was conducted in an oilseed rape greenhouse. Our RNA pesticide exhibited a certain insecticidal activity against aphids. Interestingly, the population density of aphids on leaf backs showed a significant reduction, indicating that the RNA pesticide could be taken up by plants and exhibit lethal effects upon ingestion. To determine whether the up-take of dsRNA was improved with the SPc, we detected the dsRNA content in leaves. The cycle numbers performed from either ds*eGFP*/SPc or ds*eGFP* treatments could be used to represent the dsRNA content, which could be obtained using the standard curve created by serially-diluted ds*eGFP* solution. The amount of ds*eGFP* in leaves treated with ds*eGFP*/SPc was significantly more than that with naked ds*eGFP*, which indicated that our nanocarrier could efficiently deliver dsRNA into plant roots and leaves. To reduce the experimental error as much as possible, each treatment was repeated 6 times. RNA extracted from leaves was incubated at 65 ℃ for 5 min to denature the secondary structure of the dsRNA. Therefore, we did not introduce any housekeeping genes for calibration.

The total dropping rate revealed that our RNA pesticide should be sprayed every 3 days to prevent the population growth of aphids compared to 6 days of thiamethoxam. Although not as good as thiamethoxam, the control efficacy of RNA pesticide was comparable with some botanical pesticides that we previously tested (Yan et al., 2019). The highest control efficacy of D-limonene occurred on 5 d post-treatment (67%), while that of pyrethrin was observed with 56% on 3 d post-treatment. The insecticidal activity of RNA pesticide should be further improved to meet the actual demands. Firstly, more effective

RNAi target genes with high mortality should be selected. Secondly, the optimal condition of the pET28-BL21(DE3) RNase Ⅲ- system for expressing hpRNA should be further investigated to increase the concentration of expressed hpRNA. Finally, the combination of SPc with matrine could decrease its particle size to improve the toxicity against aphids (Yan et al., 2019). The application of SPc to deliver botanical pesticides and RNA pesticides simultaneously could improve the control effect.

The first attempt to apply nanocarrier-mediated RNAi was conducted to silence chitin synthase genes in *Anopheles gambiae* (Zhang et al., 2010). Similarly, the nanoparticle-mediated RNAi was further tested in *Spodoptera frugiperda* via a synthetic cationic polymer (Parsons et al., 2018), and *Spodoptera exigua* via a guanylated polymer (Christiaens et al., 2018), which exhibited an improved RNAi efficiency. The production cost and potential risk of RNA pesticide showed great commercial interest for the agribusiness. The polycation SPc was constructed based on cheap material sources by a simple synthetic process, which decreased its production cost to $1.7/g (Li et al., 2019). Meanwhile, the sonication method was applied to obtain dsRNA from a bacterial expression system, which simplified the operation and decreased the cost. A series of nanoparticles constructed by our group showed good biocompatibility *in vitro* and *in vivo*, with much lower cytotoxicity than polyetherimide (Xu et al., 2014; You et al., 2014; Gao et al., 2016). RNAi has been regarded as a safe strategy in pest management due to its sequence specificity (Mezzetti et al., 2020; Papadopoulou et al., 2020). However, the introduction of nanoparticles may bring some potential risks for humans and environmental health (Kah, 2015). Further studies are required to investigate the environmental risk of RNA pesticides.

Declarations

Author contributions

JS, SY, ZM and XD conceived and designed research. ZM, SY, YZ, ML and ZC conducted experiments. ZM and SY analyzed data. ZM, SY and JS wrote the manuscript. All authors read and approved the manuscript.

Funding This research was supported by the National Natural Science Foundation of China (31900363) and the Beijing Natural Science Foundation (6204043).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This research does not contain any studies with human participants or animals.

References

Ahn S, Donahue K, Koh Y, Martin RR, Choi M (2019) Microbial-based double-stranded RNA production to develop cost-effective RNA interference application for insect pest management. Inter J Insect Sci 11: 1645133984

Avila LA, Chandrasekar R, Wilkinson KE, Balthazor J, Heerman M, Bechard J, Brown S, Park Y, Dhar S, Reeck GR, Tomich JM (2018) Delivery of lethal dsRNAs in insect diets by branched amphiphilic peptide capsules. J Control Release 273: 139-146

Basnet S, Kamble ST (2018) RNAi-mediated knockdown of vATPase subunits affects survival and reproduction of bed bugs (Hemiptera: Cimicidae). J Med Entomol 55: 540-546

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T, Roberts J (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25: 1322-1326

Bellés X (2010) Beyond Drosophila: RNAi in vivo and functional genomics in insects. Annu Rev Entomol 55: 111

Bolognesi R, Ramaseshadri P, Anderson J, Bachman P, Clinton W, Flannagan R, Ilagan O, Lawrence C, Levine S, Moar W, Mueller G, Tan J, Uffman J, Wiggins E, Heck G, Segers G (2012) Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). Plos One 7: e47534

Christiaens O, Tardajos MG, Martinez Reyna ZL, Dash M, Dubruel P, Smagghe G (2018) Increased RNAi efficacy in *Spodoptera exigua* via the formulation of dsRNA with guanylated polymers. Front Physiol 9: 316

Cooper AM, Silver K, Zhang J, Park Y, Zhu KY (2019) Molecular mechanisms influencing efficiency of RNA interference in insects. Pest Manag Sci 75: 18-28

Dalakouras A, Wassenegger M, McMillan JN, Cardoza V, Maegele I, Dadami E, Runne M, Krczal G, Wassenegger M (2016) Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. Front Plant Sci 07: 1327.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811

Gao Z, Huang W, Zheng Y, Lu Y, You S, Shen J, Yin M (2016) Facile synthesis of core–shell magnetic-fluorescent nanoparticles for cell imaging. Rsc Adv 6: 46226-46230

He B, Chu Y, Yin M, Müllen K, An C, Shen J (2013) Fluorescent nanoparticle delivered dsRNA toward genetic control of insect pests. Adv Mater 25: 4580-4584

Hogenhout SA, Ammar E, Whitfield AE, Redinbaugh MG (2008) Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol 46: 327-359

Huang CY, Wang H, Hu P, Hamby R, Jin H (2019) Small RNAs - big players in plant-microbe interactions. Cell Host Microbe 26: 173-182

Kah M (2015) Nanopesticides and nanofertilizers: Emerging contaminants or opportunities for risk mitigation? Front Chem 3: 64

Kumar S, Nehra M, Dilbaghi N, Marrazza G, Hassan AA, Kim K (2019) Nano-based smart pesticide formulations: Emerging opportunities for agriculture. J Control Release 294: 131-153

Li C, Xia Y (2012) Vacuolar ATPase subunit H is essential for the survival and moulting of *Locusta migratoria manilensis*. Insect Mol Biol 21: 405-413.

Li J, Qian J, Xu Y, Yan S, Shen J, Yin M (2019) A facile-synthesized star polycation constructed as a highly efficient gene vector in pest management. Acs Sustain Chem Eng 7: 6316-6322

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402-408

Ma ZZ, Zhou H, Wei YL, Yan S, Shen J (2020) A novel plasmid-*Escherichia coli* system produces large batch dsRNAs for insect gene silencing. Pest Manag Sci 76: 2505-2512

Mezzetti B, Smagghe G, Arpaia S, Christiaens O, Dietz-Pfeilstetter A, Jones H, Kostov K, Sabbadini S, Opsahl-Sorteberg H, Ventura V, Taning CNT, Sweet J (2020) RNAi: What is its position in agriculture? J Pest Sci 93: 1125-1130

Niu J, Yang WJ, Tian Y, Fan JY, Ye C, Shang F, Ding BY, Zhang J, An X, Yang L, Chang TY, Christiaens O, Smagghe G, Wang JJ (2019) Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. Pest Manag Sci 75: 2873-2881

Numata K, Ohtani M, Yoshizumi T, Demura T, Kodama Y (2014) Local gene silencing in plants via synthetic dsRNA and carrier peptide. Plant Biotechnol J 12: 1027-1034.

Papadopoulou N, Devos Y, Álvarez-Alfageme F, Lanzoni A, Waigmann E (2020) Risk Assessment considerations for genetically modified RNAi plants: EFSA's activities and perspective. Front Plant Sci 11: 445

Parsons KH, Mondal MH, McCormick CL, Flynt AS (2018) Guanidinium-functionalized interpolyelectrolyte complexes enabling RNAi in resistant insect pests. Biomacromolecules 19: 1111-1117

Petek M, Coll A, Ferenc R, Razinger J, Gruden K (2020) Validating the potential of double-stranded RNA targeting colorado potato beetle *Mesh* gene in laboratory and field trials. Front Plant Sci 11: 1250

Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA (2011) Silencing of aphid genes by dsRNA feeding from plants. Plos One 6: e25709

Price DRG, Gatehouse JA (2008) RNAi-mediated crop protection against insects. Trends Biotechnol 26: 393-400

Rai M, Ingle A (2012) Role of nanotechnology in agriculture with special reference to management of insect pests. Appl Microbiol Biot 94: 287-293

San Miguel K, Scott JG (2016) The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. Pest Manag Sci 72: 801-809

Sato K, Miyata K, Ozawa S, Hasegawa K (2018) Systemic RNAi of V-ATPase subunit B causes molting defect and developmental abnormalities in *Periplaneta fuliginosa*. Insect Sci 26: 721-731

Shen D, Zhou F, Xu Z, He B, Li M, Shen J, Yin M, An C (2014) Systemically interfering with immune response by a fluorescent cationic dendrimer delivered gene suppression. J Mater Chem B 2: 4653-4659

Singh IK, Singh S, Mogilicherla K, Shukla JN, Palli SR (2017) Comparative analysis of double-stranded RNA degradation and processing in insects. Sci Rep-Uk 7: 17059

Taning CN, Arpaia S, Christiaens O, Dietz Pfeilstetter A, Jones H, Mezzetti B, Sabbadini S, Sorteberg HG, Sweet J, Ventura V, Smagghe G (2019) RNA-based biocontrol compounds: current status and perspectives to reach the market. Pest Manag Sci 76: 841-845

Tariq K, Ali A, Davies TGE, Naz E, Naz L, Sohail S, Hou M, Ullah F (2019) RNA interference-mediated knockdown of voltage-gated sodium channel (MpNav) gene causes mortality in peach-potato aphid, *Myzus persicae*. Sci Rep-Uk 9: 5291

Tassetto M, Kunitomi M, Andino R (2017) Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in *Drosophila*. Cell 169: 314-325

Thakur N, Upadhyay SK, Verma PC, Chandrashekar K, Tuli R, Singh PK (2014) Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-ATPase A gene. Plos One 9: e87235

Wang K, Peng Y, Pu J, Fu W, Wang J, Han Z (2016) Variation in RNAi efficacy among insect species is attributable to dsRNA degradation in vivo. Insect Biochem Molec 77: 1-9

Whangbo JS, Hunter CP (2008) Environmental RNA interference. Trends Genet 24: 297-305

Wightman L, Kircheis R, R Ssler V, Carotta S, Ruzicka R, Kursa M, Wagner E (2001) Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. The Journal of Gene Medicine 3: 362-372

Xu Z, He B, Wei W, Liu K, Yin M, Yang W, Shen J (2014) Highly water-soluble perylenediimide-cored poly(amido amine) vector for efficient gene transfection. J Mater Chem B 2: 3079-3086

Yan S, Hu Q, Li J, Chao Z, Cai C, Yin M, Du X, Shen J (2019) A star polycation acts as a drug nanocarrier to improve the toxicity and persistence of botanical pesticides. Acs Sustain Chem Eng 7: 17406-17413

Yan S, Qian J, Cai C, Ma Z, Li J, Yin M, Ren B, Shen J (2020b) Spray method application of transdermal dsRNA delivery system for efficient gene silencing and pest control on soybean aphid *Aphis glycines*. J Pest Sci 93: 449-459

Yan S, Ren B, Zeng B, Shen J (2020c) Improving RNAi efficiency for pest control in crop species. Biotechniques 68: 283-290

Yan S, Ren BY, Shen J (2020d) Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. Insect Sci 28: 21-34

Yan S, Yu J, Han M, Michaud JP, Guo L, Li Z, Zeng B, Zhang Q, Liu X (2020a) Intercrops can mitigate pollen-mediated gene flow from transgenic cotton while simultaneously reducing pest densities. Sci Total Environ 711: 134855

Yan S, Zhu J, Zhu W, Li Z, Shelton AM, Luo J, Cui J, Zhang Q, Liu X (2015) Pollen-mediated gene flow from transgenic cotton under greenhouse conditions is dependent on different pollinators. Sci Rep-Uk 5: 15917

Yan S, Zhu W, Zhang B, Zhang X, Zhu J, Shi J, Wu P, Wu F, Li X, Zhang Q, Liu X (2018) Pollen-mediated gene flow from transgenic cotton is constrained by physical isolation measures. Sci Rep-Uk 8: 2862

You S, Cai Q, Zheng Y, He B, Shen J, Yang W, Yin M (2014) Perylene-cored star-shaped polycations for fluorescent gene vectors and bioimaging. Acs Appl Mater Inter 6: 16327-16334

Zha W, Peng X, Chen R, Du B, Zhu L, He G (2011) Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. Plos One 6: e20504

Zhang J, Khan SA, Heckel DG, Bock R (2017) Next-generation insect-resistant plants: RNAi-mediated crop protection. Trends Biotechnol 35: 871-882

Zhang X, Zhang J, Zhu KY (2010) Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*). Insect Mol Biol 19: 683-693

Zhang Y, Xu L, Li S, Zhang J (2019) Bacteria-mediated RNA interference for management of *Plagiodera versicolora* (Coleoptera: Chrysomelidae). Insects 10: 415

Zheng Y, Hu Y, Yan S, Zhou H, Song D, Yin M, Shen J (2019) A polymer/detergent formulation improves dsRNA penetration through the body wall and RNAi-induced mortality in the soybean aphid *Aphis glycines*. Pest Manag Sci 75: 1993-1999

Zotti MJ, Smagghe G (2015) RNAi technology for insect management and protection of beneficial insects from diseases: Lessons, challenges and risk assessments. Neotrop Entomol 44: 197-213.

Figures

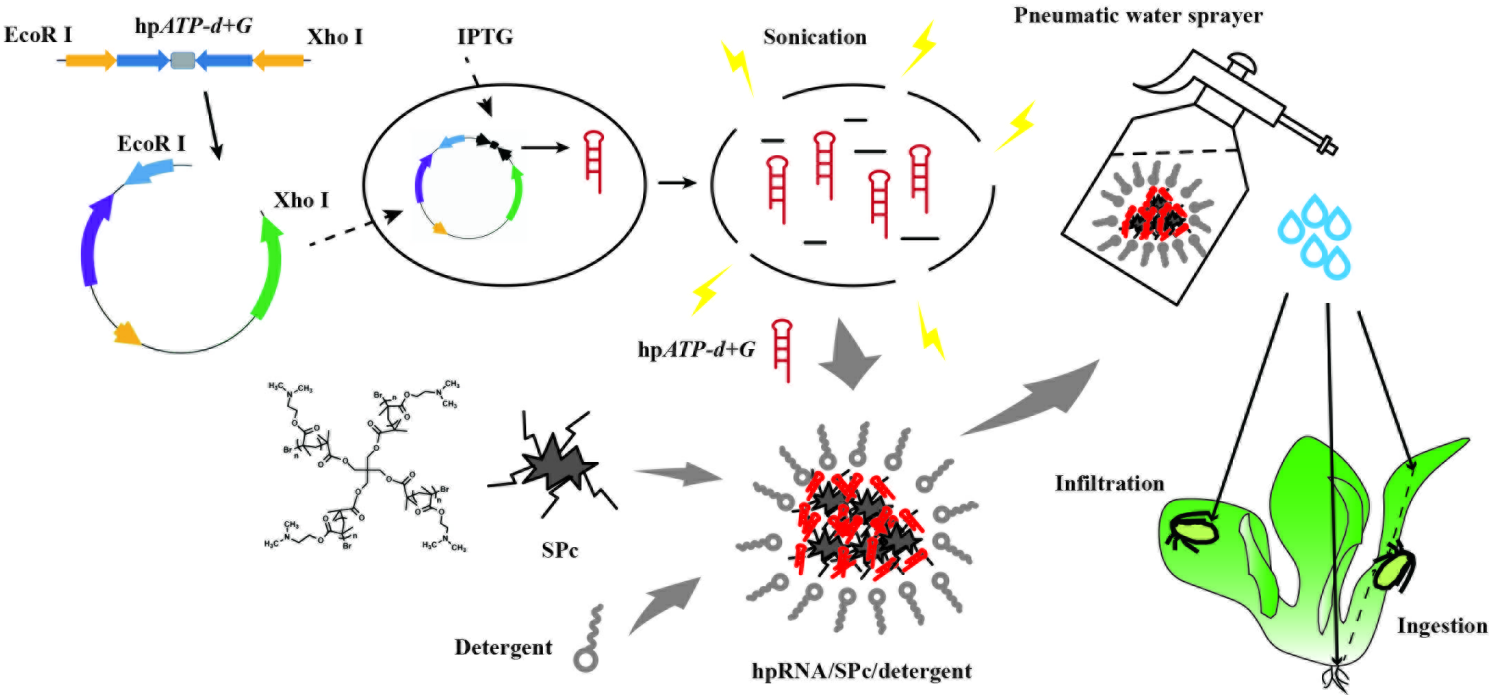

Figure 1

Schematic diagram of bacteria-expressed and nanocarrier-delivered RNA pesticide to control aphids. The hp*ATP-d+G* expressed in pET28-*ATP-d+G*-BL21(DE3) RNase III- expression system was induced by IPTG. The bacterial cultures were sonicated to obtain hpRNA, which was mixed with polycation SPc and detergent to form hpRNA/SPc/detergent formulation. The RNA pesticide was sprayed directly to the oilseed rapes infested with aphids.

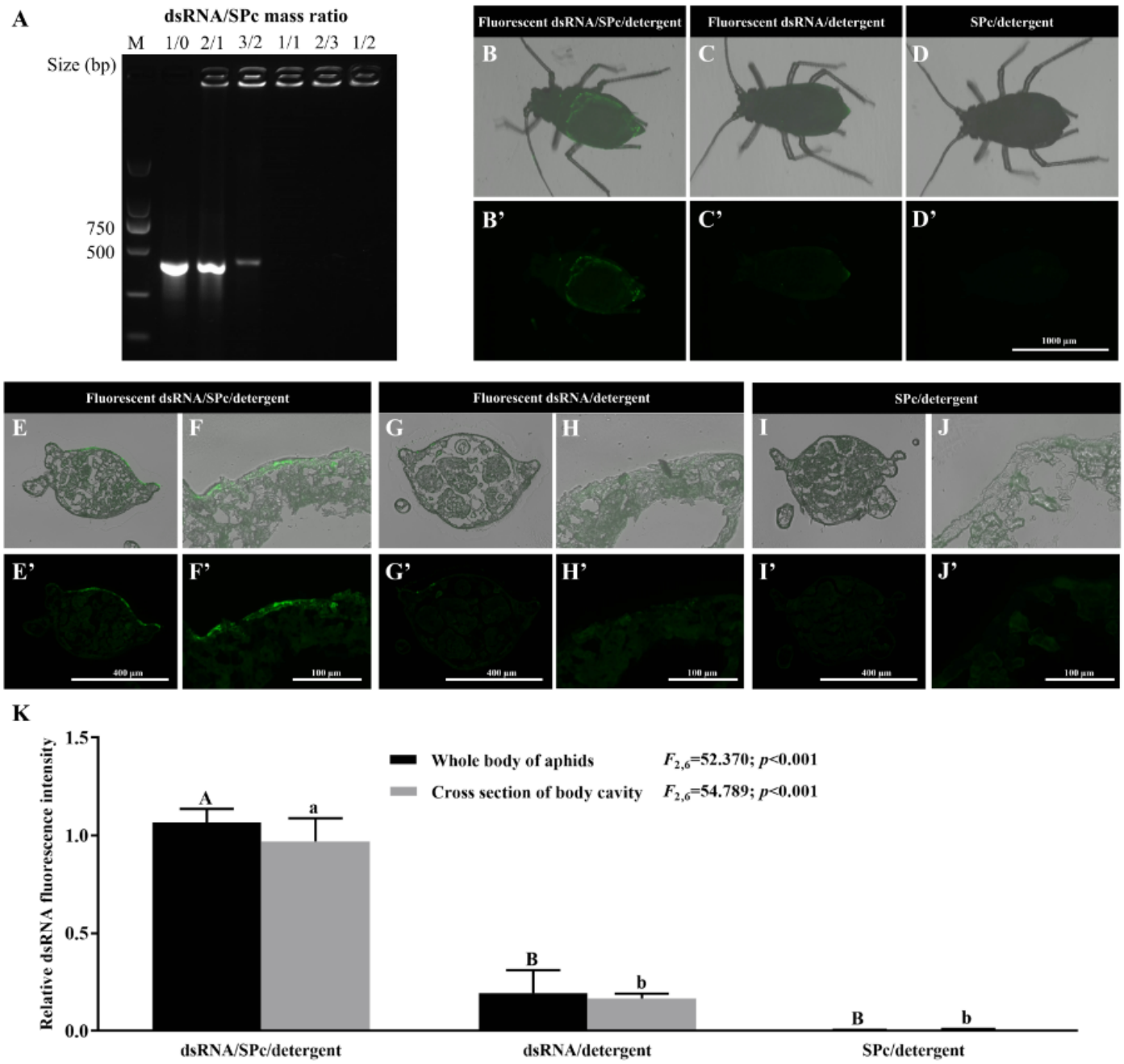

Figure 2

Infiltration effect of nanocarrier-based dsRNA delivery system in green peach aphids. (A) Agarose gel electrophoresis of ds*eGFP*/SPc complexes at various mass ratios to determine degree of complexation. The dseGFP/SPc mass ratios were 1:0, 2:1, 3:2, 1:1, 2:3, and 1:2. Among them, the ds*eGFP* mass was 1 μg. M: DNA marker. (B-D') The merged images (B, C, D) and fluorescence images (B', C', D') of aphids at 6 h post the topical application. (E-J') The merged images (E, F, G, H, I, J) and fluorescence images (E', F', G', H', I', J') of frozen sections at 6 h after treatments. (K) The dsRNA fluorescence intensities among various treatments. The fluorescence intensity was measured using the ImageJ 1.48v from 6 independent samples. Letters above the bars represent statistical significance (Tukey's HSD test, p<0.05).

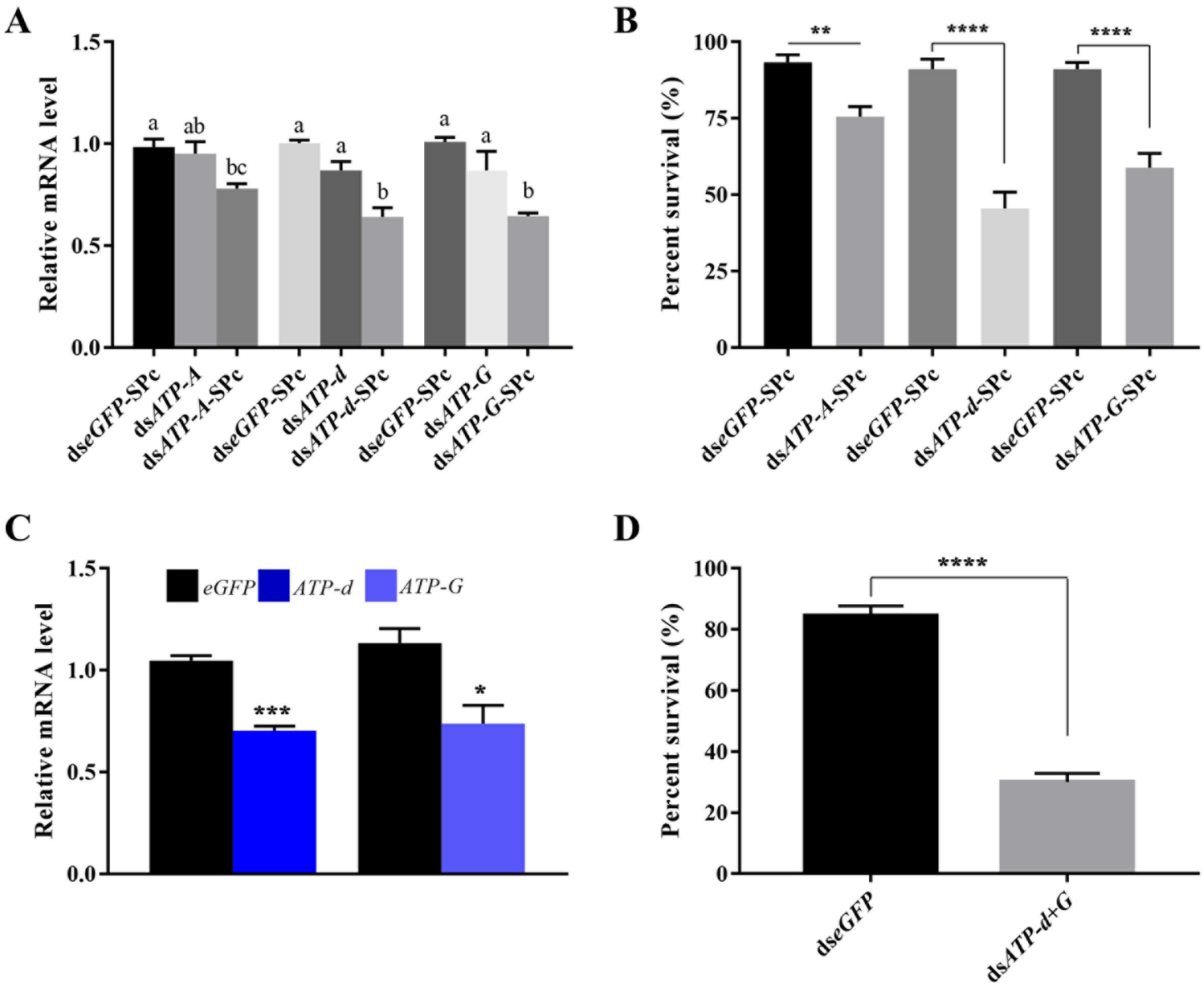

Figure 3

Silencing effect of *ATPase* genes in green peach aphids through topical application. (A) The expression level of *ATP-A*, *ATP-d* and *ATP-G* after different RNAi treatments. The dsRNA was diluted to a final concentration of 720 ng/μL. (B) Lethal effect after different RNAi treatments. (C) The expression level of *ATP-d* and *ATP-G* after their co-silencing. Final dsRNA concentration of each gene was 360 ng/μL. (D) Lethal effect of co-silencing of *ATP-d* and *ATP-G*. The 0.2 μL of different formulations was applied on the notum of 4th instar aphids. Gene expression level was detected at 24 h, and the percent survival of aphids was recorded on 3 d post the topical application. Letters above the bars represent statistical significance (Tukey's HSD test, $p<0.05$). Asterisk indicates significant difference in treatments compared to ds*eGFP* (*$p<0.05$, **$p<0.01$, ***$p< 0.001$, ****$p<0.0001$).

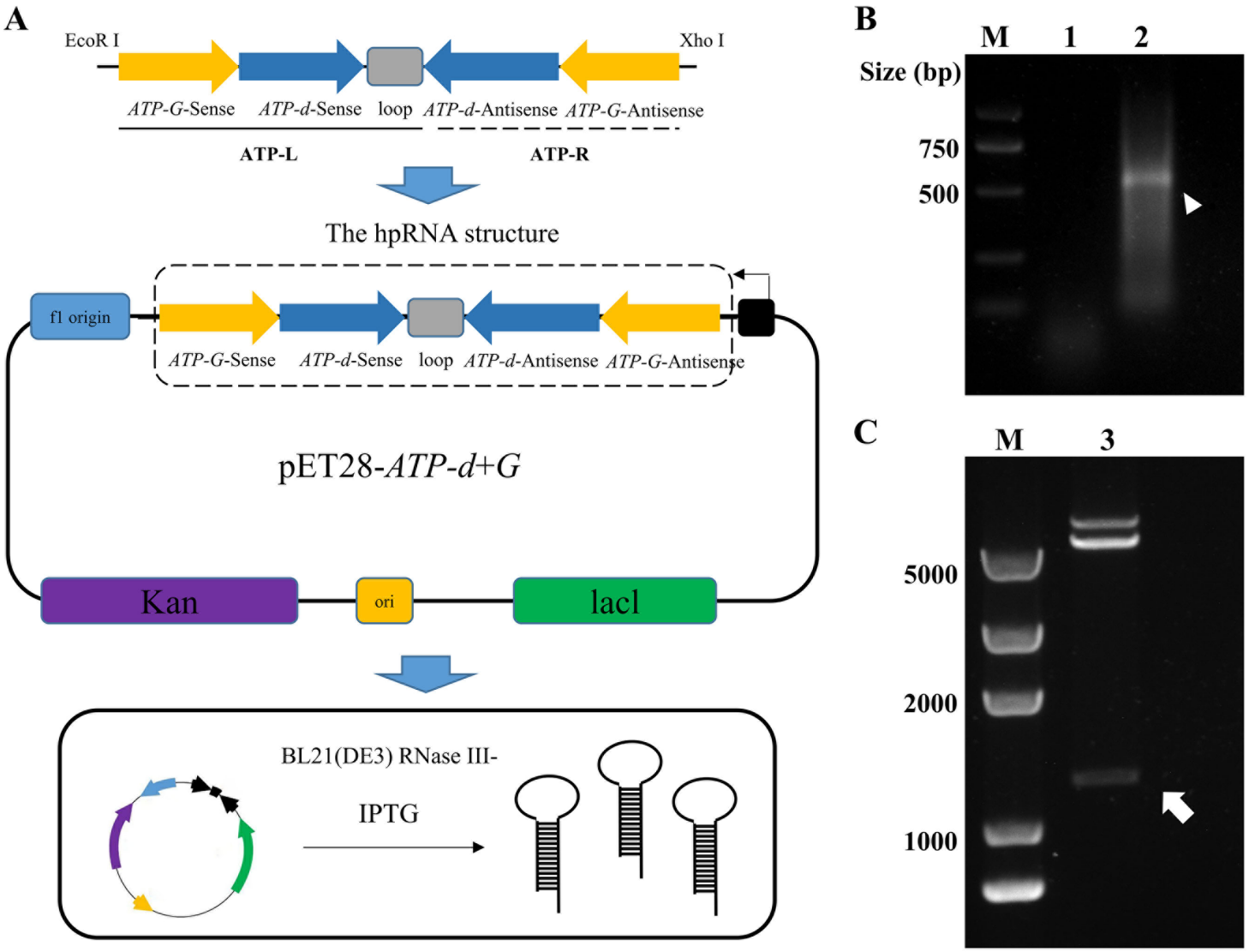

Figure 4

Construction of pET28-*ATP-d+G*-BL21(DE3) RNase III- expression system. (A) A 1463 bp of *ATP-d+G* gene fragment was inserted in the multiple-cloning site of the expression vector pET-28, which was then transformed into the BL21(DE3) RNase III- cells. RNA transcription mediated by T7 promoter was induced by IPTG. (B) A 684 bp of hp*ATP-d+G* produced by BL21(DE3) RNase III- was evaluated by 1% agarose gel, whereas pET28-EV-BL21(DE3) RNase III- system produced no dsRNA. White arrowhead indicates the expressed hpRNA. M: DNA Marker; 1: pET28-EV-BL21(DE3) RNase III-; 2: pET28-*ATP-d+G*-BL21(DE3) RNase III-. (C) Identification of the pET28-ATP-*d+G* vector by digestion with EcoR□-Xho□ to generate the 1463 bp hpATP-*d+G*, indicated by white arrow. M: DNA Marker; 3: Restriction enzyme identification of pET28-*ATP-d+G*.

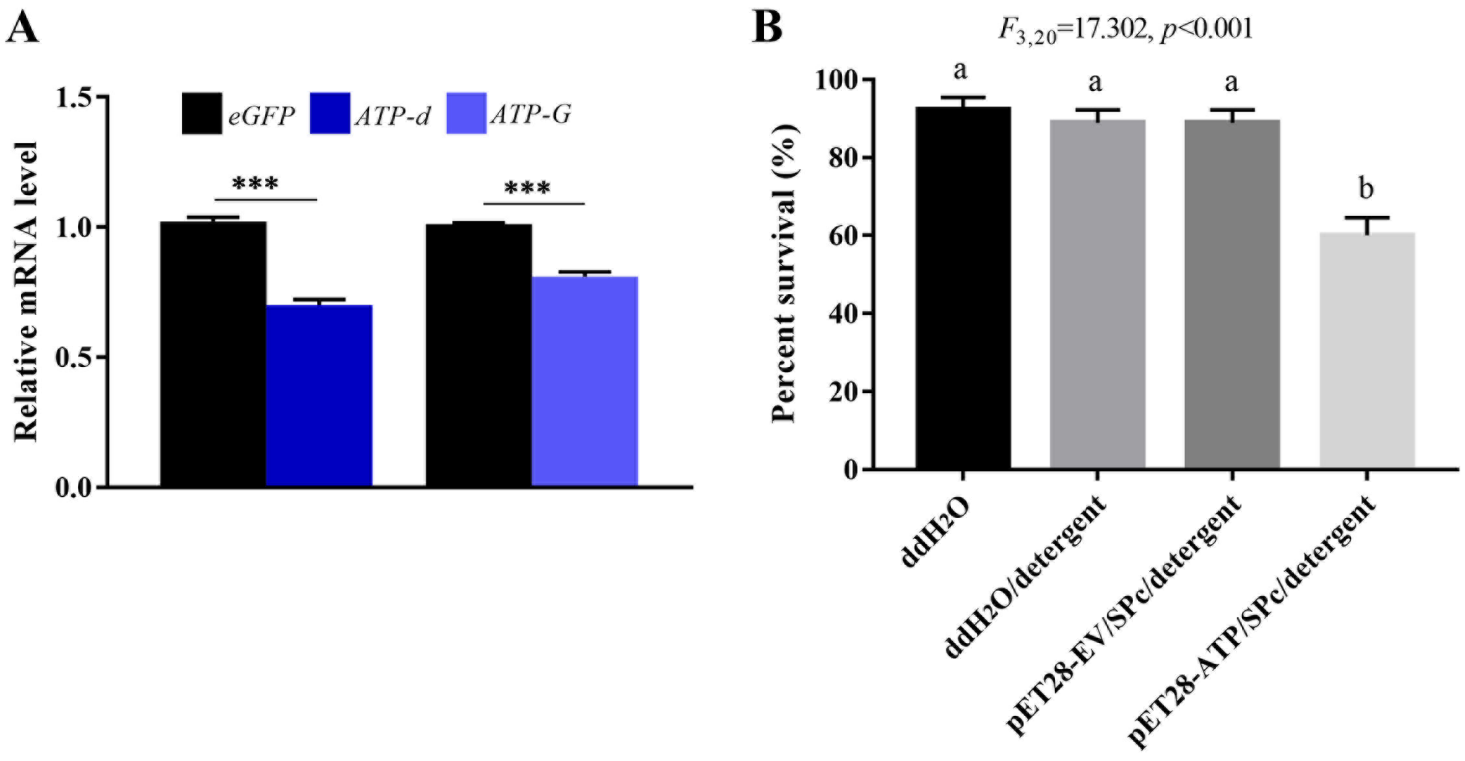

Figure 5

RNAi effects of hpRNA expressed in BL21(DE3) RNase III- system. (A) Gene silencing effect of two target genes. Asterisks indicate the significant differences (***p< 0.001). (B) Lethal effect of co-silencing of *ATP-d* and *ATP-G*. The 0.2 μL of different formulations was applied on the notum of 4th instar aphids. Gene expression level was detected at 24 h, and the percent survival of aphids was recorded on 3 d post the topical application. Letters above the bars represent statistical significance (Tukey's HSD test, p<0.05).

Figure 6

Control efficacy of bacteria-expressed and nanocarrier-delivered RNA pesticide against green peach aphids on top of leaves and leaf backs. (A-C') Lethal phenotype of aphids on top of leaves (A-C) and leaf backs (A'-C') after the treatment. The black circle indicates dead aphids. (D) Dropping rate of the aphids on top of leaves. (E) Dropping rate of the aphids on leaf backs. (F) Control efficacy of the aphids on top of leaves. (G) Control efficacy of the aphids on leaf backs. The thiamethoxam was applied as a positive

control, and the water was applied as a negative control. The amount of applied formulation was 100 ml/m^2. Ten plants from each plot were selected as ten replicates to record the aphid number.

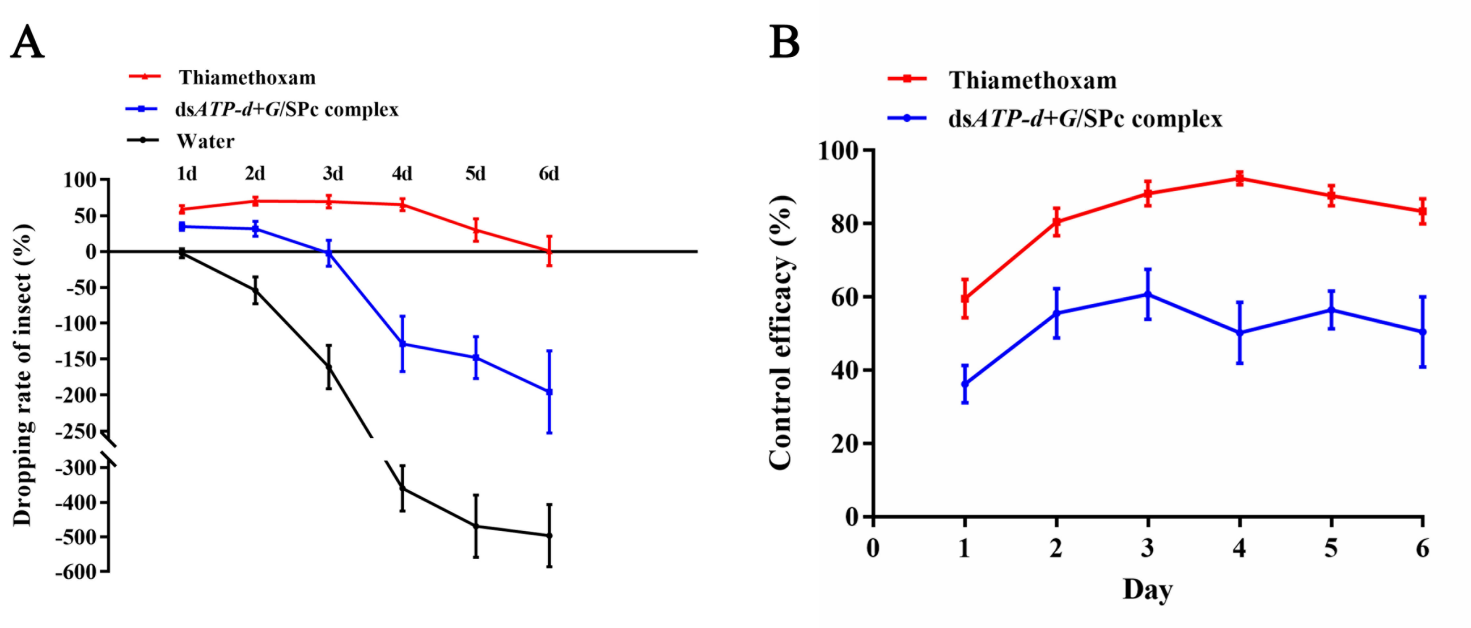

Figure 7

Total control efficacy of bacteria-expressed and nanocarrier-delivered RNA pesticide. (A) Dropping rate of insect. (B) Control efficacy of RNA pesticide against aphids.

Supplementary Files

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- Supplementarydata.docx