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Horizontally transferred genes as RNA interference targets for aphid and whitefly control

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Summary

RNA interference (RNAi)-based technologies are starting to be commercialized as a new approach for agricultural pest control. Horizontally transferred genes (HTGs), which have been transferred into insect genomes from viruses, bacteria, fungi or plants, are attractive targets for RNAi-mediated pest control. HTGs are often unique to a specific insect family or even genus, making it unlikely that RNAi constructs targeting such genes will have negative effects on ladybugs, lacewings and other beneficial predatory insect species. In this study, we sequenced the genome of a red, tobacco-adapted isolate of *Myzus persicae* (green peach aphid) and bioinformatically identified 30 HTGs. We then used plant-mediated virus-induced gene silencing (VIGS) to show that several HTGs of bacterial and plant origin are important for aphid growth and/or survival. Silencing the expression of fungal-origin HTGs did not affect aphid survivorship but decreased aphid reproduction. Importantly, although there was uptake of plant-expressed RNA by *Coccinella septempunctata* (seven-spotted ladybugs) via the aphids that they consumed, we did not observe negative effects on ladybugs from aphid-targeted VIGS constructs. To demonstrate that this approach is more broadly applicable, we also targeted five *Bemisia tabaci* (whitefly) HTGs using VIGS and demonstrated that knockdown of some of these genes affected whitefly survival. As functional HTGs have been identified in the genomes of numerous pest species, we propose that these HTGs should be explored further as efficient and safe targets for control of insect pests using plant-mediated RNA interference.

Introduction

Aphids, whiteflies and other hemipteran insect pests cause considerable damage to agricultural crops. Although current control strategies, in particular chemical pesticides, provide some level of protection, insects continuously develop new tolerance or resistance mechanisms (Bass *et al*., 2014; Koch *et al*., 2018). Genetically engineered *Bt* crops have been shown to be effective in controlling specific insect pests, without long-term environmental persistence (Mendelsohn *et al*., 2003; Sears *et al*., 2001; Sujii *et al*., 2013; Tian *et al*., 2015). However, there has been only limited success in the development of *Bt* toxins that target hemipteran pests (Chougule and Bonning, 2012; Lu *et al*., 2010; Pessoa *et al*., 2016; Sujii *et al*., 2013). Thus, there is a need to develop novel and environmentally friendly transgenic plant approaches for the control of phloem-feeding insects such as aphids and whiteflies.

RNA interference (RNAi)-based gene expression silencing has emerged as a novel and powerful strategy for agricultural pest control. As phloem feeders, hemipteran pests are less likely to take up surface sprays of RNAi constructs, which can be deployed against chewing herbivores such as Colorado potato beetles (*Leptinotarsa decemlineata*; Rodrigues *et al*., 2021). However, plant-mediated RNAi has been effective for targeting hemipteran gene expression (Chung *et al*., 2021). Several classes of aphid genes have been successfully inhibited by plant-mediated RNAi, including *C002* (Coleman *et al*., 2015; Mutti *et al*., 2006; Pitino *et al*., 2011), receptor of activated kinase C (*Rack-1*) (Coleman *et al*., 2015; Pitino *et al*., 2011), *MpPInt01* and *MpPInt02* (Coleman *et al*., 2015), *Mp55* (Elzinga *et al*., 2014); and those encoding aquaporin, sucrase-transglucosidase, and sugar transporters (Tzin *et al*., 2015), V-ATPase E and tubulin folding cofactor D (Guo *et al*., 2014), serine protease (Bhatia *et al*., 2012), hunchback (Mao and Zeng, 2014), carboxylesterase CbE E4, juvenile hormone binding protein, calreticulin, and cathepsin, carboxylesterase (Xu *et al*., 2014), salivary sheath protein (Abdellatef *et al*., 2015), and a zinc finger protein (Xie *et al*., 2022). Similarly, genes that have been effectively targeted using plant-mediated RNAi for controlling *Bemisia tabaci* (whiteflies) include cytochrome P450 genes *cyp315a1* and *cyp18a1* (Luan *et al*., 2013), and those encoding ecdysone receptor (EcR) and acetylcholinesterase (AchE) (Malik *et al*., 2016), aquaporin and α-glucosidase (Raza *et al*., 2016), v-ATPase (Ibrahim *et al*., 2017; Thakur *et al*., 2014), cyclophilin B (CypB) and heat shock protein 70 (hsp70) (Kanakala *et al*., 2019), and phenolic glucoside malonlyltransferase (*BtPMaT1*) (Xia *et al*., 2021).

As part of the regulatory approval process for commercialization, RNAi-based transgenic plants should be assessed for biological risks, which include effects on non-target insect species with similar gene sequences (Casacuberta *et al*., 2015).

Essential genes that are potential targets for RNAi-mediated pest control, including many of those described above for the control of aphids and whiteflies, are often highly conserved across the insect phylogeny. As it is not currently feasible to examine the genomes of all potential non-target insects, bioinformatic analysis alone is not sufficient for the design of RNAi constructs that are unlikely to have off-target effects on beneficial insect species.

Horizontally transferred genes (HTGs), which are often taxon-, genus- or even species-specific (Wybouw *et al*., 2016), are an attractive option for limiting potential off-target effects during RNAi-mediated pest control. HTGs, including those transferred from prokaryotes to prokaryotes, prokaryotes to eukaryotes, and eukaryotes to eukaryotes, have been described in all branches of life (Husnik and McCutcheon, 2018; Soucy *et al*., 2015). Importantly, the integration, expression and maintenance of HTGs in the recipient genome suggest that their presence provides a selective advantage (Soucy *et al*., 2015). There are many hurdles for HTGs to become functional in the recipient genome, particularly in the case of genes that are transformed from prokaryotes to eukaryotes. For instance, the presence or absence of introns, variable GC contents, codon usage preferences, and differences in transcriptional promoters can limit successful gene expression in a recipient species (Husnik and McCutcheon, 2018). Nevertheless, functional HTGs have been described in many species, including aphids and whiteflies (Chen *et al*., 2016; Chung *et al*., 2018; Luan *et al*., 2015; Novakova and Moran, 2012; Parker and Brisson, 2019; Sloan *et al*., 2014).

Prior to this study, a few HTGs with different origins have been investigated in aphid genomes. Carotenoid biosynthetic genes in aphids were shown to be horizontally transferred from a fungus, including the highly duplicated carotene desaturase (*Tor*) and the carotenoid cyclase-carotenoid synthase (*CarRP*) (Novakova and Moran, 2012). One carotenoid desaturase gene copy was present only in a red *Myzus persicae* (green peach aphid) isolate, and a point mutation in this gene, which arose in a lab culture, resulted in the loss of red colour in the aphids (Moran and Jarvik, 2010). Similar red colour reduction was also observed in *Acyrthosiphon pisum* (pea aphid) nymphs when silencing a carotenoid desaturase in the parental aphids (Ding *et al*., 2020). These results indicated that carotenoid biosynthetic genes of fungal origin remain functional in the recipient aphid genomes and confer to the aphid red-green colour polymorphism, which in turn influence their susceptibility to natural enemies (Moran and Jarvik, 2010). More recently, 2 *A. pisum* HTGs of bacterial origin, *amiD* and *ldcA1*, were targeted using RNAi constructs that were supplied in artificial diet (Chung *et al*., 2018). Enriched *amidD* and *ldcA1* expression in *A. pisum* bacteriocytes, which harbour *Buchnera aphidicola* endosymbionts, coupled with the fact that *amiD* and *ldcA* were lost when the peptidoglycan biosynthetic genes were not present in the symbiont *Buchnera* genome (Smith *et al*., 2022), suggested that amiD and ldcA1 function in degrading bacterial peptidoglycan, thereby protecting *Buchnera* from host attack (Chung *et al*., 2018). Consistent with this hypothesis, knockdown of *amiD* and *ldcA1* by RNAi caused a significant reduction in *Buchnera* abundance and inhibited aphid growth. In addition to HTGs from fungi and bacteria, HTGs that originated from viruses have also been found in aphids. For example, the cytolethal distending toxin subunit B (*cdtB*) found in the genome of *M. persicae* strain G006 was suggested to be involved in aphid resistance to a predatory wasp (Verster *et al*., 2019). In the pea aphid genome, two HTGs from a densovirus have been described and demonstrated to modulate the aphid wing plasticity (Parker and Brisson, 2019).

Our previous analysis of the *B. tabaci* MEAM1 genome identified 142 HTGs from bacteria, fungi and plants (Chen *et al*., 2016). Several of these genes were proposed to contribute to broad host range and insecticide resistance of whiteflies. Interestingly, a recent study found that a detoxifying gene in whiteflies, phenolic glucoside malonyltransferase (*BtPMaT1*), likely originated from plants and was able to neutralize host plant defensive metabolites (Xia *et al*., 2021). Plant-mediated silencing of *BtPMaT1* expression impairs whitefly detoxification functions and confers full whitefly resistance in tomato plants, confirming the utility of this HTG for whitefly control (Xia *et al*., 2021).

To further explore HTGs as potential RNAi targets for aphid control, we assembled the genome of a red, tobacco-adapted strain of *M. persicae* (Ramsey *et al*., 2007, 2014), conducted a genome-wide annotation of HTGs, and compared this to the HTG profiles of other aphid species. Then, using plant-mediated virus-induced gene silencing (VIGS), we tested the RNAi effects of these HTGs on aphid survival. Additionally, we conducted RNAi of a subset of the HTGs that have been annotated in the *B. tabaci* MEAM1 (Chen *et al*., 2016). We found that knockdown of these HTGs using plant-mediated RNAi can significantly affect insect performance. Importantly, the knockdown of the aphid HTGs did not affect the survival of *Coccinella septempunctata* (seven-spotted ladybugs) that fed on these aphids. Our results suggest that HTGs will be effective and safe targets for plant genetic engineering to control aphid populations in the field.

Materials and methods

Insect and plant cultures

The tobacco-adapted *M. persicae* strain USDA-Red (Ramsey *et al*., 2007, 2014) was maintained on *Nicotiana tabacum* (tobacco) plants in a growth room at 23 °C with a 16:8 h light:dark photoperiod. A *B. tabaci* MEAM1 colony was obtained from Angela Douglas (Cornell University) and maintained on an acylsugar-deficient *asat2-1* mutant *Nicotiana benthamiana* lineage (Feng *et al*., 2022) at 23 °C with a 16:8 h light:dark photoperiod. A *C. septempunctata* colony was maintained on *A. pisum* on *Vicia faba* (faba bean). Mealybug ladybird beetle (*Cryptolaemus montrouzieri*) adults were purchased from Amazon (www.amazon.com). *Nicotiana benthamiana* wild type and *asat2-1* mutant plants (Feng *et al*., 2022) for aphid, whitefly and ladybug experiments were grown in Cornell Mix [by weight 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer (Scotts, Marysville, OH), and 1% Unimix (Scotts)] in a Conviron growth chamber with a photosynthetic photon flux density of 200 mmol/m/s and a 16:8 h day:night photoperiod, at 23 °C with 50% relative humidity.

Sequencing, assembly and annotation of the USDA-Red *M. persicae* genome

To produce a chromosome-scale genome assembly of the USDA-Red *M. persicae* strain, we combined PacBio long-read, Illumina short-read and Hi-C sequencing of genomic DNA. To aid in gene

prediction, the *M. persicae* transcriptome was sequenced using Illumina strand-specific RNA-Seq and PacBio Iso-Seq platforms. Details for the sequencing, assembly and annotation of the *M. persicae* genome are elaborated in the Supplemental Methods.

Identification of horizontally transferred genes

To identify HTGs in *M. persicae* strain USDA-Red, we compared protein sequences in the USDA-Red genome to six databases of complete proteomes in UniProt, including archaea, bacteria, fungi, plants, metazoa (excluding proteins from species in the Arthropoda), and other eukaryotes (the remaining eukaryotes excluding fungi, plants, and metazoa). The index of horizontal gene transfer, h, was calculated by subtracting the bitscore of the best metazoan match from that of the best non-metazoan match as described by Crisp *et al*. (2015). We defined candidate HTGs as those with h ≥ 30 and the bitscore of the best non-metazoan match hit ≥100. The corresponding genome sequences of these candidates were compared against NCBI nt database to exclude contamination. We then performed phylogenetic analyses to validate HTGs. For each candidate gene, its protein sequence was compared by BLASTP against the protein databases of six taxa (archaea, bacteria, fungi, plants, metazoan and other eukaryotes). The top five hits from each taxon were extracted, and aligned with the protein sequence of the candidate gene using MUSCLE (Larkin *et al*., 2007). Each alignment was trimmed to exclude regions where gaps were more than 20% of sequences. Phylogenetic trees were constructed with PhyML (Guindon *et al*., 2009) using a JTT model with 100 bootstraps. HTGs were considered validated if the genes were monophyletic with taxa of plants, bacteria, fungi or other microorganisms.

To identify HTGs in *M. persicae* strain G006 (Mathers *et al*., 2017; Ramsey *et al*., 2007), we implemented *de novo* HTG annotation following the same protocol as for *M. persicae* strain USDA-Red. We conducted a homology search using the HTGs that we found in *M. persicae* strain USDA-Red and other genes previously reported in aphids (Parker and Brisson, 2019; Verster *et al*., 2019). The HTG homologues in other aphid species (*A. pisum*, *Aphis glycines*, *Aphis gossypii*, *Daktulosphaira vitifoliae*, *Diuraphis noxia*, *Eriosoma lanigerum*, *Myzus cerasi*, *Pentalonia nigronervosa*, *Rhopalosiphum maidis*, and *Rhopalosiphum padi*) were identified using reciprocal homology blast (Table 1; gene IDs in other aphid species are listed in Table S1). The newest-version genomes and annotations of all aphid species were downloaded from AphidBase (https://bipaa.genouest.org/is/aphidbase/) and used in this study. In a previous publication, we described the identification of whitefly HTGs (Chen *et al*., 2016).

Design of dsRNA and cloning of target HTG sequences in the TRV-VIGS vector

For each target gene, dsRNA fragments with a length of 150–300 bp were designed using the ERNAi website (https://www.dkfz.de/signaling/e-rnai3/; Horn and Boutros, 2010). To reduce the chances of off-target silencing, the designed fragments were checked against the reference genomes of the experimental species, *M. persicae* strain USDA-Red (this study), *B. tabaci* MEAM1 (Chen *et al*., 2016), *N. benthamiana* (Bombarely *et al*., 2012), and *C. septempunctata*, using a VIGS tool (Fernandez-Pozo *et al*., 2015) and local BLAST (Mount, 2007) to eliminate dsRNA fragments that contain any perfect matches of ≥19 nt for HTGs of bacterial and plant origin and >21 nt for HTGs of fungal origin to the reference genomes. The retained dsRNA fragments were cloned into the TRV-VIGS vector (Senthil-Kumar and Mysore, 2014) using the Invitrogen Gateway recombination cloning system (Invitrogen). Briefly, dsRNA fragments were first PCR-amplified from aphid cDNA using primers designed based on the dsRNA fragments (Table S2). PCR products were then cloned into pDONR207 using the Gateway BP clonase, dsRNA fragments in pDONR207 were swapped into Gateway compatible TRV2 plasmid (pTRV2) by recombination using Gateway LR clonase. The final constructs were named TRV2-GOI (gene of interest). In parallel, we obtained a construct TRV2-PDS, which carries a dsRNA fragment targeting *N. benthamiana* phytoene desaturase as a positive control for expression silencing (Velasquez *et al*., 2009). As negative controls, we used TRV2-GFP or TRV2-GUS, which carry dsRNA fragments targeting Green Fluorescent Protein (GFP) or *Escherichia coli* beta-glucuronidase (GUS) genes, respectively, but not sequences that are found in either the target insects or *N. benthamiana* (Vaghchhipawala *et al*., 2011), as well as a TRV2-EV (empty vector).

Transient agrobacterium infection of *N. benthamiana* for TRV-based VIGS

To infiltrate plants, plasmids (TRV1 and TRV2 carrying the gene of interest) were transformed into *Agrobacterium tumefaciens* and then infiltrated into *N. benthamiana* as previously described (Senthil-Kumar and Mysore, 2014). Briefly, the TRV1 and TRV2-GOI plasmids were transformed into *A. tumefaciens* strain GV3101. Each TRV2-GOI *Agrobacterium* culture was mixed with one carrying TRV1 and adjusted to a final OD_{600} of 0.3 with cell suspension buffer [final concentrations at 0.01 M MES (2-(N-morpholino)ethanesulfonic acid), 0.01 M $MgCl_2$, and 0.2 mM acetosyringone]. After 3 h of incubation at 23 °C, the *Agrobacterium* TRV mixtures were infiltrated to saturate three leaves of v4-stage *N. benthamiana* using 1-mL needleless syringes. Inoculated plants were kept in a growth chamber for 2–3 weeks, at which point plants infiltrated with TRV-PDS showed photobleaching symptoms that indicated viral spread. The TRV-GOI plant leaves were assayed for the presence of TRV1 and the expression of GOI dsRNA using PCR (Figure S1). Leaf samples were collected for RNA extraction using the SV Total RNA Isolation system (Promega, Fitchburg, WI) and cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). The synthesized cDNA samples were amplified by PCR using Phusion™ High-Fidelity DNA Polymerase (ThermoFisher Scientific, Waltham, MA) and the VIGS primers (Table S2, Figures S2 and S3). A 20 μL PCR reaction contains 4 μL betaine, 4 μL 5× High-Fidelity buffer, 1 μL 2.5 mM dNTPs, 0.2 μL Phusion polymerase, 1 μL each of 10 mM forward and reverse primers, 1 μL cDNA and 7.8 μL of ddH_2O. The PCR program was 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final step of 72 °C for 5 min. The confirmed TRV-GOI plants were then used for aphid bioassays.

Aphid bioassays

For each GOI, 5 or 6 infiltrated plants were used for aphid bioassays, and two aphid cages were attached to the adaxial side of fully developed young leaves on each plant. To set up the bioassays, 10 adult aphids were added to each cage and allowed to produce nymphs for 24 h. The adults were removed, and 25 newborn nymphs were scored for survival every 24 h for up to

Table 1 Horizontally transferred genes identified in aphid genomes

ID in *M. persicae* USDA-Red	Annotation	Origin	*M. persicae G006*	*A. pisum*	*A. glycines*	*A. gossypii*	*D. vitifoliae*	*D. noxia*	*E. lanigerum*	*M. cerasi*	*P. nigronervosa*	*R. maidis*	*R. padi*
MPE127429	Putative LD carboxypeptidase	Bacteria	y	y	y	y	n	y	y	y	y	y	y
MPE127428	ATP binding protein	Bacteria	y	y	y	y	y	y	y	y	y	y	y
MPE015894	N-acetylmuramoyl-L-alanine amidase	Bacteria	y	y	n	n	n	y	n	y	n	n	n
MPE005086	Cytolethal distending toxin subunit B	Viruses	y	n	n	n	n	n	n	y	n	n	n
MPE025920	Non-structural protein NS1 superfamily	Viruses	y	y	y	n	y	n	n	y	y	n	y
MPE014463	Non-structural protein NS1 superfamily	Viruess	y	n	n	n	n	n	n	n	n	n	n
MPE006690	Non-structural protein NS1 superfamily	Viruses	y	n	n	n	n	n	n	n	n	n	n
MPE006487	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006492	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006495	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006496	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006497	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006498	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006500	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006501	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006504	Lycopene cyclase/phytoene synthase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE006493	Carotenoid desaturase	Fungi	y	y	y	y	y	y	y	y	y	y	n
MPE006499	Carotenoid desaturase	Fungi	y	y	y	y	y	y	y	y	y	y	n
MPE006502	Carotenoid desaturase	Fungi	y	y	y	y	y	y	y	y	y	y	n
MPE006505	Carotenoid desaturase	Fungi	y	y	y	y	y	y	y	y	y	y	y
MPE002740	Unknown with ankyrin repeats	Plants	y	y	y	y	n	n	y	y	y	y	y
MPE008575	Unknown with ankyrin repeats	Plants	y	n	n	n	n	n	n	y	n	n	n
MPE008576	Unknown with ankyrin repeats	Planst	y	n	n	n	n	n	n	y	n	n	n
MPE010373	Unknown with ankyrin repeats	Plants	y	y	n	n	n	n	n	y	y	y	y
MPE011636	Reverse transcriptase domain-containing protein	Plants	y	n	n	n	n	n	n	y	n	n	y
MPE013670	Unknown with ankyrin repeats	Plants	y	n	n	n	n	n	n	y	n	n	n
MPE016359	Unknown with ankyrin repeats	Plants	y	y	y	y	n	y	n	y	y	y	y
MPE018635	Unknown with ankyrin repeats	Plants	y	n	n	n	n	n	n	n	n	n	n
MPE018636	Unknown with ankyrin repeats	Plants	y	y	n	n	n	n	n	n	n	n	y
MPE023973	Unknown with ankyrin repeats	Plants	y	n	y	n	n	y	n	y	n	y	y

5 days. After 5 days, 10–15 adult aphids were collected and flash-frozen in liquid nitrogen for target gene expression analyses using quantitative reverse transcriptase-PCR (qRT-PCR) (Primers see Table S2, Figures S2 and S3).

To test the effects of silencing bacterial and plant-origin HTGs on aphid reproduction, immediately after the 5th-day survival check, the aphid cages were moved to previously uninfested leaves and five adult aphids were left in each cage to measure aphid reproduction for a week. To test the effects of dsTor and dsCarRP on aphid reproduction, one adult aphid was added to each cage for 24 h, and then the adult aphid was removed. Nymphs that were deposited by the adult aphids were collected after 7 days, the number of progeny and the survival of the nymphs was scored, and adults were collected for qRT-PCR analysis.

Ladybug bioassays

On wildtype *N. benthamiana* plants, *M. persicae* prefer feeding on older, senescing leaves but the TRV virus tends to move to younger and new leaves. To generate enough aphids for the ladybug bioassays, we performed additional VIGS experiments and maintained a larger aphid population on *asat2-1* mutant *N. benthamiana* plants (Feng *et al*., 2022), which are deficient in acyl sugars and allow efficient *M. persicae* feeding on the TRV-infected younger leaves.

For ladybug bioassays, 2-day-old *C. septempunctata* larvae were used for all experiments. Each ladybug larva was individually caged in a vented plastic cup and aphids from VIGS *N. benthamiana* plants were served every other day. The survival of each ladybug was tracked for 7 days, and 10 ladybugs were monitored for each of the VIGS treatments. The experiments were repeated twice using wildtype *N. benthamiana* and twice with *asat2-1* mutant *N. benthamiana*. To confirm the tri-trophic persistence of RNAi signals, we also conducted assays using *C. montrouzieri* adults.

Whitefly bioassays

As *B. tabaci* strain MEAM1 could not survive on wildtype *N. benthamiana*, we used *asat2-1* mutant *N. benthamiana* (Feng *et al*., 2022) for bioassays. For each experiment, 3 or 4 TRV-GOI infiltrated plants were used for each gene of interest, and three cages were attached to each plant. Ten young adult whiteflies (emerged within the past week) were placed in each cage. After 7 days, the survival rates of whiteflies were assessed and compared across different treatments. The surviving whiteflies were collected for q-RT-PCR analyses of target gene expression. The whitefly VIGS bioassays were repeated three times with similar results. Two replicates of a separate experiment were set up to collect whiteflies after 1 day for gene expression analyses.

mRNA expression and qPCR analysis

We used q-RT-PCR to measure the expression of target GOIs in treated aphids and whiteflies. Total RNA was extracted from 10 to 20 *M. persicae* using the SV Total RNA Isolation system (Promega) and RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). cDNAs were then diluted 10-fold and used for qPCR reactions. Each qPCR reaction contained 5 μL of the PowerUp™ SYBR™ Green PCR master mix (Applied Biosystems), with 1 μL of each qPCR primer (Table S2) and 1 μL of cDNA. qPCR primers were designed to not overlap with the selected dsRNA in each of the targeted genes to avoid detecting signals from ingested dsRNAs. PCR reactions were initiated with an incubation at 95 °C for 30 s, followed by a 40 cycles of 95 °C for 5 s, 60 °C for 1 min and a melting curve was collected after the reaction. The Ct values were used to quantify and analyse gene expression according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Ubiquitin and/or *EF1a* were used as the internal control genes in each qPCR experiment for HTGs of bacterial and plant origin and *RpL7* was used as an internal control for *Tor* and *CarRP*. Changes in the expression levels of each gene were calculated by comparing the ratio of the $\Delta\Delta C_T$ values of samples from aphids and whiteflies fed on TRV2-GOI plants to those fed on TRV2 empty vector control plants.

Data analyses and statistics

For both insect bioassays and gene expression assays, data were pooled from multiple experiments for statistical analyses. We tested for differences using the univariate analysis of variance with a fixed factor of treatments and a random factor of experiment, followed by a Bonferroni *post hoc* test for multiple comparison corrections using SPSS v.25 (IBM, Armonk, NY). Differences in ladybug survival were tested using the Cox mixed-effect model (Therneau and Grambsch, 2000) followed by a Tukey *post hoc* test using R Studio v 1.3.959 (RStudio Team, 2020). For dsTor and dsCarRP, the aphid reproduction data were tested using ANOVA followed by the Tukey's HSD test. For gene expression data, Mann–Whitney *U*-tests were used to test for differences.

Results

Chromosome-scale assembly of the *M. persicae* genome

As the genome of the USDA-Red strain of *M. persicae* had not been sequenced, we generated a chromosome-scale genome assembly by combining PacBio long-read, Illumina short-read and Hi-C sequencing. The assembled *M. persicae* genome has a total length of 383.0 Mb and consists of 331 contigs with an N50 length of 4.5 Mb. A total of 364.4 Mb (95.1% of the assembly) are clustered into six chromosomes (Figure 1), which is consistent with the commonly observed 2n = 12 karyotype of *M. persicae* (Blackman, 1980). To evaluate the completeness of the *M. persicae* genome assembly, the Illumina sequencing reads were aligned to the assembly, allowing up to three mismatches using BWA-MEM (Li and Durbin, 2009), resulting in a mapping rate of 95.2%, indicating that most of the acquired reads were successfully assembled into the genome. Furthermore, BUSCO (Simao *et al*., 2015) showed that 94.2% of the core eukaryotic genes were at least partially captured by the genome assembly and 92.6% were completely captured. Combining evidence from *ab initio* predictions, protein homology and transcripts derived from Illumina strand-specific RNA-Seq and PacBio Iso-Seq data of the USDA-Red strain, we predicted a total of 27 430 protein-coding genes in the *M. persicae* genome. The assembled genome has been deposited in GenBank under PRJNA888091.

Horizontally transferred genes (HTGs) in *M. persicae* str. USDA-Red

We identified 30 genes that were predicted to be horizontally transferred from bacteria, viruses, fungi or plants into *M. persicae* strains USDA-Red and G006 (Table 1 and Table S1). Genes of bacterial origin include those encoding an uncharacterized

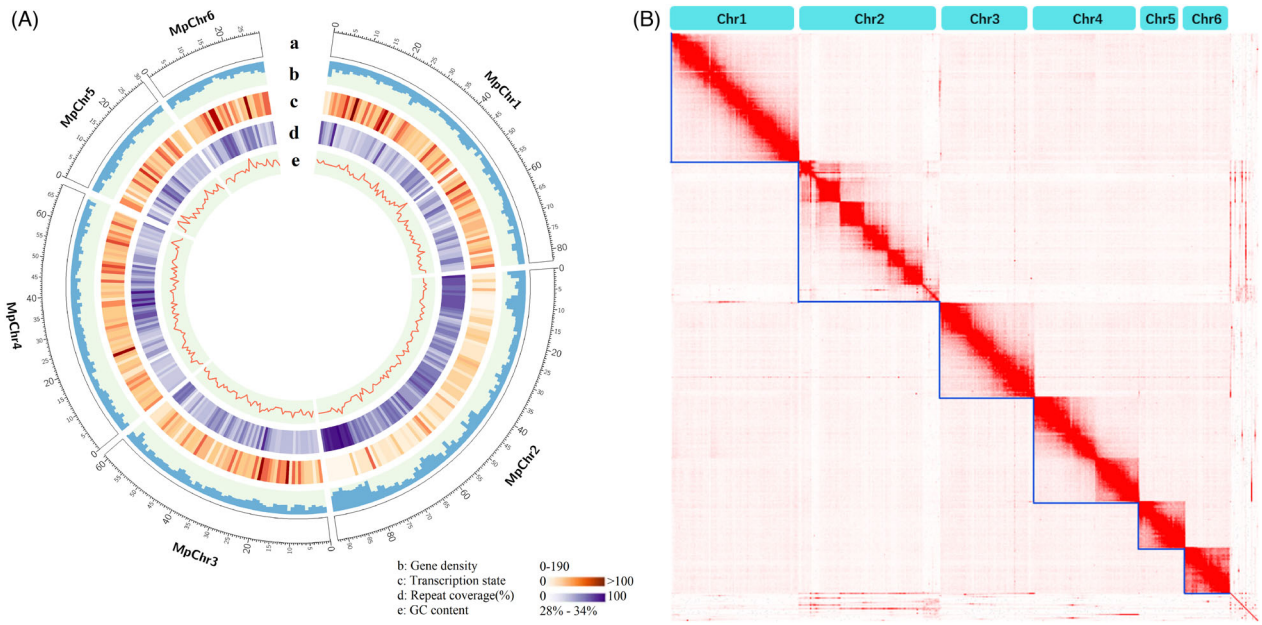

Figure 1 Chromosome-scale genome assembly of a tobacco-adapted red *Myzus persicae* strain. (A) *M. persicae* genome landscape. (a) Ideogram of the 6 *M. persicae* pseudochromosomes at the Mb scale. (b) Gene density is represented as number of genes per Mb. (c) Transcription state. The transcription level was estimated by read counts per million mapped reads in 1-Mb windows. (d) Percentage of coverage of repeat sequences per Mb. (e) Guanine-cytosine (GC) content in 1-Mb windows. (B) Heatmap showing the frequency of HiC contacts along the *M. persicae* genome assembly. Blue lines indicate chromosome-scale scaffolds.

protein with a predicted ATP binding function, an N-acetylmuramoyl-L-alanine amidase (amiD) and a microcin c7 self-immunity protein, which is a homologue of the *A. pisum* murein tetrapeptide carboxypeptidase (ldcA1, ACYPI009109) (Table 1). Genes of viral origin were all identified based on homology search using previously reported HTGs (Parker and Brisson, 2019; Verster *et al*., 2019). For the non-structural protein NS1 superfamily genes, three copies were identified in the *M. persicae* USDA-Red genome, whereas either one or two copies were present in other species, including *A. pisum* in which these HTGs were initially identified (Parker and Brisson, 2019). Genes of fungal origin were mainly predicted to be involved in carotenoid biosynthesis, with nine copies of lycopene cyclase-phytoene synthase and four copies of carotenoid desaturase genes (Table 1). Genes of plant origin are most similar to those from microalgae and encode uncharacterized proteins with predicted ankyrin repeats, that is, *MPE002740*, *MPE008575*, *MPE008576*, *MPE010373*, *MPE013670*, *MPE016359*, *MPE018635*, *MPE018636*, *MPE023973* (Table 1). In addition, one other plant-origin HTG, *MPE011636*, was identified from our *de novo* annotation (Table 1).

Genomic comparisons across different aphid species (*A. pisum*, *A. glycines*, *A. gossypii*, *D. vitifoliae*, *D. noxia*, *E. lanigerum*, *M. cerasi*, *P. nigronervosa*, *R. maidis*, and *R. padi*) showed that most HTGs from *M. persicae* strains USDA-Red and G006 are present in multiple other aphid species (Table 1). For instance, the fungal-origin lycopene cyclase/phytoene synthase gene and all of its duplicates were found in all assessed aphid species. By contrast, one of the plant-origin uncharacterized ankyrin repeat protein genes (*MPE0018635*) and the two additional copies of non-structural protein gene (*MPE014463* and *MPE006690*) were found only in *M. persicae* strains G006 and USDA-Red (Table 1).

Tri-trophic persistence of RNA

To examine the possibility of tri-trophic persistence of RNAi signals, we used two ladybug species, *C. septempunctata* larvae (Figure 2) and *C. montrouzieri* adults (Figure S4). A dsRNA fragment (dsGFP), targeting the GFP gene, which it is not naturally present in plants, aphids or ladybugs, was expressed in *N. benthamiana*. The plant-expressed dsRNA of GFP fragments were consistently detected in both ladybug species that were fed on aphids that fed on dsGFP plants (Figure 2 and Figure S4). As the aphids were removed from the plants for feeding *C. septempunctata* larvae and *C. montrouzieri*, we could rule out direct transfer of the RNA from plants to beetles. Given the observed transfer of RNA from plants to beetles via aphids, we proposed using RNAi to target HTGs that are specific to insect pests, thereby avoiding negative impacts on non-target species, in particular beneficial insects like *C. septempunctata* larvae and *C. montrouzieri*.

Silencing of *M. persicae* bacterial-origin HTGs using VIGS

To evaluate the effects of silencing bacterial-origin HTGs (amidase, ATP binding protein, and *ldcA*) on both plant-feeding aphids and the ladybugs consuming these aphids, we knocked down gene expression in *M. persicae* using TRV VIGS. Expression of all three VIGS-targeted genes was significantly reduced relative to aphids feeding on TRV2-EV, TRV2-*GUS*, and/or TRV2-*GFP* control plants ($P < 0.05$) (Figure 3). For the amidase gene, we observed a significantly lower aphid survival rate as early as 24 h after the initiation of aphid feeding on VIGS plants, compared to both TRV2-*GUS* and EV controls ($P < 0.05$). In the case of the ATP binding protein gene, aphid survival rates decreased significantly on the TRV2-*ATPb* VIGS plants compared to TRV2-*GFP* (only initially at 24 h post feeding) or EV plants (throughout the time

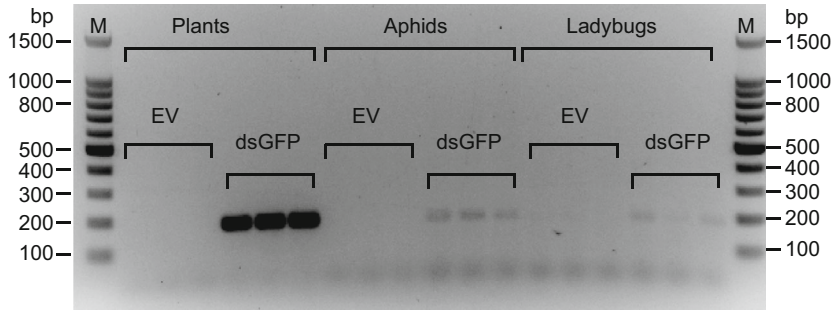

Figure 2 Evidence of tri-trophic persistence of dsRNA. Agarose gel image showing the detection of dsGFP in *Coccinella septempunctata* ladybug larvae after consuming *Myzus persicae* aphids that had fed on *Nicotiana benthamiana* infiltrated with TRV2-*GFP*. The expected amplicon size is 180 bp, which is consistent to the band size shown in the gel. PCR from cDNA samples of the following treatments are shown in each lane: Plants: plants that infiltrated with TRV2 empty vector (EV) or TRV2-*GFP* vector (dsGFP); Aphids: aphids fed on EV and dsGFP VIGS plants; Ladybugs: ladybugs fed on aphids that were fed on EV and dsGFP VIGS plants. Each treatment was performed with three biological replicates (shown as three lanes), and this experiment was repeated twice. M: Promega 100 bp DNA ladder.

period monitored, from 24 to 120 h post-feeding ($P < 0.05$). In the case of *ldcA*, we observed a significantly lower aphid survival rate on TRV2-*ldcA* plants compared to both TRV2-*GFP* and EV controls ($P < 0.05$). We did not observe significant effects on aphid reproduction with any of the three tested genes. Furthermore, there were no significant effects on the survival of *C. septempunctata*, when using either wildtype or *asat2-1* mutant *N. benthamiana* plants for the VIGS experiments.

VIGS-mediated silencing of *M. persicae* plant-origin HTGs

After 120 h of feeding, expression of five plant-origin HTGs, all of which encode uncharacterized proteins with ankyrin repeat domains (Table 1), was significantly down-regulated by VIGS in comparison to *M. persicae* on TRV2-*GFP* and/or EV control plants ($P < 0.05$) (Figure 4). The effects on gene expression varied from a 20% decrease for *MPE018635* to a 50% decrease for *MPE013670*. We observed a significantly lower aphid survival rate on the VIGS plants compared to both TRV2-*GFP* and EV controls ($P < 0.05$) (Figure 4). The decreases in aphid survival ranged from 30% for *MPE018635* to 40% for *MPE010373* after 120 h of aphid feeding (Figure 4). The significant decreases in aphid survival were observed starting from 24 h after initiation of aphid feeding and continuing to decrease over time, with the exception of *MPE023973*, for which significant decreases were observed only starting at 96 h (Figure 4). Similar to the bacterial-origin HTGs, we did not observe significant effects on aphid reproduction with any of these five plant-origin HTGs. Similarly, there were no significant effects on the survival of *C. septempunctata*, when using either wildtype or *asat2-1* mutant *N. benthamiana* plants for the VIGS experiments. For three additional genes of plant origin, *MPE018636*, *MPE002740* and *MPE016359*, we did not find significant effects on aphid survival. In the case of dsMPE018636 VIGS plants, we observed a significant decrease in aphid survival in only one of five VIGS experiments. However, *MPE018636* expression was not significantly decreased in that experiment.

VIGS-mediated silencing of the fungal-origin Tor and CarRP genes

Carotene desaturase (Tor) is required for the production of the red carotenoids that provide the colour of *M. persicae* strain USDA-Red. Carotenoid cyclase–carotenoid synthase (CarRP) catalyses both the committed step for carotenoid biosynthesis and later steps involving the formation of cyclic carotenoids (Hansen and Moran, 2011; Novakova and Moran, 2012). We conducted *Tor* and *CarRP* VIGS experiments and checked their expression at a time point of 7 days after aphids were born on VIGS plants. Compared to aphids on dsGFP control plants, the expression levels of both genes were reduced by approximately 50% ($P < 0.05$) in aphids feeding on TRV2-*Tor* and/or TRV2-*CarRP N. benthamiana* plants (Figure 5A,B). No apparent macroscopic phenotypic changes (particularly loss of red colour) were observed among the groups of aphids. With only about 50% reduction in gene expression in the VIGS experiments, the lack of a visible colour change in the aphids could be due to residual carotenoid accumulation. Nevertheless, progeny production by aphids that were reared on the TRV2-*Tor* or TRV2-*CarRP* VIGS plants over 8 days was significantly reduced compared to the TRV2-*GFP* control (Figure 5C).

VIGS-mediated silencing of *M. persicae* viral-origin *cdtB*

We tested one of the viral-origin HTGs encoding the cytolethal distending toxin B (*cdtB*), which was suggested to be involved in aphid resistance to a predatory wasp (Verster *et al*., 2019). Somewhat unexpectedly, *cdtB* gene expression was consistently observed to be up-regulated in the TRV-*cdtB* plants relative to TRV-*GFP* and EV controls (Figure 6a). This may be indicative of unexplained up-regulation of *cdtB* expression as a compensatory response to the attempted RNA interference. We did not observe any effects on aphid survival in our plant-mediated VIGS experiments, which were conducted in the absence of wasp predation (Figure 6b).

VIGS-mediated silencing of whitefly HTGs

Bemisia tabaci MEAM1 has at least 142 HTGs from bacteria and fungi (Chen *et al*., 2016). As VIGS targets, we chose five *B. tabaci* HTGs, encoding a squalene synthase (*SQS*, *Bta11043*), a diaminopimelate decarboxylase (*DAPDC*, *Bta03593*), an argininosuccinate synthase (*ASS*, *Bta00062*), a cyanate hydratase (*CYN*, *Bta20016*), and an aromatic peroxygenase (*APO*, *Bta04808*), respectively. In parallel, we chose three essential whitefly genes as positive controls, including acetylcholine esterase (*AchE*) and toll-like receptor 7 (*TLR7*) genes, which have been targeted previously by RNAi (Malik *et al*., 2016), and the tubulin-specific chaperon D gene (*TBCD*), which has been targeted by VIGS in aphids (Guo

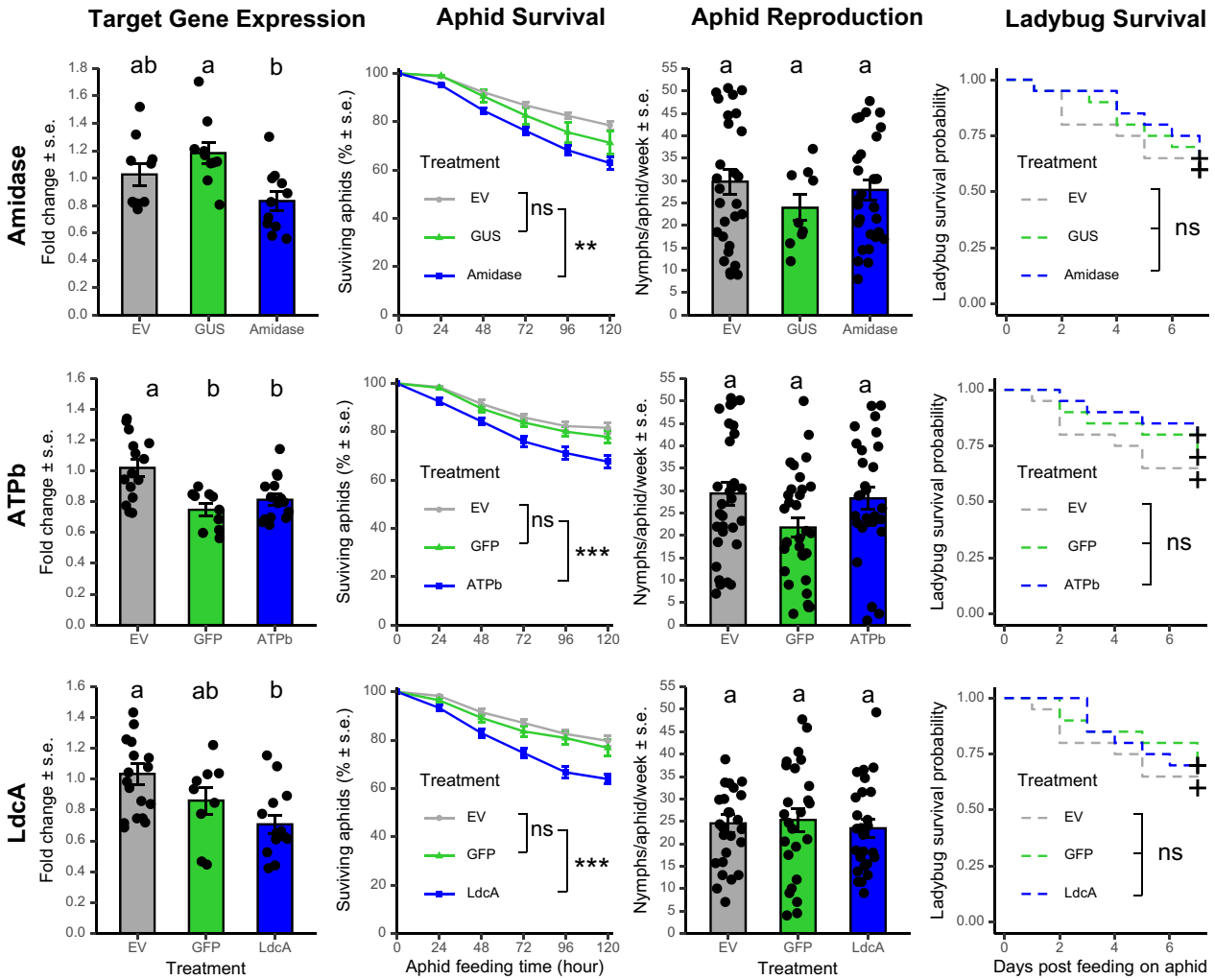

Figure 3 Knockdown of bacterial-origin horizontally transferred genes reduced aphid survival with no effects on ladybug survival. Bioassay experiments were repeated at least 5 times, with 5–8 plants for each construct as biological replicates in each experiment. Dots on top of each bar graph represent individual data points collected from multiple experiments. Letters above the bars indicate significant differences between treatments by ANOVA ($P < 0.05$) with a block effect (experiment). Significant differences in the aphid survival between treatments are indicated for the last time point (*i.e.* 120 h), $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

et al., 2014). We initially conducted VIGS experiments with wildtype *N. benthamiana*, but the low survival rate of *B. tabaci* on these plants made it impossible to reliably assess the effectiveness of gene expression silencing and interpret the results (Figure 7a). Instead, we conducted whitefly VIGS experiments with *asat2-1* mutant *N. benthamiana*, which allows improved whitefly reproduction and survival (Feng *et al.*, 2022). In VIGS experiments with *asat2-1* plants, *AchE* and *SQS* expression was reduced after 1 and 7 days (Figure 7b,c), which resulted in significantly reduced whitefly survival (Figure 7d). Although *TLR7* and *TBCD* gene expression was reduced by VIGS only on day 1 when feeding on *asat2-1* plants (Figure 7b), there was nevertheless a negative effect on whitefly survival over 7 days (Figure 7d). Two possible explanations for this observation are: (i) There was a survivor bias in that we could only measure gene expression levels in surviving whiteflies, and perhaps all whiteflies with efficient *TLR7* and *TBCD* expression silencing were dead after 7 days; or (ii) there may be gene expression compensation at the whole-insect level over time, but not in specific tissues that affect insect survival. Quantitative PCR of fractionated whiteflies would be necessary to determine the time course of *TLR7* and *TBCD* expression silencing and whether VIGS primarily affects gene expression in specific tissue types.

Discussion

Identification of HTGs in insect pests

With the increasing number of available genome sequences, HTGs have been identified in all branches of life, including a broad range of agricultural insect pests such as aphids and whiteflies (Husnik and McCutcheon, 2018; Soucy *et al.*, 2015). Many of those HTGs have been shown to be expressed and functional after the incorporation into the recipient genomes (Husnik and McCutcheon, 2018; Wybouw *et al.*, 2016), providing the recipient species benefits such as nutrition, protection and adaptation to environmental stresses (Husnik and McCutcheon, 2018; Soucy *et al.*, 2015). HTGs can help the recipient insects to digest plant cell wall components that would otherwise

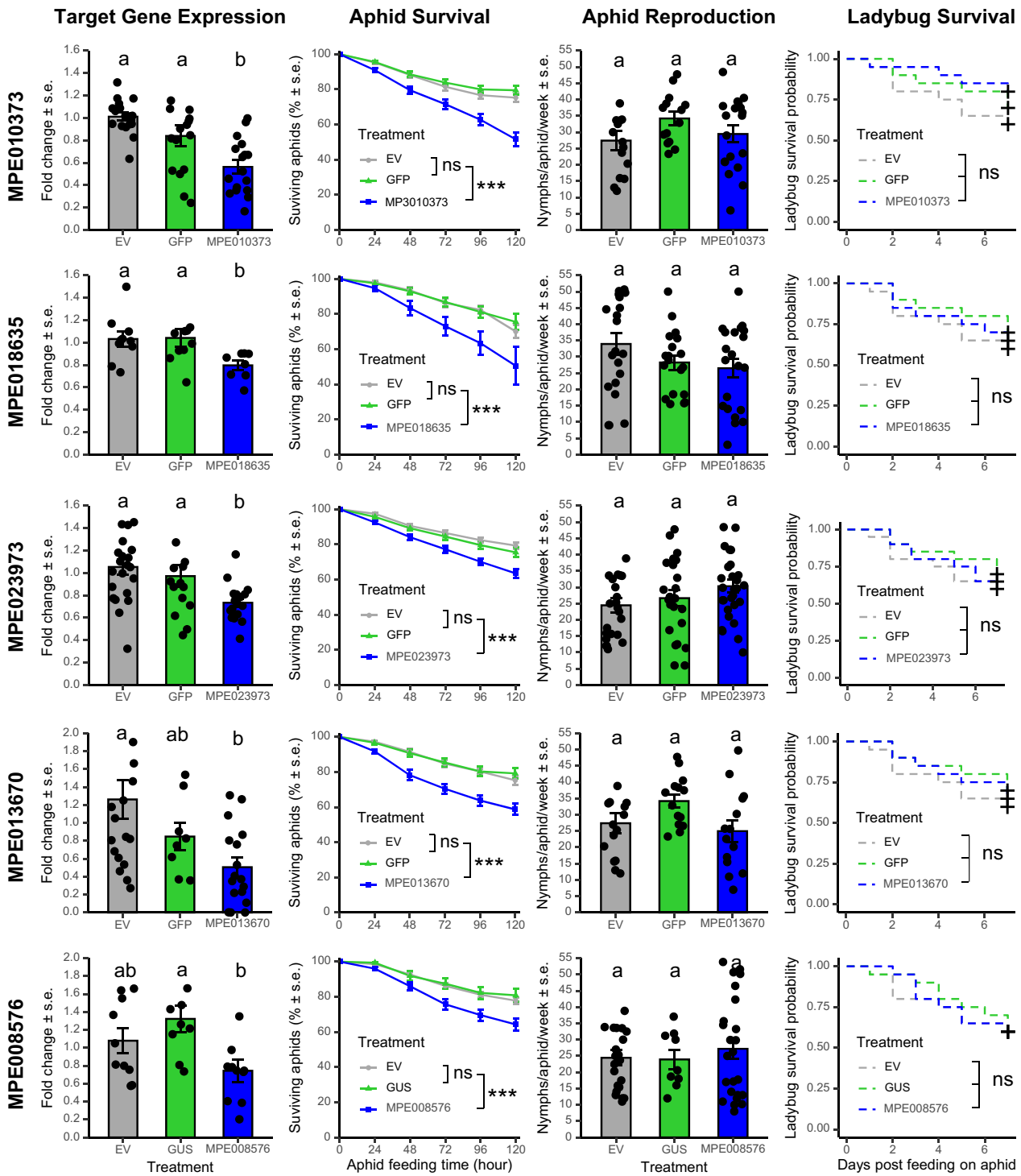

Figure 4 Knockdown plant-origin horizontally transferred genes reduced aphid survival with no effects on ladybug survival. Bioassay experiments were repeated at least 5 times, with 5–8 plants for each construct as biological replicates in each experiment. Dots on top of each bar graph represent individual data points collected from multiple experiments. Letters above the bars indicate significant differences between treatments by ANOVA ($P < 0.05$) with a block effect (experiment). Significant differences of the aphid survival between treatments are indicated for the last time point (*i.e.* 120 h), $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

be non-digestible (Kirsch *et al.*, 2014; Pauchet *et al.*, 2014; Pauchet and Heckel, 2013; Zhao *et al.*, 2014). For example, the pectin-degrading polygalacturonases found in herbivorous beetles are ancient HTGs from fungi that help beetles to digest cell wall components such as pectin, cellulose and hemicellulose (Kirsch *et al.*, 2014). HTGs can also help recipient insects detoxify plant defensive metabolites (Daimon *et al.*, 2008). For instance, mulberry latex, such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-

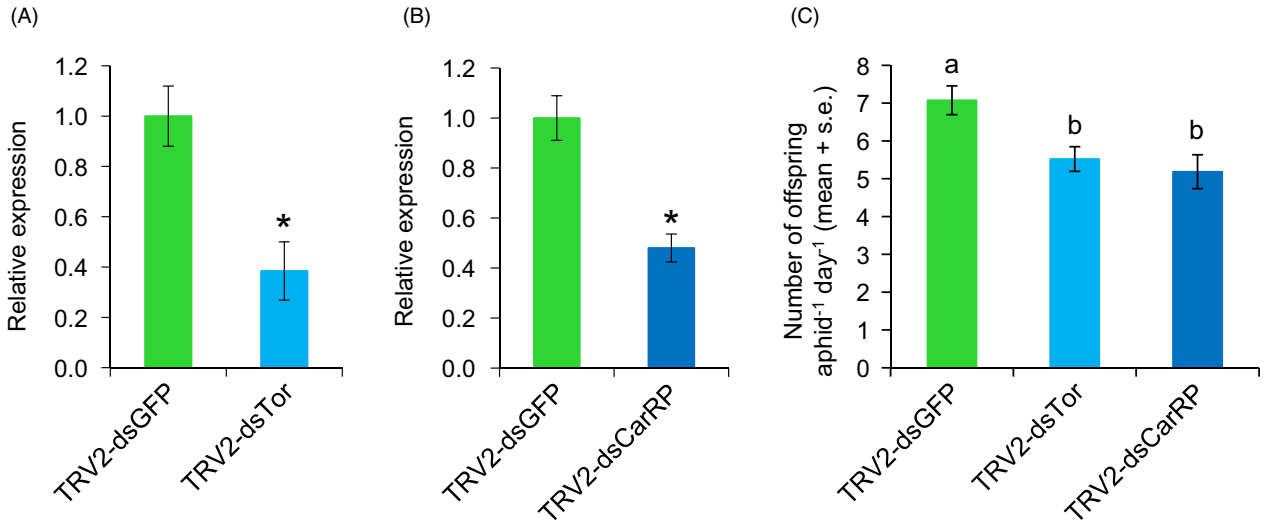

Figure 5 Silencing of *Tor* and *CarRP* genes reduces aphid reproduction. (A) *Tor* gene and (B) *CarRP* gene expression after 7 days exposure of *Myzus persicae* to TRV-VIGS in *Nicotiana benthamiana*. Data shown are the mean ± standard error of 5 or 6 biological replicates, with three aphids pooled for each replicate. The *Y*-axis has arbitrary units, with gene expression in the TRV-GFP strain normalized as 1. **P* < 0.05, Mann–Whitney *U*-test relative to the control. (C) Reproductive output per aphid over 8 days of *M. persicae* reared on *N. benthamiana* plants with the two RNAi constructs. ANOVA results are shown, with different letters referring to treatments with significantly different expression by Tukey's HSD test.

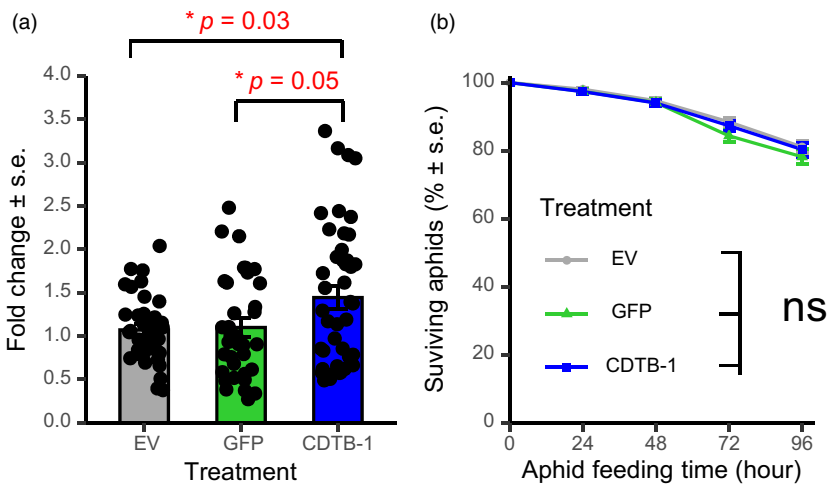

Figure 6 VIGS up-regulated *cdtB* (virus-origin HTG) gene expression (a), but did not affect aphid survival (b). Bioassays for were replicated 4 times with similar results. Each experiments included 5–8 plants for each construct as biological replicates. Significant differences between groups were tested using ANOVA with a block effect (experiment), followed by multiple comparisons using the Bonferroni method. **P* < 0.05, ns, not significant.

AB1) and 1-deoxynojirimycin (DNJ), are highly toxic to non-mulberry specialist insects, but present no harm to the mulberry specialist silkworm, *Bombyx mori*. A beta-fructofuranosidase gene that was horizontally transferred from bacteria is highly expressed in the *B. mori* gut and helps in digesting plant-defensives alkaloidal sugars (Daimon *et al.*, 2008). In sap-feeding insects, HTGs from bacteria have been found to complement the function of their obligate endosymbiont bacteria in the biosynthesis of essential nutrients, such as essential amino acids and vitamins (Husnik *et al.*, 2013; Luan *et al.*, 2015; Sloan *et al.*, 2014). Some HTGs have been proposed to be involved in protecting recipient insects from pathogen or predators (Verster *et al.*, 2019). For example, the *cdtB* gene of bacteriophage origin in the vinegar flies and aphids may function in defending these insects against natural enemies (Verster *et al.*, 2019). As these nutritional and protective functions shown above are critical to insect development, survival and reproduction, and most of them are species- or genus-specific, those HTGs in insect pests should be further explored as efficient and specific targets for the control of pest species.

HTGs in aphids and their potential for aphid control

Many HTGs were described in aphid genomes as aphid genomic resources started becoming available (Ding *et al.*, 2020; International Aphid Genomics Consortium, 2010; Moran and Jarvik, 2010; Nikoh *et al.*, 2010; Nikoh and Nakabachi, 2009; Parker and Brisson, 2019; Verster *et al.*, 2019). Notably, aphids acquired carotenoid biosynthesis genes, which determine body colour through horizonal transfer from fungi. The absence of visible changes in aphid body colour after *Tor* and *CarRP* transcriptional silencing indicates that the observed 50% reduction in gene expression (Figure 5) does not cause a complete loss of carotenoid production. Additionally, aphid body colour is influenced by the environment, including abiotic (*e.g.* temperature) and biotic environmental (*e.g.* predation and endosymbionts) factors (Tsuchida, 2016). Thus, the colour changes from

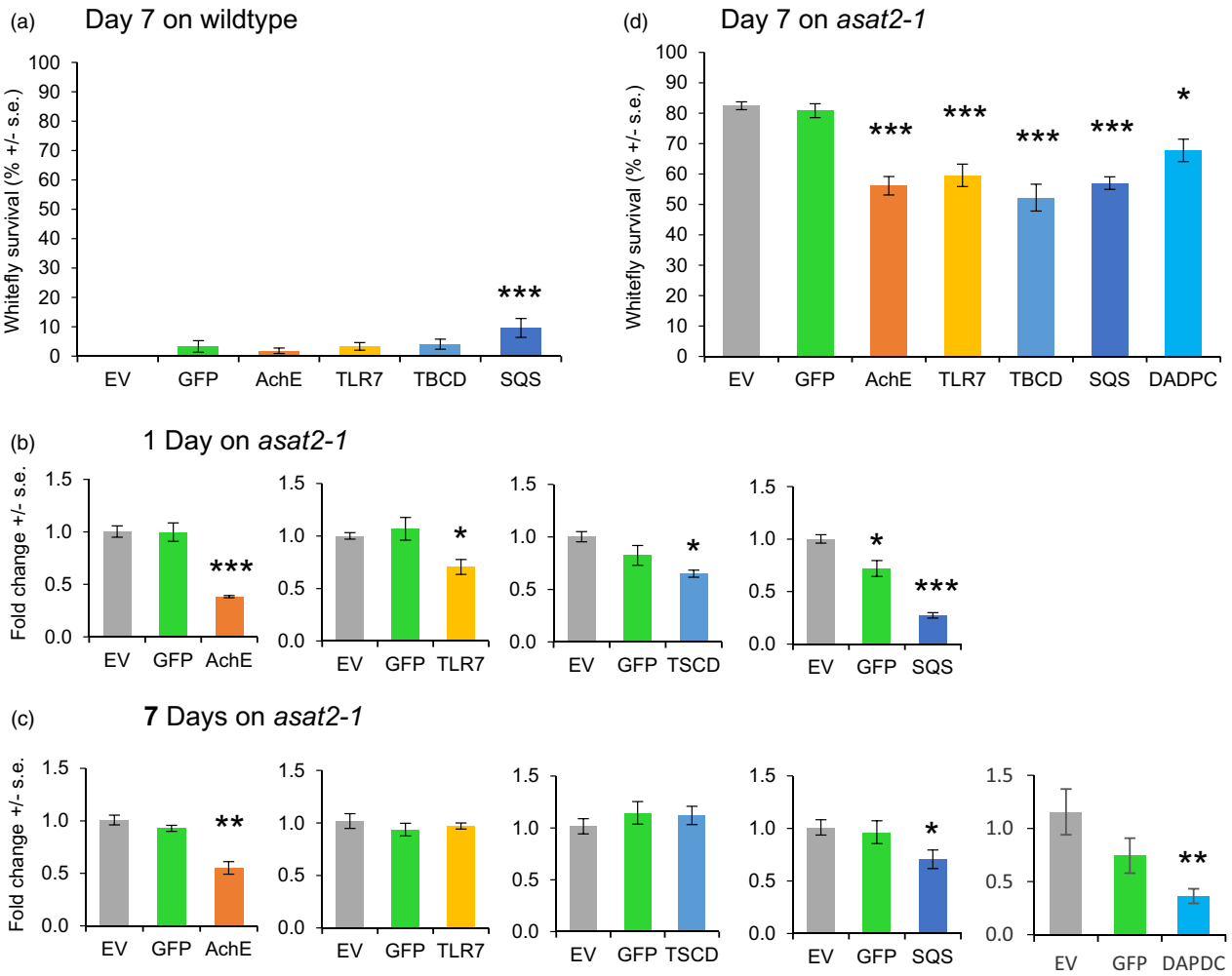

Figure 7 Virus induced gene silencing (VIGS) of whitefly genes using tobacco rattle virus (TRV). *Bemisia tabaci* (whiteflies) were placed on wildtype (a) and *asat2-1* (b–d) *Nicotiana benthamiana* plants infected with TRV expressing VIGS constructs, and gene expression was measured after one (b) and 7 days (c). In each experiment, the empty virus vector (EV) and *GFP*-carrying virus were used as controls. Gene expression was normalized to 1 for EV control plants. Whitefly survival was assessed on TRV-infected plants after 7 days of feeding on (a) wildtype plants or (d) *asat2-1* plants. GFP/AchE/SQS/TLR7/TBCD/DADPC: VIGS plants with TRV expressing RNA constructs targeting GFP, *AchE*, *SQS*, *TLR7*, *TBCD*, and *DADPC*, respectively; AchE: acetylcholinesterase, SQS: squalene synthase, TLR7: toll-like receptor 7, TBCD: tubulin-specific chaperon D, DADPC: diaminopimelate decarboxylase. The survival and the 7-day qPCR experiments were repeated three times. The 1-day qPCR experiments were repeaetd twice. Significant differences were determined using one-way ANOVA with a fixed factor of treatments and a block effect of experiment, followed by a Dunnett's *post hoc* test for comparing VIGS constructs to the empty vector (EV) control. Mean ± SE of $n = 3$ (a), $n = 9$ (b), and $n = 27$ (c, d). $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

the knockdown of *Tor* and *CarRP* could be concealed by other factors that were not a focus in our RNAi experiments.

Aphid body colour further influences the ecological interactions between aphids and their predators, such as wasps (Moran and Jarvik, 2010). Due to reduced survival and/or reproduction, these predator interactions can be costly for aphids, (Nelson, 2007). Consistent with previous research, our plant-mediated VIGS experiments showed negative effects on aphid performance, in this case progeny production. Although Tor and CarRP are believed to be dispensable during aphid development, the changes in *Tor* and *CarRP* gene expression could be perceived by the aphids as a metabolic disturbance, thereby incurring the observed reproductive costs.

We used plant-mediated VIGS to determine that three HTGs of bacteria origin influence *M. persicae* survival. Transiently knocking down *ATPb*, *amiD* and *LdcA* expression significantly reduced aphid survival (Figure 3). This result for *amiD* and *LdcA* in *M. persicae* is consistent with an investigation of these two genes in *A. pisum*, in which knocking down *amiD* and *ldcA* by dsRNAs in artificial diet reduced aphid growth (Chung *et al.*, 2018). In the case of *ATPb*, the VIGS construct caused no significant expression knockdown relative to the GFP control but there was nevertheless a decrease in aphid survival. There are several possible explanations for this: (i) Expression silencing is tissue-localized and we do not observe a significant effect when analysing whole insects, (ii) Transient silencing and/or compensatory gene expression may be occurring, which can nevertheless have longer-term negative effects, (iii) The constructs may be targeting multiple genes, with significant effects not being observed when analysing an individual gene, or (iv) the GFP construct may have unexpected negative effects on the expression of some aphid genes.

All of the plant-derived HTGs that we identified in *M. persicae* encode uncharacterized proteins with ankyrin repeat domains. The ankyrin repeat domains consists of ~33 amino acid tandem motifs, which have been well documented in protein–protein interaction studies (Sedgwick and Smerdon, 1999). Since the first report of ankyrin repeat proteins in yeast (*Saccharomyces cerevisiae*), fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) (Breeden and Nasmyth, 1987), ankyrin repeat proteins have been identified in numerous organisms, ranging from viruses and bacteria to humans (Sedgwick and Smerdon, 1999). Bacterial ankyrin repeat proteins are mainly found in species that are obligately or facultatively associated with eukaryotic hosts (Siozios *et al.*, 2013). Different ankyrin repeat proteins have been identified that regulate host-pathogen and host-symbiont interactions (Kumagai *et al.*, 2007; Nguyen *et al.*, 2014; Pan *et al.*, 2008). Aphids are well known for their interactions with associated obligate and facultative symbionts, as well as being vectors for many plant viruses, and expression of horizontally acquired ankyrin repeat proteins was detected previously in aphid bacteriocyte and/or gut transcriptomes (Duncan *et al.*, 2016; Feng *et al.*, 2018). The functions of these proteins in mediating interactions between aphids and their symbionts will require further investigation. Nevertheless, our results show that these horizontally transferred ankyrin repeat proteins are important for aphid survival (Figure 4).

Selection of non-specific controls for RNAi experiments

In several previous insect RNAi experiments, researchers chose a fragment of GFP as the negative control (Dong *et al.*, 2022; Jain *et al.*, 2022; Pitino *et al.*, 2011; Tariq *et al.*, 2019; Ye *et al.*, 2019; Yoon *et al.*, 2020), presumably because this sequence is not present in their target insects and plants. However, our experiments show that a GFP fragment may lead to non-specific changes in the expression of some targeted genes. For instance, we observed that our target gene expression was similarly repressed by the negative control and TRV-*GFP* (*e.g.* the results for *ATPb* in Figure 3). This was not the case when we used TRV-*GUS* as the non-specific negative control, as in the case of the amidase gene in Figure 3 and *MPE008576* in Figure 4. Therefore, gene fragments used as negative controls for RNAi experiments will need to be cautiously selected and tested for their effects on the specific target genes of interest.

HTGs in whiteflies

In the whitefly genome, 64 genes were predicted to be horizontally transferred from bacteria and 78 genes were predicted to be horizontally transferred from fungi (Chen *et al.*, 2016). Here, we have targeted five whitefly HTGs, which were chosen based on their predicted metabolic functions, using plant-mediated VIGS and demonstrated that knocking down HTGs can significantly reduce whitefly survivorship (Figure 7). These results suggest that HTGs could be targets for the purpose of whitefly control.

Given that horizontal transfer of functionally expressed microbial genes into insect germlines is rare on an evolutionary timescale, there is likely a selective advantage to having these genes expressed in whiteflies. This was confirmed by the observation that VIGS of both *SQS* and *DAPDC* reduced whitefly survival relative to control plants (Figure 7d). Transient expression knockdown of *SQS* and *DAPDC*, as well as other horizontally transferred genes, will enable future research to study the functions of these genes in whitefly metabolism. Due to their importance for whitefly survival, as well their absence in beneficial insects such as ladybugs and lacewings, horizontally transferred genes also are attractive targets for controlling whiteflies by RNA interference.

Avoiding negative effects in beneficial species

Despite the reduction in aphid reproduction and/or survival when HTGs were targeted by RNAi, we did not observe any negative effects on the survival of *C. septempunctata* larvae that were feeding on these aphids. This is consistent with the lack of target sequence homology in the beetles, which do not have the HTGs. However, it is also possible that there are indirect negative effects on predatory insects feeding on aphids that were targeted with RNAi constructs. For instance, silencing cathepsin L gene expression in *M. persicae* reduced their protein content and made the less suitable as prey for *C. septempunctata* (Rauf *et al.*, 2019).

Conclusions

The well-established *N. benthamiana* VIGS system, which allows rapid cloning of target-specific constructs (Liu *et al.*, 2002), will allow screening of other horizontally transferred genes to identify those that are most suitable for hemipteran pest control. Such assays will be facilitated by the use of *asat2* mutant *N. benthamiana*, which improves aphid growth and allows *B. tabaci* survival (Feng *et al.*, 2022). For the majority of tested *M. persicae* and *B. tabaci* HTGs, knocking down expression by means of plant-mediated VIGS decreased survival and/or reproduction. Importantly, no negative effects were observed on *C. septempunctata*. Together, our results indicate that HTGs have potential as efficient and biologically safe targets for hemipteran pest control by plant-mediated RNA interference.

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Conflict of interest

The authors declare that they have no conflicts of interest related to this research.

Author contributions

GJ, HF and VT designed experiments. HF, VT, SS, SH and FA: cloned genes, constructed virus vectors, measured gene expression, and conducted insect bioassays. HF, WC and GJ analysed data. HF and GJ wrote the manuscript. ZF, TU and GJ obtained funding and provided other essential resources. All authors approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Examples of planta expression of dsRNAs that targeting horizontally transferred genes.

Figure S2 Carotenoid desaturase nucleotide sequence alignment.

Figure S3 Carotenoid cyclase synthase nucleotide alignment.

Figure S4 Tri-trophic persistence of dsRNA using a dsRNA targeting GFP.

Table S1 Homologous annotation of HTGs across aphid species.

Table S2 Primers used for aphid and whitefly VIGS.