



Flock house virus as a vehicle for aphid Virus-induced gene silencing and a model for aphid biocontrol approaches

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Abstract

Due to their high specificity and efficacy, RNA interference (RNAi)-based strategies have been used for fundamental functional genomics studies in a number of insects. However, its potential for translational applications in pest management is also of great interest. The lack of suitable RNAi triggering approaches, however, so far has largely precluded the implementation of RNAi-based approaches to target aphids. In this work, we first demonstrate that Flock House virus (FHV), an insect virus, can infect multiple aphid species, including the green peach aphid, *Myzus persicae* (*M. persicae*), the corn leaf aphid, *Rhopalosiphum maidis* (*R. maidis*), and the bird cherry-oat aphid, *Rhopalosiphum padi* (*R. padi*), by both microinjection and oral feeding. Using green fluorescent protein (GFP) as an indicator, we showed that the defective interfering RNA (DI-634) of FHV RNA2, which is generated autonomously during wild-type (WT) virus replication, can carry foreign sequences, and further for their functional expression. More importantly, the engineered DI-634 was incorporated into virus particles in co-infections with WT FHV. Using FHV virions containing genetically modified DI-634, the accumulation levels of the *M. persicae* mRNAs for *Cathepsin L* (*CatL*) and *Sugar Transporter 4* (*ST4*), were decreased by ~35% and ~30–50%, respectively when virions were injected intrathoracically into aphids. Finally, and of more practical relevance, oral acquisition of these engineered FHV virions caused lethality of *M. persicae*. In summary, as a proof-of-concept, our work demonstrates that FHV can be a valuable RNAi tool for fundamental research, and suggests opportunities for using engineered insect viruses as biological agents for aphid pest control.

Keywords Aphid · Virus-induced gene silencing (VIGS) · Biocontrol · Flock house virus (FHV) · RNA interference (RNAi)

Key message

- FHV is infectious in multiple aphid species, including the green peach aphid, *Myzus persicae*, the corn leaf aphid, *Rhopalosiphum maidis*, and the bird cherry-oat aphid,

Rhopalosiphum padi, both by microinjection and oral feeding.

- FHV DI-634 is tolerant of target nucleotide sequence insertions and can be encapsidated into virus particles in the presence of wild-type (WT) FHV RNA1, and RNA2 co-infection.
- Engineered FHV DI-634 can trigger RNAi, cause lethality, and suggests opportunities for using engineered insect viruses as biocontrol agents of aphid pest.

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Introduction

Aphids are soft-bodied, phloem-feeding hemipteran insects that are among the most destructive insect pests, and cause substantial yield losses in a wide variety of crop species worldwide (Valenzuela and Hoffmann 2015; Zapata et al. 2018). Aphid feeding depletes nutrients that are critical for

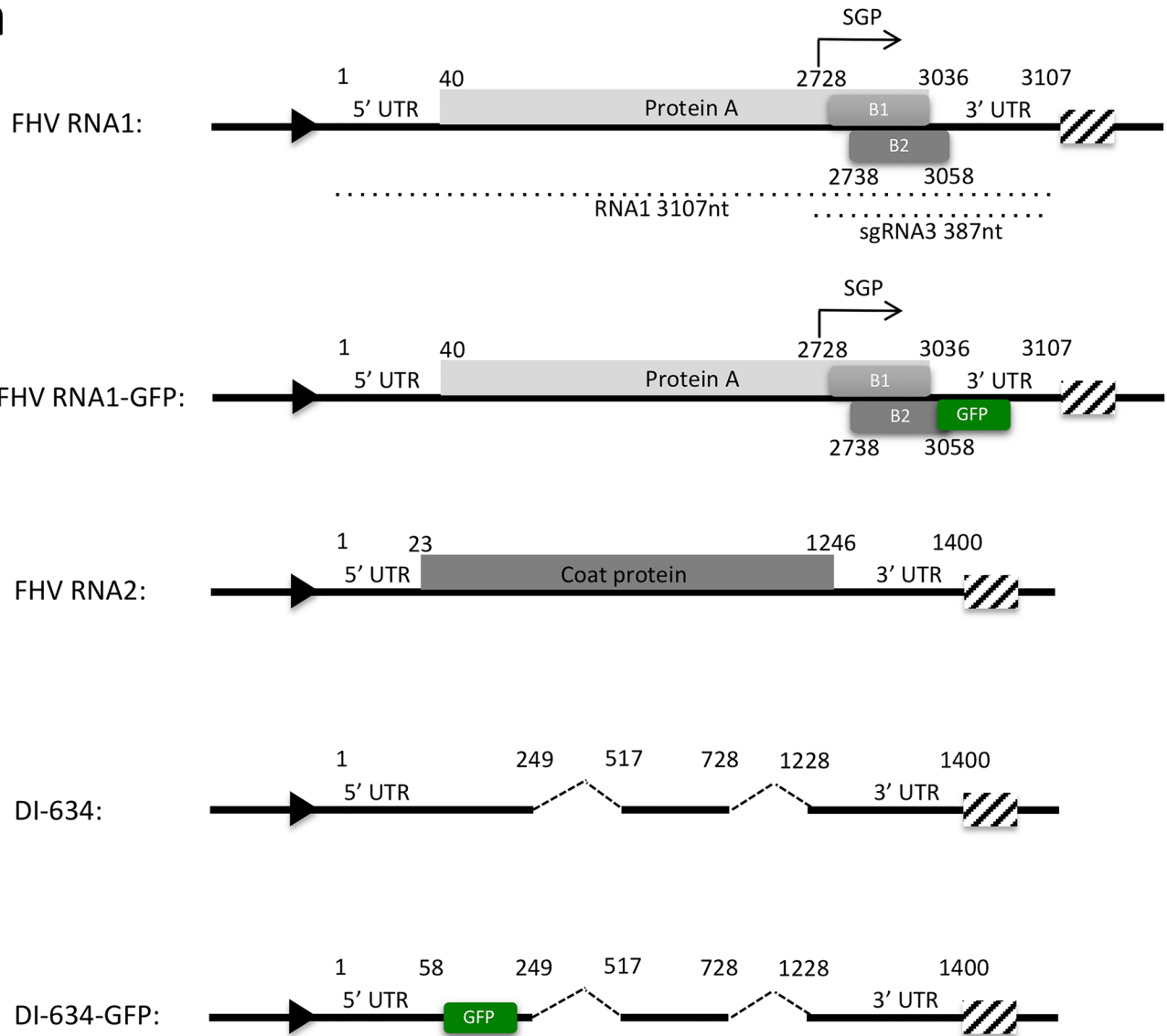
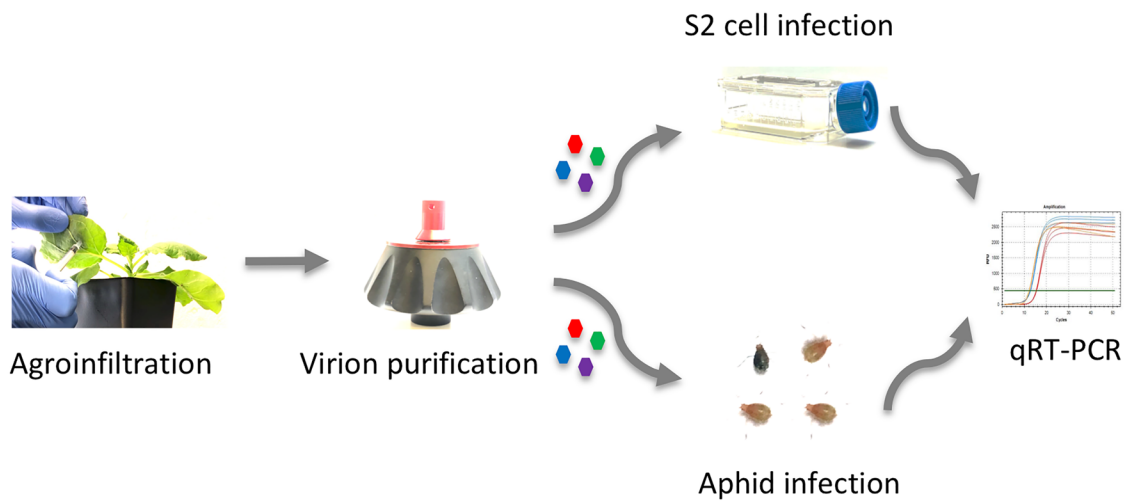
a**b**

Fig. 1 Schematic illustration of FHV constructs used in this study and the experimental design. **a** FHV RNA1 has a 39 nt 5' untranslated region (5' UTR; nt 1–39) and a 49 nt 3' UTR (nt 3059–3107). The coding region of Protein A (also known as viral RNA dependent RNA polymerase), protein B1, and the silencing suppressor B2, are at nt 40–3036, 2728–3036, and 2738–3058, respectively. Protein A is encoded by FHV RNA1, while protein B1 and B2 are encoded by subgenomic RNA3 (sgRNA3). The two dashed lines represent the 3107 nt RNA1 and 387 nt sgRNA3; To engineer FHV RNA1, the GFP coding sequence, or other inserts, were inserted into FHV RNA1 near the 3' end of and in frame with the B2 ORF. FHV RNA2 (1,400 nts) encodes the viral coat protein (nt 23–1246), with a 22 nt 5' UTR (nt 1–22) and a 154 nt 3' UTR (nt 1247–1400). The three regions of RNA2, nt 1–249, 517–728, and 1228–1400, retained in the defective interfering RNA2 (DI-634) are shown. The GFP coding sequence, or other inserts, represented by the green box, were introduced into DI-634 at nt position 59. All the viral sequences are cloned into the binary vector pJL89, under control of the CaMV 35S promoter (represented by the black triangles) and followed with the HDV ribozyme sequence (indicated by rectangular boxes with diagonal lines). SGP: Subgenomic promoter. **b** WT and engineered FHV virions were isolated from agroinfiltrated *Nicotiana benthamiana* plants in this study. To check if the engineered virions are replication-competent, S2 cells were infected and processed for RT-qPCR analysis (the upper branch). Aphids were further infected by microinjection with virions isolated from agroinfiltrated leaves, and the RNAi effect was checked by RT-qPCR (the lower branch)

plant growth, introduces phytotoxic saliva which affects plant development, and aphids of several species are efficient vectors of plant-infecting viruses, leading to even more severe damage (Riedell and Kieckhefer 1995; Ng and Perry 2004; Dedryver et al. 2010). Their broad host range, parthenogenetic reproduction, dense populations, aggregated feeding behavior, and flexibility in adapting to different environmental conditions are key features that make aphids a challenging agricultural pest to control. Additionally, aphids mostly occur in temperate regions, but due to global warming, their geographic distribution is expanding, increasing the urgent need to develop effective aphid management strategies.

Chemical pesticides are commonly used to target aphids and have shown great success in helping to manage aphid populations. However, due to their potential negative impact on the environment as well as the emergence of pesticide-resistant aphid species, non-chemical pesticide-based control strategies are highly desired (Silva et al. 2012; Bass et al. 2014). In this regard, RNA interference (RNAi)-based pest management strategies have been increasingly investigated (Baum and Roberts 2014; Mamta and Rajam 2017; Zhu and Palli 2020). RNAi is a conserved biological process occurring in all eukaryotic organisms (Hannon 2002). The endoribonuclease Dicer recognizes and cleaves double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) or microRNAs, which are incorporated into the RNA-induced silencing complex (RISC) containing Argonaute proteins; this complex then targets and cleaves complementary messenger RNAs (mRNAs) or virus RNAs (vRNAs) within

the cell, thereby inhibiting their translation into proteins. Since the discovery that exogenously introduced dsRNAs can induce sequence-specific gene silencing in the nematode *Caenorhabditis elegans*, it has rapidly emerged as powerful biotechnology for targeted downregulation of gene expression (Fire et al. 1998), and provides a potentially powerful approach for pest management.

Laboratory scale demonstrations have proven that strong RNAi effects can be triggered by dsRNA in aphids. For example, microinjection of dsRNA into the pea aphid, *Acyrtosiphon pisum*, triggers RNAi, leading to reduced expression of the calcium-binding protein Calreticulin and cysteine protease Cathepsin L (Jaubert-Possamai et al. 2007); topically applied dsRNA can also induce RNAi effects in aphids (Niu et al. 2019). Furthermore, feeding aphids on transgenic plants that stably express dsRNAs or derived siRNAs can also induce RNAi effects in aphids (Pitino et al. 2011), and the downregulation of essential endogenous aphid genes can eventually cause aphid lethality (Mutti et al. 2006). However, to translate these findings into field-based applications, obvious drawbacks must first be addressed. For instance, microinjection is very laborious and impossible to perform on the vast numbers of aphids in the field; dsRNA delivered by oral feeding, spraying, or other similar approaches requires a large amount of dsRNA, for which the cost of in vitro production can be prohibitive, although commercial efforts aiming at a lower cost are promising; establishing transgenic plants is time-consuming and transformation methods for many economically important crops are not yet well established. Consequently, exploring additional RNAi approaches that are more cost-effective and easier to administer is a primary objective for making the use of RNAi-based aphid control methods a reality.

Viruses are powerful inducers of RNAi responses, and recombinant plant viruses have been used as tools to induce RNAi effects in plant-feeding insects, including aphids. The recombinant viruses have been engineered to contain short sequences corresponding to specific insect mRNAs. When the engineered viruses infect plants, the plant RNAi response generates siRNAs against the vRNAs, including the inserted sequences. The latter are acquired by the target insect feeding on these plants and RNAi effects have been demonstrated (Wuriyangan and Falk 2013; Khan et al. 2013; Feng and Jander 2022). Recombinant plant viruses thus offer the potential for targeting insects such as aphids, however, recombinant insect viruses would be more desirable. They would induce systemic RNAi effects in the infected insects, and for translational applications, they may even be able to spread among the target insect population. However, so far, no insect viruses have been developed for inducing RNAi effects in aphids.

Flock House virus (FHV), an insect virus, is classified in the *Alphanodavirus* genus of the *Nodaviridae* family (Scotti

et al. 1983). Different from many other viruses, FHV can cross kingdom barriers and infect not only insects, but also plants, yeasts, and nematodes (Dasgupta et al. 2007). The viral genome consists of two positive-sense single-stranded RNAs [(+) ssRNAs], which are both packaged into a single, nonenveloped, icosahedral virion. RNA1 is 3,107 nucleotides (nts) and encodes protein A, the viral RNA-dependent RNA polymerase (RdRP). The 1,400 nts RNA2 has only one open reading frame (ORF), which encodes the viral coat protein (CP). The subgenomic RNA3 (sgRNA3; 387 nts), which is not encapsidated, is generated from RNA1 during virus infection and encodes protein B1, the function of which is unknown, and encodes the silencing suppressor B2 protein (Fig. 1a). Additionally, FHV replication generates defective interfering RNAs (DI-RNAs), which can also be encapsidated, and for which replication is dependent on the wild-type (WT) virus (Zhong et al. 1992; Jovel and Schneemann 2011). Like many other (+) RNA viruses, the replication of FHV RNA forms dsRNA intermediates for the synthesis of progeny (+) vRNA, and host recognition of this dsRNA induces the production of siRNAs and RNAi effects, and as such FHV can be used as vectors to trigger RNAi in cultured S2 cells (Hongwei et al. 2002; Taning et al. 2018).

In this study, we investigated whether FHV could infect aphids, and then be used to induce RNAi effects in aphids. First, a plant-based FHV virion production platform, which is relatively low cost compared with cell-based platforms, was established. Subsequently, it was determined that the green peach aphid, *Myzus persicae* (*M. persicae*), the corn leaf aphid, *Rhopalosiphum maidis* (*R. maidis*), and the bird cherry-oat aphid, *Rhopalosiphum padi* (*R. padi*), can be infected by FHV through both microinjection and oral feeding. We also found that DI-634 (Fig. 1a), a defective interfering RNA derived from FHV RNA2, is tolerant of target nucleotide sequence insertions. Using virions containing engineered DI-634, we showed down-regulation of the *M. persicae* mRNAs for *Cathepsin L* (*CatL*) and *Sugar Transporter 4* (*ST4*) by ~35 and ~30–50%, respectively. Oral feeding of these virions further caused the mortality of *M. persicae*. In summary, this work demonstrates that engineered FHV can be used as Virus-induced gene silencing (VIGS) vector and suggests the potential of using recombinant insect viruses to induce RNAi effects in target aphids.

Materials and methods

Plants, insect cell cultures, and aphids

Nicotiana benthamiana plants were kept in a growth chamber (24 °C, 16:8 h light/dark), and were used for inoculation at 4 weeks old. *Drosophila melanogaster* (S2) cells (Thermo Fisher Scientific, Waltham, MA, USA) were maintained at

28 °C in Schneider's *Drosophila* Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum, Heat Inactivated (Sigma-Aldrich, St. Louis, MO, USA) and 1 × Penicillin–Streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) using standard laboratory procedures. *M. persicae* were reared on radish (*Raphanus raphanistrum* subsp. *sativus*) plants, while *R. maidis* and *R. padi* colonies were maintained on barley (*Hordeum vulgare*) and oat (*Avena sativa*) plants, respectively. All colonies were kept in an air-conditioned room (25 °C, 16:8 h light/dark).

Plasmid construction

To construct pJL89/FHV RNA1 and pJL89/FHV RNA2, the full-length genomic segments of FHV RNA1 and RNA2 were amplified by polymerase chain reaction (PCR) using plasmid pMT/FHV RNA1 and pMT/FHV RNA2 as the template, respectively (Hongwei et al. 2002) (Fig. S1a); The vector pJL89 that carries the cauliflower mosaic virus (CaMV) 35S promoter and hepatitis delta virus (HDV) ribozyme sequence was PCR amplified using the carrot mottle virus (CMoV) infectious clone pJL89/CMoV as the template (Jiang et al. 2021) (Fig. S1a). Agarose gel-purified PCR products were then mixed with 2 × Gibson Assembly® Master Mix (NEB, Ipswich, MA, USA) as per the manufacturer's instructions, and positive colonies were selected after *E. coli* competent cell transformation.

Four overlapping DNA fragments were assembled to make construct pJL89/FHV DI-GFP (Fig. S1b). The largest fragment, with a truncation in region nt 59–1228 of FHV RNA2, was PCR amplified from plasmid pJL89/FHV RNA2. The other two fragments, nt 59–249 and nt 517–728 of FHV RNA2 were PCR amplified using pJL89/FHV RNA2 as the template, and the coding sequence of green fluorescent protein (GFP) was amplified from plasmid pEAQ-HT/P26:GFP (Qiao et al. 2018). Using a similar strategy, the GFP coding sequence of pJL89/FHV DI-GFP was replaced with a 200 bp fragment derived from the *M. persicae* *CatL* gene (accession number AJ496197), a 289 bp fragment derived from the *NaV* gene (accession number FN601405), a 478 bp fragment derived from the *Vha8* gene (accession number EC387265.1), or a 264 bp fragment derived from *ST4* gene (accession number KR047102), to produce the constructs pJL89/FHV DI-CatL, pJL89/FHV DI-NaV, pJL89/FHV DI-Vha8, and pJL89/FHV DI-ST4, respectively.

To make construct pJL89/FHV RNA1-GFP, the GFP coding sequence was introduced into pJL89/FHV RNA1 as described by Maharaj et al. (Maharaj et al. 2014) (Fig. S1c). Constructs pJL89/FHV RNA1-CatL, pJL89/FHV RNA1-NaV, pJL89/FHV RNA1-Vha8, and pJL89/FHV RNA1-ST4 were constructed similarly, and the DNA fragments for gene targeting were amplified from the corresponding

pJL89/FHV DI clones as mentioned above. The sequences of all plasmids constructed in this study were confirmed by sanger sequencing. The cloning strategies described above are shown in Fig. S1. The primers used in this study are listed in Table S1.

Virus inoculation

FHV infection in *N. benthamiana* plants was established by agroinfiltration as described previously (Sparkes et al. 2006). *Agrobacterium tumefaciens* GV3101 containing pJL89/FHV RNA1, pJL89/FHV RNA2, or their derived clones, were selected on LB kanamycin-rifampicin agar plates. To prepare *A. tumefaciens* suspensions for agroinfiltration, fresh cell cultures were centrifuged at 5000 rpm for 10 min, and the cell pellets were resuspended in agroinfiltration buffer (10 mM MgCl₂, 150 μM acetosyringone). After incubation at room temperature for 2–4 h, the concentration of all cell suspensions was adjusted to 0.6 at an optical density at 600 nm. To establish WT virus infection, plants were agroinfiltrated with *A. tumefaciens* suspensions containing pJL89/FHV RNA1 and pJL89/FHV RNA2 at 1:1 ratio; for infection of DI-RNAs, *A. tumefaciens* suspensions of pJL89/FHV RNA1, pJL89/FHV RNA2, and pJL89/FHV DI-RNAs were mixed at a ratio of 1:1:10. To infect plants with engineered FHV RNA1 clones, an equal volume of *A. tumefaciens* suspensions containing engineered pJL89/FHV RNA1 clones and WT pJL89/FHV RNA2 were mixed to infiltrate plants. Virions were purified from plants then passed through a filter (0.22 μm); and used to infect S2 cells, the titer of filtered virion preparations was adjusted to 1×10^5 copies per μL (copies/μL), and simply introduced into the cell culture medium.

Virion purification

FHV virion purification was done as described by Routh et al (2012) with some modifications. Leaves infected with FHV were ground in 4 volumes (w/v) of extraction buffer (50 mM HEPES, pH 7.0), and filtered through three layers of cheesecloth. The extract was then centrifuged twice at 8000 rpm for 10 min. For FHV infected S2 cells, NP40 was added to a final concentration of 1% (v/v). After incubation on ice for 30 min, the lysate was subjected to two rounds of centrifugation at 8000 rpm for 10 min. RNase A was added to the lysate at a final concentration of 10 μg/mL and was incubated at room temperature for 30 min. For ultracentrifugation, the extract/lysate was laid on top of a 30% sucrose cushion (made in 50 mM HEPES buffer, pH 7.0), and centrifuged at 50,000 rpm for 1.5 h. The pellet was then resuspended in an appropriate amount of HEPES buffer and stored at 4 °C.

Transmission electron microscopy (TEM)

TEM was done as described by Matsumura et al. (Matsumura et al. 2019). Briefly, FHV virion preparations were loaded onto Formvar-carbon-coated grids for 2 min, after which the excess fluid was wicked away with filter paper. After staining with 1% uranyl acetate, the grids were air-dried. TEM observation was done with a JEOL 2100F transmission electron microscope at 200 kV accelerating voltage.

Aphid microinjection and membrane feeding assays

Synchronized aphids at 5 days old were used for all assays. Aphid microinjection was done essentially as described by Tamborindeguy et al. (2008). Briefly, aphids were transferred with a paintbrush and placed in a homemade vacuum-operated aphid holder, with their backs against the holder. The virion preparation was then loaded into a glass needle, and injection was done with a Nanoject III (Drummond Scientific, Broomall, PA, USA) in the middle of the aphid abdomen between the second and third abdominal segments. After injection, aphids were maintained with membrane feeding on an artificial diet (Wille and Hartman 2008); the diet was changed daily and nymphs were removed.

For the membrane feeding assay, sucrose was mixed with the virion preparation to a final sucrose concentration of 20%. The titer of the virion preparation was adjusted to 1×10^7 copies/μL for the feeding assay with WT FHV virions, and to 3×10^7 copies/μL for all feeding assays with the engineered DI-RNA virions. The titer was determined so that we could supply the most virions to the aphids as possible, thus increasing the chance of aphids becoming infected by FHV, especially when the virions were delivered orally. An aliquot (100 μL) of the virion-sucrose mixture was loaded onto a piece of parafilm stretched over the opening of a plastic vial (38 × 84 mm). This was then overlaid with another piece of stretched parafilm to ensure the solution spread evenly. For each feeding assay, a group of 20 aphids was placed in the plastic vial. After feeding for 48 h, the aphids were maintained on a virion-free artificial diet. The virion-free artificial diet was changed and nymphs were removed daily. All aphids were kept in a room at 25 °C, 16:8 h light/dark.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Aphid samples were ground in liquid nitrogen using a bead beater prior to RNA extraction. Total RNA extraction was done with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA samples were further treated with RQ1 RNase-free DNase I (Promega, Madison, WI, USA), and purified by

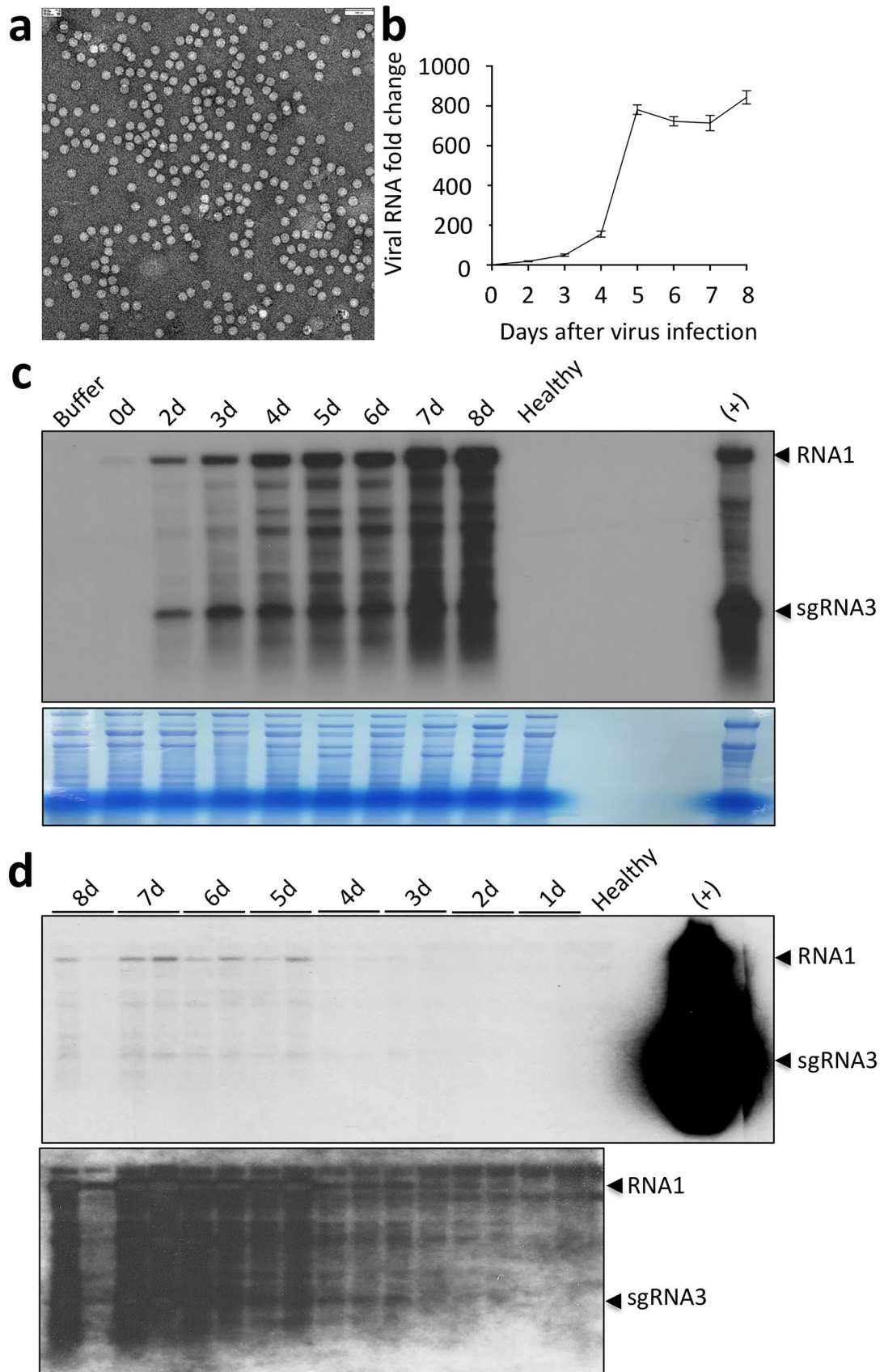


Fig. 2 FHV infection in aphid *M. persicae*. **a** *N. benthamiana* plants were infiltrated with equal volumes of *Agrobacterium tumefaciens* harboring pJL89/FHV RNA1 and pJL89/FHV RNA2. FHV virions were isolated from agroinfiltrated leaf tissues at 4 d.p.i and used to re-inoculate S2 cells. Virions were further isolated from infected S2 cells and observed under a transmission electron microscope. Scale bar = 100 nm. The virions were delivered into *M. persicae* by micro-injection (**b** and **c**) and oral feeding (**d**), and groups of ten aphids were collected daily, up to 8 d.p.i. Total RNA was extracted and processed for RT-qPCR analysis to quantify the accumulation of FHV RNA1. The *M. persicae Actin* gene was used as the internal control. Viral RNA fold changes (compared to the RNA level at 0 d.p.i) are shown (**b**). RNAs were further applied for northern blotting to detect the presence of sgRNA3. The probe binds to both sgRNA3 and RNA1, and the expected bands are indicated with arrows (**c** and **d**). The methylene blue-stained membrane is shown in the lower panel, indicating equal loading of the RNA samples (**c**). Buffer: aphids were injected with HEPES buffer (**c**); Healthy: non-infected aphids (**c** and **d**); (+): RNA isolated from the above-agroinfiltrated plants was used as a positive control (**c** and **d**). Shorter time (4 h; top) and longer time exposure (36 h; bottom) of the blot are presented. For longer exposure, the positive control lane was cropped (**d**)

phenol–chloroform extraction. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), and diluted 40 times prior to RT-qPCR. The RT-qPCR reaction mix contained the following: 2 μ L of diluted cDNA, 500 nM each of forward and reverse primers, 5 μ L of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Foster City, CA, USA), and water to a total volume of 10 μ L. The thermocycling conditions were as follows: 95 °C for 30 s, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s.

To measure the titer, RNA was extracted from 100 μ L of purified FHV virion preparations. RNA extraction was done with TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized, diluted, and used for RT-qPCR as described above. The copy number of WT RNA1 and engineered DI-RNAs was quantified to determine the titer of WT and engineered FHV DI-RNA virion preparations, respectively. The corresponding plasmids pJL89/FHV RNA1 and pJL89/FHV DI-RNAs at different dilutions were used as template to generate the standard curve for RT-qPCR.

Northern blotting

RNA samples were denatured with glyoxal solution (35 μ L DMSO; 10 μ L glyoxal; 7 μ L 10 \times HEPES-EDTA buffer) at 55 °C for 30 min and were transferred to a Hybond-NX nylon membrane (GE Amersham, Piscataway, NJ, USA) after being separated by electrophoresis on a 1% agarose gel. The RNAs were fixed to the nylon membrane using a UV Crosslinker and the blots were stained with methylene blue to visualize RNA integrity, then soaked in 1 M Tris–HCl and microwaved for 30 s. Hybridization was done

with antisense probes targeting the FHV sgRNA3 (also targeting genomic RNA1). The [α - 32 P] UTP labeled probe was made with the MAXIscript Kit (Ambion, Austin, TX, USA). The membrane was then washed once with 2 \times SSC (0.30 M NaCl, 0.03 M sodium citrate)/0.1% SDS for 15 min, once with 0.5 \times SSC/0.1% SDS for 15 min, and a final wash with 0.1 \times SSC/0.1% SDS at 65 °C for 15 min. The signal was observed using Premium X-Ray film (Phenix Research Products, Candler, NC, USA).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For virion preparations and cell pellets, the samples were mixed with 2 \times SDS sample loading buffer and boiled for 5 min. For plant samples, the tissue was ground into fine powder in liquid nitrogen prior to mixing with 2 volumes of protein extraction buffer (100 mM Tris–HCl, pH 7.5; 100 mM EDTA, pH 8.0; 5 mM dithiothreitol; 150 mM NaCl; 0.1% Triton X-100; Cocktail proteinase inhibitor), the samples were then boiled for 10 min, and the supernatant was mixed with an equal volume of 2 \times SDS sample loading buffer after centrifuging at 15,000 rpm for 10 min. The samples were then loaded on 12% SDS-PAGE gels, and the gels were stained with Coomassie Brilliant Blue R-250 (Fisher Biotech, Fair Lawn, NJ, USA) after electrophoresis. For western blotting, the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Foster City, CA, USA). The antisera were used at the following dilutions: anti-GFP (Invitrogen, Carlsbad, CA, USA) at 1:2,000, Goat Anti-Rabbit IgG (H+L)-HRP Conjugate at 1:10,000 (Bio-Rad, Hercules, CA, USA).

Results

Establishment of a plant-based FHV virion production platform

FHV virions can be recovered from cDNA clone-transfected insect cell lines, such as S2 cells (Hongwei et al. 2002). However, we found the yield of virions to be fairly low when FHV was recovered from transfected cells, and a subsequent round of S2 cell reinoculation with the recovered virions was essential to obtain a sufficient number of virions for subsequent experiments (data not shown). Taking advantage of the various plant expression systems we have in our laboratory, we sought to develop a plant-based FHV virion production platform. Thus, the cDNA sequence of FHV RNA1 and RNA2 were individually cloned into a pJL89 vector under the CaMV 35S promoter to drive the transcription of both RNAs, followed by the self-cleaving HDV ribozyme sequence to ensure

production of the authentic viral 3' terminus (Fig. 1a and Fig. S1a). The resulting constructs were termed pJL89/FHV RNA1 and pJL89/FHV RNA2. The success of this strategy has been demonstrated in the recovery of other viruses in our lab (Jiang et al. 2021), and a similar approach to recover FHV virions in plants has been shown by Padmanaban et al. (2008). Leaves of the model plant *N. benthamiana* were then infiltrated with a mixture of *A. tumefaciens* GV3101 containing pJL89/FHV RNA1 and pJL89/FHV RNA2 to initiate FHV infection. FHV virions were then isolated from agroinfiltrated leaf tissues 4 days later. Although the amount of virions is enough for our experiments, to obtain sufficient pure virions for TEM observation, S2 cells were inoculated with the virions isolated from plants and subjected to virion purification (Fig. 1b). TEM observation showed the diameter of virions to be about 33.25 ± 2.26 nm ($N=20$) (Fig. 2a), consistent with what has been reported by others for FHV virions (Kimi et al. 2021), confirming the success of this plant-based FHV virion production platform.

FHV infects different aphid species by both microinjection and oral feeding

We next assessed whether FHV was infectious to aphids. For an initial test, the titer of the purified FHV virions was quantified by RT-qPCR, and the final titer was adjusted to 1×10^5 copies/ μ L. *M. persicae* aphids were injected intrathoracically with 45 nanoliters (nLs) of the FHV virion preparation per aphid, and the level of FHV RNA1 accumulation was determined by RT-qPCR from 0 to 8 days post-injection (d.p.i). We found the vRNA accumulated rapidly in *M. persicae* until 5 d.p.i, and then remained at a stable level with slight increase (Fig. 2b). As shown in Fig. 2c, northern blot analysis clearly showed the production of FHV sgrNA3, which is not present in the FHV inoculum, and is only produced during FHV replication, further confirming that FHV was infectious and replicating in *M. persicae*. FHV virions (at a titer of 1×10^7 copies/ μ L) also were fed to healthy *M. persicae* by membrane feeding for 2 days, after which the aphids were maintained by membrane feeding on a virus-free artificial diet, and then total RNAs were extracted and used for northern blotting. Similarly, we observed the production and accumulation of sgrNA3 at later time points compared to intrathoracic injection, from days 3 to 8 (Fig. 2d). We further tested if FHV can infect two other aphid species, *R. maidis* and *R. padi*, which are important plant pests and plant virus vectors. Our data showed that FHV can infect *R. maidis* and *R. padi* after microinjection (Fig. S2a and S2c), and can also infect *R. maidis* by oral feeding (Fig. S2b). Due to poor survival of *R. padi* on the artificial diet, oral infection of FHV in *R. padi* was not tested further.

Both RNA1 and DI-634 can be engineered for GFP expression, but only DI-634-GFP is encapsidated in the presence of WT FHV infection

To explore the potential of using FHV as a VIGS vector for RNAi in aphids, we aimed to determine whether either genomic segment RNA1, RNA2, or defective interfering RNAs (DI-RNAs) were amenable to engineering, and at which nucleotide position in the RNAs an exogenous sequence could be introduced. Duane et al. and Maharaj et al. have demonstrated the expression of GFP when its coding sequence was introduced in the near 3' end of the B2 ORF in FHV RNA1 (Duane et al. 2000; Maharaj et al. 2014). Dasgupta et al. have successfully engineered FHV DI-634 to express GFP when the coding sequence was inserted at nt position 59 (Fig. 1a) (Dasgupta et al. 2003). However, those demonstrations were done in either insect or mammalian cell lines, and whether the engineered GFP-expressing RNAs can be encapsidated into FHV virions has not been confirmed in plants. For our purpose, we inserted the GFP coding sequence into pJL89/FHV RNA1 and pJL89/FHV DI-634, producing constructs pJL89/FHV RNA1-GFP and pJL89/FHV DI-634-GFP, respectively (Fig. 1a, Fig. S1b and Fig. S1c). GFP expression will indicate the feasibility of these two approaches, and a stronger silencing effect can be expected if insertion of exogenous sequences as large as the GFP coding sequence (720 nts) can be tolerated. *N. benthamiana* leaves were agroinfiltrated with either *A. tumefaciens* containing pJL89/FHV RNA1-GFP and pJL89/FHV RNA2 or pJL89/FHV RNA1, pJL89/FHV RNA2, and pJL89/FHV DI-634-GFP. In both cases, agroinfiltrated leaves showed slight fluorescence when visualized under a UV lamp at 3 d.p.i (Fig. S3a and 3a). In order to confirm GFP expression in leaves, we performed western blotting which clearly showed the accumulation of GFP (Fig. S3b and 3b). Co-inoculation of pJL89/FHV DI-634-GFP plus pJL89/FHV RNA1 showed the greatest GFP accumulation, while the addition of pJL89/FHV RNA2 showed reduced GFP accumulation (Fig. 3b). FHV virions were isolated from agroinfiltrated leaves at 4 d.p.i, and were used to inoculate S2 cells. We observed GFP expression at 2 d.p.i when S2 cells were infected with virions purified from pJL89/FHV DI-634-GFP, pJL89/FHV RNA1, and pJL89/FHV RNA2 co-agroinfiltrated plants (Fig. 3c), while no fluorescence was observed when cells were infected with virions isolated from pJL89/FHV RNA1-GFP and pJL89/FHV RNA2 co-agroinfiltrated plants (data not shown). Coomassie blue staining further confirmed the GFP-expressing recombinant FHV RNA1 was not encapsidated (Fig. S3c). To stimulate increased encapsidation of the GFP expressing DI-634 RNA (DI-634-GFP, referred to as DI-GFP hereafter), we tested different ratios of *A. tumefaciens* suspensions containing pJL89/FHV RNA1, pJL89/FHV RNA2, and pJL89/FHV DI-634-GFP. We found

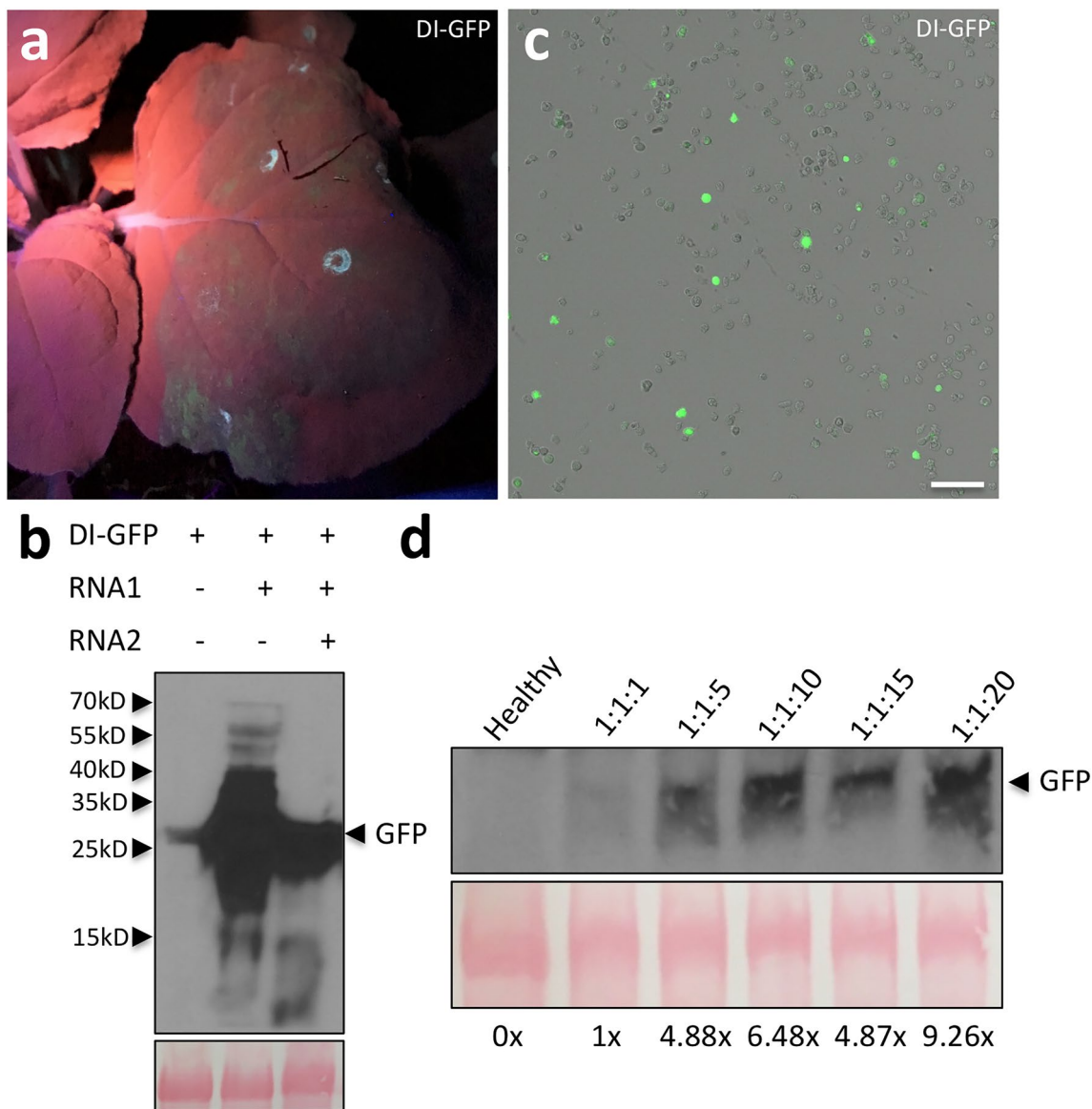


Fig. 3 GFP expression and encapsidation of DI-GFP. *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* harboring pJL89/FHV DI-GFP alone, or with equal volumes of pJL89/FHV DI-GFP and pJL89/FHV RNA1, or with pJL89/FHV DI-GFP, pJL89/FHV RNA1, and pJL89/FHV RNA2 at a ratio of 1:1:1. Leaves expressing RNA1, RNA2, and DI-GFP were observed under a UV lamp at 3 d.p.i (a), and then further processed for western blotting (b). Virions were isolated from leaves expressing RNA1, RNA2, and DI-GFP, and used to infect S2 cells. The cells were checked under a fluorescent microscope at 2 d.p.i (c). Using a similar approach,

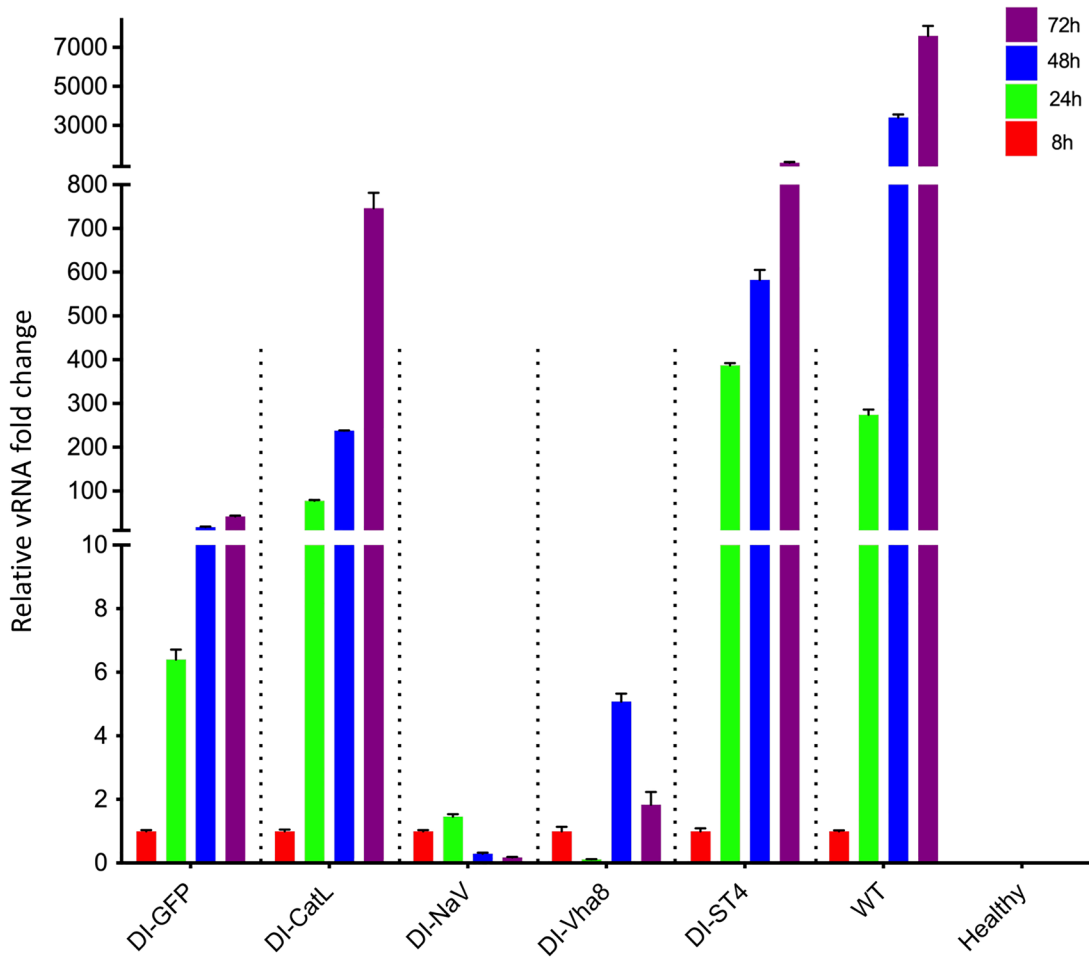
virions were isolated from plants agroinfiltrated with *Agrobacterium tumefaciens* to express RNA1, RNA2, and DI-GFP at different ratios, and the GFP expression in re-inoculated S2 cells was checked by western blotting (d). The ratios of RNA1: RNA2: DI-GFP are shown on top, and the relative protein expression levels are shown at the bottom. The Ponceau S stained Rubisco large subunit (b) and *Drosophila* lamina protein (d) serve as a loading control. The band intensity was quantified with ImageJ. The Western blotting was done with anti-GFP antibodies (b and d). Scale bar = 100 μm (c)

a relatively high level of GFP expression was achieved when a ratio of pJL89/FHV RNA1: pJL89/FHV RNA2: pJL89/FHV DI-634-GFP = 1:1:10 was used (Fig. 3d). Consequently, this ratio was used for all following experiments. These data suggest FHV DI-634 is tolerant of insertions and DI-GFP can be incorporated into virus particles when co-inoculated with WT FHV RNA1 and RNA2.

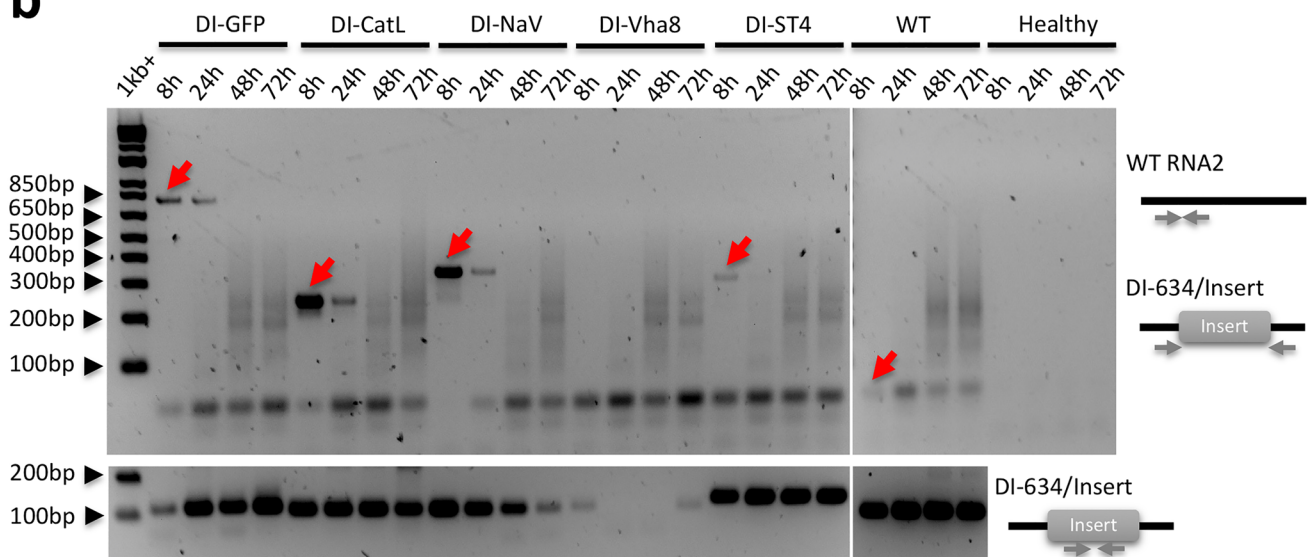
Engineering DI-634 to target aphid mRNAs

Rauf et al. demonstrated that expressing a 200 bp fragment of the aphid gene *Cathepsin L* (*CatL*) from transgenic plants can cause up to 80% mortality of adult *M. persicae* (Rauf et al. 2019). Alternatively, using the plant virus tobacco rattle virus (TRV) as a VIGS vector, Tzin et al.

a



b



demonstrated siRNAs derived from a 264 bp fragment of the gene *Sugar Transporter 4 (ST4)* can cause downregulation of the same endogenous *M. persicae* gene at the

mRNA level (Tzin et al. 2015). Bilgi et al. have shown the ingestion of dsRNA synthesized from a 478 bp fragment of the gene *Vacuolar H⁺-ATPase 8 (Vha8)* reduced

Fig. 4 Infection of engineered DI-CatL, DI-NaV, DI-Vha8, and DI-ST4 in S2 cells. *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* harboring pJL89/FHV RNA1, pJL89/FHV RNA2, and the engineered pJL89/FHV DIs at a ratio of 1:1:10. Virions were isolated from agroinfiltrated leaves and were used to re-inoculate S2 cells. The cells were then collected at 8, 24, 48, and 72 h after inoculation, the accumulation of DI-RNAs was quantified (a) and the retention of inserts was checked by RT-PCR (b). The RT-qPCR in (a) and RT-PCR in (b, the lower panel) were done with primers corresponding to the interior of each inserted sequence of the DI-RNAs. The expected size of PCR products was 111 bp for DI-GFP, 103 bp for DI-CatL, 103 bp for DI-NaV, 106 bp for DI-Vha8, 126 bp for DI-ST4, and 109 bp for WT FHV; The RT-PCR in (b, the upper panel) was done with primers corresponding to the FHV RNA2 backbone. The primers could bind to the WT RNA2 and the DI-RNAs. The expected size of PCR products was 760 bp for DI-GFP, 240 bp for DI-CatL, 329 bp for DI-NaV, 518 bp for DI-Vha8, 304 bp for DI-ST4, and 40 bp for WT FHV. The S2 cell gene *Tubulin84B* was used as the internal control (a). Healthy: S2 cells inoculated with extracts from healthy plants; WT: S2 cells were inoculated with extracts from WT FHV infected plants. Mean values \pm SDs of three independent experiments are shown (a). The expected PCR products, of the expected size, are indicated by red arrows (b, the upper panel)

target gene expression (Bilgi et al. 2017); Similarly, Tariq et al. confirmed that oral feeding of a 289 bp *Voltage-gated sodium channel (NaV)* dsRNA caused significant mortality in *M. persicae* (Tariq et al. 2019). Thus, to test the potential of using FHV and DI-634 as a VIGS vector, the above target sequences were introduced into pJL89/FHV DI-634, thereby yielding the constructs pJL89/FHV DI-634-CatL, pJL89/FHV DI-634-ST4, pJL89/FHV DI-634-Vha8, and pJL89/FHV DI-634-NaV. *N. benthamiana* leaves were agroinfiltrated with *A. tumefaciens* harboring pJL89/FHV RNA1, pJL89/FHV RNA2, together with the above clones, at a ratio of 1:1:10. Virions were isolated from agroinfiltrated leaves, and S2 cells were inoculated with the virions. RT-qPCR was performed with primers corresponding to the interior of each inserted sequence and showed significant accumulation of engineered DI-634 at 48 and 72 h after infection, especially with constructs harboring DI-GFP, DI-CatL, and DI-ST4 (Fig. 4a). We further checked the stability of the target sequences, to ensure the sequences were not truncated or excluded during virus infection. RT-PCR was done with primers corresponding to the RNA2 backbone. In this case, the primers could bind to WT RNA2 and the engineered DI-RNAs. As shown in Fig. 4b (upper panel), the full-length sequences of GFP, CatL, NaV, and ST4 were detected in the samples tested at 8 and 24 h, while the Vha8 insert was barely detectable. None of the inserts were detected at later time points (48 and 72 h) for any of the constructs, probably due to preferential binding of the PCR primers to the WT RNA2. To confirm this possibility, we further carried out RT-PCR with the same primers used in the above RT-qPCR assay. As shown in Fig. 4b (lower panel), the inserts could be detected at later time points (48 and 72 h). These

results suggest FHV DI-634 can retain these aphid-specific cDNA sequences, and potentially serve as a VIGS vector to induce RNAi in aphids.

Engineered DI-634 downregulates mRNA levels and causes mortality of *M. persicae*

We then tested if DI-CatL, DI-NaV, and DI-ST4 can downregulate the corresponding *M. persicae* mRNA levels in vivo. DI-GFP was used as a negative control as no aphid mRNA will be targeted. The titers of purified virions isolated from agroinfiltrated plants were quantified by RT-qPCR and adjusted to 1×10^7 copies/ μ L. 45 nLs of the purified virions were microinjected into each aphid. Aphids were collected at 24, 48, and 72 h after intrathoracic injection, total RNAs were extracted, and the titer of DI-634 vRNAs and the accumulation level of each target mRNAs were quantified. As shown in Fig. 5a, compared with the titer at 24 h post injection, the titer of DI-GFP, DI-CatL, DI-NaV, and DI-ST4 vRNA reached a higher level at 72 h post injection, indicating that DI-634 vRNAs were replicating in aphid. RT-qPCR analysis showed the mRNA accumulation levels of CatL and ST4 were downregulated by $\sim 35\%$ and $\sim 30\text{--}50\%$, respectively (Fig. 5b). However, the mRNA level of NaV showed only a slight decrease of $\sim 20\%$ at 24 h, but no effect was observed at 48 and 72 h after injection. Due to the mechanical damage, microinjection may cause the death of *M. persicae*. Consequently, to better evaluate the mortality effects caused by these engineered DI-634 RNAs, oral feeding experiments were performed. Sucrose was mixed with purified virions at a titer of 3×10^7 copies/ μ L to a final concentration of 20% sucrose. For each feeding assay, a total of 100 *M. persicae* aphids were fed for 2 days, after which the aphids were maintained on the virus-free artificial diet, and aphid mortality was recorded daily. WT FHV did not induce significant mortality when compared to the buffer control (Fig. S4). As shown in Fig. 5c, the engineered DI-ST4 induced the greatest mortality, followed by DI-CatL. When compared to the non-target control DI-GFP, DI-NaV did not cause any significant difference in aphid mortality.

Discussion

In this work, we have demonstrated that the nodavirus, FHV, has a host range including at least the three aphid species tested here (Figs. 2 and S2). We also show that recombinant FHV can be produced in plants and virions isolated from plants can then be used for downstream aphid VIGS experiments to induce aphid mortality (Fig. 5). By simply mixing *A. tumefaciens* containing the binary vector pJL89 carrying FHV RNA1 and RNA2 genomic segments (Fig. 1 and Fig. S1), virions can be isolated from infiltrated plants. It is

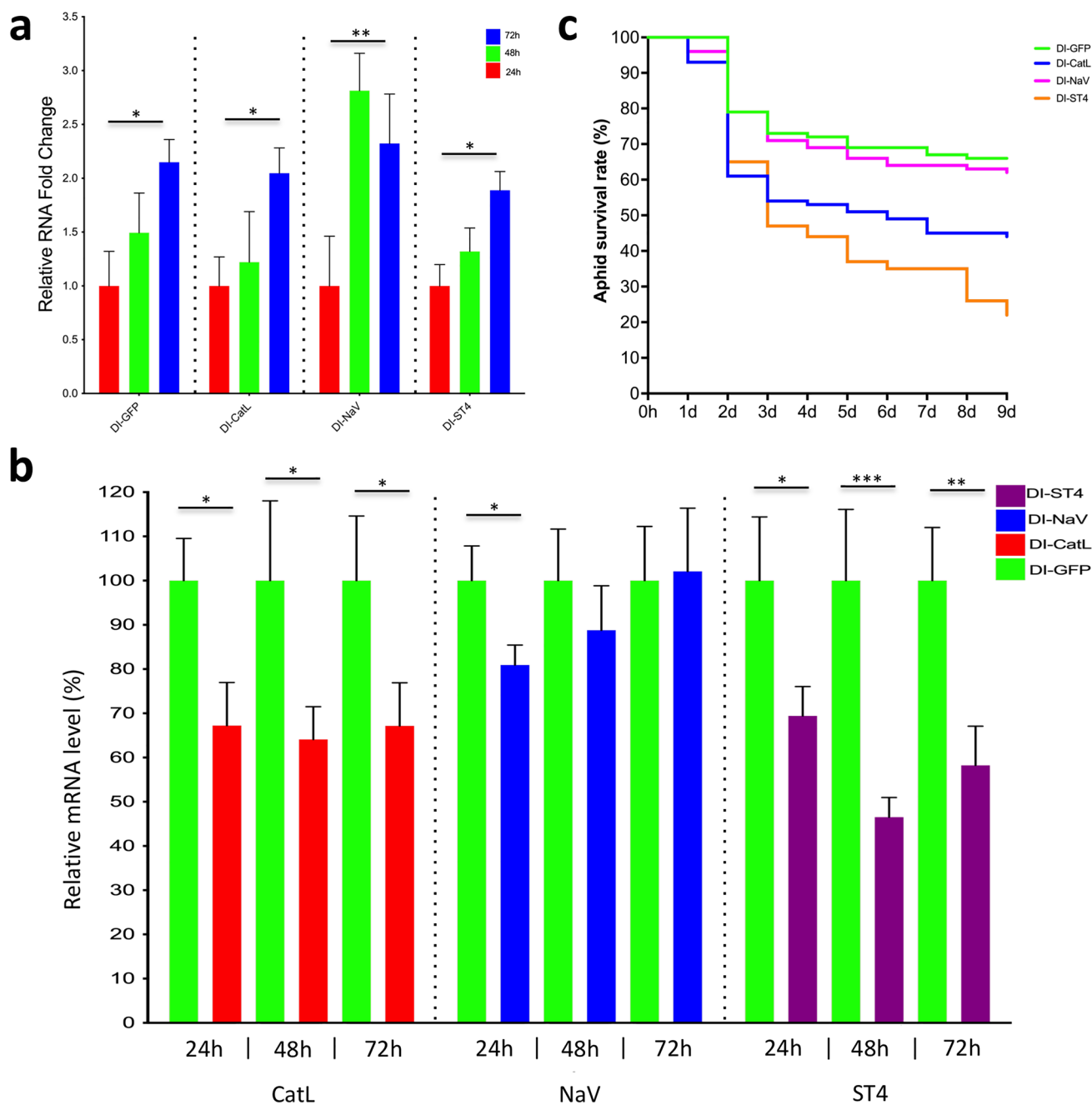


Fig. 5 Infection of engineered DI-CatL, DI-NaV, and DI-ST4 in aphids *M. persicae*. Virions were isolated from *N. benthamiana* plants expressing WT RNA1, RNA2, and DI-GFP, or DI-CatL, or DI-NaV, or DI-ST4, and were microinjected into (a and b) or orally fed to (c) *M. persicae* aphids. The accumulation levels of DI-RNAs in aphids were checked at 24, 48, and 72 h, and were compared to their level at 24 h (a). The mRNA accumulation levels of the endogenous genes *CatL*, *NaV*, and *ST4* were quantified at 24, 48, and 72 h, and were compared to their corresponding levels in DI-GFP infected

controls. The *M. persicae Actin* gene was used as the internal control. For each treatment, ten individual aphids were analyzed in each independent experiment (a and b); For the survival assay, each treatment was started with 100 aphids. Aphids were fed with virions for 48 h and then maintained on a virus-free artificial diet (c). Significant difference is indicated by asterisks (Student's *t*-tests: ***, $P < 0.001$; **, $0.001 < P < 0.01$; *, $0.01 < P < 0.05$). Mean values \pm SDs from three independent experiments are shown

known that DI-634, one of the defective interfering RNAs of RNA2, can be packaged into virions. Using GFP as an indicator protein, we showed that when its coding sequence

was introduced into DI-634 at nt position 59, the engineered DI-GFP was packaged into virions (Figs. 1 and 3). This was achieved by co-agroinfiltration of *N. benthamiana* plants

with *A. tumefaciens* to induce expression of WT RNA1, RNA2, and DI-GFP. We further optimized the encapsidation rate of DI-GFP by adjusting the ratio of *A. tumefaciens* suspensions of RNA1, RNA2, and DI-GFP (Fig. 3). An advantage of this plant-based system is that the ratio of RNA1:RNA2:DI-RNA introduced into each cell is more controllable compared to the cell-based system, thus promoting the incorporation of engineered DI-634 into virus particles.

It seems that the insertion of the GFP coding sequence into RNA1 resulted in encapsidation when a cell-based system was used (Taning et al. 2018). However, when we attempted to insert sequences into RNA1, when using the plant-based system, the engineered RNA1 could not be encapsidated (Fig. S3). We also noticed that the profile of RNAs incorporated into virions, of this plant- and cell-based system, appears to be different. S2 cells exhibited GFP expression when they were inoculated with virions isolated from pJL89/DI-634-GFP, pJL89/FHV RNA1, and pJL89/FHV RNA2 co-agroinfiltrated plants (Fig. 3). However, when cDNA clones of FHV RNA1, RNA2, and DI-GFP were co-transfected into S2 cells, the GFP expression could not be reconstituted in the second round of inoculation with virions isolated from the primary transfected cells (data not shown). Furthermore, the expression of GFP in S2 cells was not recovered when cells were inoculated with virions isolated from the first generation of DI-GFP-infected S2 cells (the first generation of S2 cells were infected with virions isolated from pJL89/DI-634-GFP, pJL89/FHV RNA1, and pJL89/FHV RNA2 co-agroinfiltrated plants). This is probably because the presence of WT FHV RNA1 and RNA2, which can replicate efficiently, outcompete with the DI-GFP. Consequently, the plant-based system was our preferred choice here and only the first generation of virions that were isolated from the agroinfiltrated leaf tissues were used in our following assays. However, the exact RNA composition of the recombinant virions, whether the DI-GFP is encapsidated with WT FHV RNA1 into a single virion, is not yet known.

The infectivity of FHV virions via microinjection was confirmed in multiple aphid species, including *M. persicae*, *R. maidis*, and *R. padi*, which are all important plant pests as well as vectors of plant-infecting viruses (Fig. 2 and S2). When using FHV as a VIGS vector to target aphids, microinjection is very tedious. Consequently, we further confirmed that FHV virions can infect *M. persicae* and *R. maidis* by oral feeding (Figs. 2 and S2). We wanted to test the oral acquisition of FHV by *R. padi*, however, these aphids did not perform well when they were maintained on the artificial diet. As we have mentioned, FHV has a broad host range. It is reasonable to suspect that FHV can infect many other aphid species. In addition, WT FHV can infect aphids robustly (Figs. 2 and S2) and does not seem to cause any pathogenic effects (Fig. S4). In another project, we have

confirmed that FHV can infect another important agricultural hemipteran insect pest *Diaphorina citri* (unpublished). This paves the road for engineering FHV as a wide-spectrum virus for VIGS in aphids and possibly other hemipteran insects.

To develop FHV as a VIGS vector to trigger RNAi, we tried several approaches. We engineered FHV RNA1, RNA2, and DI-634, with consideration of various strategies that have been reported by other researchers. The introduction of the foreign sequences into the 3' region of the B2 ORF did not affect the vRNA replication, however, these engineered RNAs could not be encapsidated (Fig. S3). The constraining icosahedral shape of the FHV virion significantly prevents us from increasing the sizes of genomic RNAs 1 or 2 such that they cannot be encapsidated into virions. For our purposes, we found modification of DI-634 to be a much more feasible approach. DI-634 with insertion of the GFP coding sequence (up to 720 bp) could be packaged into virions (Fig. 3). In our studies, the insertion of 200, 289, and 264 bp fragments into DI-634 to target the corresponding *M. persicae* *CatL*, *NaV*, and *ST4* genes, did not affect FHV replication or encapsidation of the engineered DI-634. It appears that the length and the nucleotide composition of the insert are somewhat flexible. This gave us the confidence that using virions containing engineered DI-634 will trigger RNAi in aphids.

Using the approach established in this work, the accumulation levels of *CatL* and *ST4* mRNAs were downregulated up to ~35% and 30–50% in *M. persicae* aphids when the respective DI-634 virions were delivered by microinjection (Fig. 5b). As early as 24 h after injection, we observed silencing effects. In comparison, the mRNA accumulation level of *NaV* was downregulated at 24 h, but not at later time points, such as 48 and 72 h (Fig. 5b). This correlates with the virus titer in S2 cells, in which the virus titer increased at 24 h, but not at 48 and 72 h (Fig. 4a). Thus, it appears to be the case that better viral performance will result in a higher silencing effect. More importantly, the oral acquisition of these engineered DI-634 induced greater mortality of *M. persicae* (Fig. 5c). In this work, using engineered FHV virions as a vehicle, as a proof-of-concept, we demonstrate a novel approach for using insect viruses to target aphids. However, because of their parthenogenetic lifestyle, aphids present a problem that must be solved for translational approaches. Our feeding assays showed that recombinant FHV induced mortality only in parental aphids. FHV was not passed on to progeny, which is produced before the RNAi shows significant effects in the parental aphids. To solve this issue, exploring RNAi approaches using endogenous aphid viruses that can be vertically transmitted to progeny as VIGS vectors may be a more stable approach. Next-generation sequencing has identified many potential aphid viruses (Kondo et al. 2020; Chang et al. 2021; Qi

et al. 2021; An et al. 2021), and the development of VIGS vectors similar to this one will surely further advance this field of study.

Author contribution

JJ and BWF conceived and designed research; JJ conducted experiments with assistance from AE, WQ, and EM; JJ and BWF analyzed data; JJ and BWF wrote the manuscript; All authors read and approved the manuscript.

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Data availability The material and tools used in the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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