Research Article

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RNAi-mediated silencing of Mediterranean fruit fly (*Ceratitis capitata*) endogenous genes using orally-supplied double-stranded RNAs produced in *Escherichia coli*

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Abstract

BACKGROUND: The Mediterranean fruit fly (medfly), *Ceratitis capitata* Wiedemann, is a major pest affecting fruit and vegetable production worldwide, whose control is mainly based on insecticides. Double-stranded RNA (dsRNA) able to down-regulate endogenous genes, thus affecting essential vital functions via RNA interference (RNAi) in pests and pathogens, is envisioned as a more specific and environmentally-friendly alternative to traditional insecticides. However, this strategy has not been explored in medfly yet.

RESULTS: Here, we screened seven candidate target genes by injecting in adult medflies gene-specific dsRNA hairpins transcribed *in vitro*. Several genes were significantly down-regulated, resulting in increased insect mortality compared to flies treated with a control dsRNA targeting the green fluorescent protein (GFP) complementary DNA (cDNA). Three of the dsRNAs, homologous to the beta subunit of adenosine triphosphate (ATP) synthase (*ATPsynbeta*), a vacuolar ATPase (*V-ATPase*), and the ribosomal protein S13 (*RPS13*), were able to halve the probability of survival in only 48 h after injection. We then produced new versions of these three dsRNAs and that of the GFP control as circular molecules in *Escherichia coli* using a two-self-splicing-intron-based expression system and tested them as orally-delivered insecticidal compounds against medfly adults. We observed a significant down-regulation of *V-ATPase* and *RPS13* messenger RNAs (mRNAs) (approximately 30% and 90%, respectively) compared with the control medflies after 3 days of treatment. No significant mortality was recorded in medflies, but egg laying and hatching reduction was achieved by silencing *V-ATPase* and *RPS13*.

CONCLUSION: In sum, we report the potential of dsRNA molecules as oral insecticide in medfly.

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

Keywords: medfly; RNA interference; double-stranded RNA; self-splicing intron; vacuolar ATPase; ribosomal protein S13

1 INTRODUCTION

The Mediterranean fruit fly (medfly) *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is a devastating agricultural pest with a wide range of unrelated host species, including hundreds of fruits and vegetables for human consumption, and an amazing capacity to disperse, adapt and invade new ecological niches in tropical and temperate areas.[1] The medfly damage is associated with egg laying inside ripe fruits and vegetables and the subsequent larval feeding, causing annual losses in the order of billions of dollars, which result from reduced production, cost of control measures, and lost markets.[2] Strict quarantine regulations have been established to limit the spread between regions.[3] Medfly control in crops has been traditionally based on chemical pesticides.[4] However, some species-specific and eco-friendly alternatives are also being used or developed as part of integrated pest management (IPM) programs.[5–7]

The induction of RNA interference (RNAi) in pests and pathogens is currently considered a promising strategy for more

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specific and environmentally-friendly crop protection. RNAi is a highly conserved mechanism in eukaryotic organisms for gene regulation and protection against harmful exogenous and endogenous genetic elements. It is efficiently triggered by different types of RNAs, including double-stranded RNA (dsRNA), highly homologous to the regulated genetic factors. Intake of gene-specific dsRNAs in pests and pathogens may induce the down-regulation of these genes, potentially affecting essential physiological processes in those organisms. In some, such as nematodes, insects, and other arthropods, cuticle and midgut cells can intake exogenously supplied dsRNA molecules present in the environment and ingested food.[8–12] Once in the cell, the dsRNAs are diced by the RNase-III-type enzyme Dicer-2 to generate small interfering RNAs (siRNAs) that guide the slice of complementary RNAs through the activity of the RNA-induced silencing complex (RISC) that contains the endoribonuclease Argonaute (Ago)-2, a process that likely results in substantial degradation of the endogenous RNA. In arthropods, the RNAi effect may spread from the initial cells to the rest of the organism producing a systemic effect,[13–15] although unlike in nematodes, fungi, or plants, the RNAi response is not amplified by endogenous RNA-dependent RNA polymerases (RdRp).[16–18]

Several lines of evidence suggest the potential susceptibility of medfly to RNAi-mediated control. Firstly, dsRNA-mediated silencing has been extensively reported in species of Tephritidae through diverse delivery systems, developmental stages, and target genes.[19] Secondly, the transcriptomic analysis of *Ceratitis capitata* revealed that key genes involved in the RNAi pathways, such as Dicer and Ago, are actively expressed.[20,21] Thirdly, the functionality of the RNAi pathway induced by exogenous RNAs has been experimentally demonstrated as sterile insects of both sexes have been obtained after knocking down sex determination genes by injecting specific dsRNA directly into embryos.[22–25] However, due to the sheltered oviposition, larval development inside the fruit and the pupation in the soil, in-field control of species from Tephritidae relies almost exclusively on adults. These strategies may be likely based on supplementing food lures in artificial baits with target-specific dsRNAs.[26] Thus, two essential aspects for using RNAi as a pesticide in *Ceratitis capitata* remain undetermined: the susceptibility of adult flies to RNAi-mediated post-transcriptional knockdown and whether or not orally supplied dsRNAs can achieve RNAi. This is especially relevant as RNAi efficiency is highly variable not only between insect orders and even close-related species but also between juvenile and adult stages[8,27–30] – larvae are usually more sensitive when subjected to equal dsRNA treatments – and delivery methods, with several reports of effective silencing by injection coupled with complete oral insensitivity.[31–33] The variation is related to insect intrinsic factors, such as nuclease-mediated dsRNA degradation in saliva, gut, and hemolymph,[33–38] variable cellular uptake and intercellular transport activities, and even differences in expression of RNAi machinery.[39–41]

An additional challenge for implementing RNAi strategies is still the cost-effective production and formulation of target-specific dsRNAs. Recent strategies involve the production of dsRNAs in microbiological factories and its encapsulation in nanoparticles to overcome the low stability of RNA against biotic and abiotic stresses and to improve their absorption.[42–45] In this regard, we recently developed a strategy to overproduce dsRNAs in *Escherichia coli* by emulating the structural characteristics of plant pathogenic RNAs, namely viroids,[46] whose high compaction and circular structure make them resistant to degradation and prone to bacterial accumulation.[47] The system also takes advantage of the autocatalytic properties of group-I introns,[48] which allows plasmid stabilization and circularization of dsRNA molecules without incorporating viroid-derived sequences in the resulting molecules.

Here, we used this dsRNA production system to analyze the effectiveness of RNAi control strategies in medfly. We screened *Snf7*, a vacuolar sorting protein; *RyR*, ryanodine receptor; *Nep4*, a metallopeptidase; *IAP2*, an inhibitor of apoptosis; *ATPsynbeta*, a component of the channel that produces adenosine triphosphate (ATP) using the energy of proton gradient; *V-ATPase*, a vacuolar proton pump that produces a proton gradient with ATP consumption; and *RPS13*, encoding a ribosomal protein that is part of the 40S subunit. Screening of seven target genes for *Ceratitis capitata* adults showed high susceptibility to three microinjected *in vitro*-produced hairpins homologous to *ATPsynbeta*, *V-ATPase* and *RPS13*. The latter two genes were also significantly and specifically repressed when evaluated via orally delivered circular dsRNA produced in *E. coli*. As a result, the fecundity and fertility of the treated females were significantly reduced. Thus, we have established a starting point for a novel medfly control strategy based on orally-delivered dsRNAs that, combined with other IPM strategies, may provide more sustainable crop protection against this devastating pest.

2 MATERIALS AND METHODS

2.1 Insect rearing and maintenance

Medfly adults came from a laboratory colony maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada (Valencia, Spain). The medfly population maintenance and diet followed the methodology previously described.[49] The environmental chamber conditions were 25 ± 2 °C, 60–70% relative humidity with a photoperiod of 14 h:10 h (light/dark). This colony is annually reinforced with regular additions of flies emerging from pupae collected on naturally infested fruit in the field. For each experiment, to obtain a cohort of adults, approximately 1000 24-h-old pupae were collected and kept in transparent polymethylmethacrylate cages (20 cm × 20 cm × 20 cm) until adult emergence. Unfed 24-h-old adults were used in all experiments.

2.2 Construction of plasmids to express dsRNA against *Ceratitis capitata*

We constructed a series of plasmids for the *in vitro* transcription of dsRNAs homologous to seven *Ceratitis capitata* genes and the enhanced green fluorescent protein (GFP) as a negative control. Total RNA from *Ceratitis capitata* adult flies, purified using silica-gel columns (Zymo Research, Irvine, CA, USA), was used as a template in reverse transcription (RT) reactions (RevertAid reverse transcriptase; Thermo Scientific, Waltham, MA, USA) using an oligo(dT) primer. The resulting complementary DNAs (cDNAs) were used in polymerase chain reactions (PCRs) with specific primers (Supporting Information Table S1) and the Phusion high-fidelity DNA polymerase (Thermo Scientific) to amplify each target sequence (400–500 bp) in sense and antisense orientations. A region of similar size of the GFP cDNA in both orientations was also obtained. In addition, we amplified the cDNA corresponding to the *Tetrahymena thermophila* 26S ribosomal RNA (rRNA) type-I intron, including 10 nt of the flanking exons (positions 43 to 475 of GenBank V01416.1). Each inverted repeat and the autocatalytic intron were inserted into the *Bpi*I-digested plasmid pMT7 (Supporting Information Dataset S1) by the Gibson assembly reaction,[50] using the NEBuilder HiFi assembly master

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mix (New England Biolabs, Ipswich, MA, USA). *Escherichia coli* DH5α was electroprated with the assembled products. Recombinant resistant clones were selected in plates of lysogeny broth (LB) medium containing ampicillin [10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (NaCl), 1.5% agar, and 50 μg/mL ampicillin], and grown in liquid LB media (as earlier minus 1.5% agar), at 37 °C with vigorous shaking (225 rpm). Plasmids were purified (NucleoSpin Plasmid; Macherey-Nagel, Dueren, Germany), and sequences were experimentally confirmed by gel electrophoresis and sequencing (3130xl Genetic Analyzer; Life Technologies). We additionally built plasmids to express circular dsRNAs in *E. coli*. For this purpose, we sequentially digested the plasmid pLPIE-LacZ (Dataset S2) with *Esp*3I and *Bpi*I and added in two consecutive Gibson assembly reactions the target gene or the GFP sequences in each orientation. We also constructed plasmids for expressing circular RNA molecules under the control of the inducible T7 promoter in bacteria with the same methodology. The complete sequences of all resulting plasmids are detailed in Dataset S2.

2.3 *In vitro* production of dsRNA

Based on our previous work on the production of dsRNA in *E. coli*,[47] we constructed a series of plasmids in which a single bacteriophage T7 promoter controls the transcription of an inverted repeat of each target gene. In these plasmids, the inverted repeats were separated by a cDNA corresponding to the self-splicing type-I *T. thermophila* 26S rRNA intron, plus 10 nt of both native flanking exons. This intron was observed to self-splice efficiently out of the primary transcript during the *in vitro* transcription reaction. While the intron cDNA separates the long-inverted repeats in the plasmids to increase stability, efficient self-splicing produces a hairpin RNA composed of two perfect complementary strands separated by a 20 nt loop derived from exon fragments (Fig. 1(A)).

For the *in vitro* synthesis of dsRNA hairpin molecules, pMT7 derivatives were linearized with *Bsa*I (BsaI-HFv2; New England Biolabs), purified in silica columns, and used as templates for *in vitro* transcription with bacteriophage T7 RNA polymerase (Roche Life Science). The quality and concentration of the produced dsRNA were electrophoretically assessed by non-denaturing polyacrylamide gel electrophoresis (PAGE) in 5% polyacrylamide gels (37.5:1 acrylamide/*N*,*N*′-methylenebisacrylamide) in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) for 1.5 h at 75 mA. Gels were stained by shaking in 200 mL of 1 μg/mL ethidium bromide for 15 min and, after washing three times with water, documented using an ultraviolet (UV) transilluminator (UVIdoc-HD2/20MX; UVITEC). Total RNAs from the *in vitro* transcription reactions were extracted by mixing with one volume of a 1:1 (*v/v*) mix of phenol (saturated with water and equilibrated at pH 8.0 with Tris–HCl, pH 8.0) and chloroform, and then re-extracted with chloroform. The aqueous phases were then precipitated overnight at −20 °C with 0.1 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of 96% ethanol. After centrifugation (15 min, 13 000 rpm at 4 °C), the RNA pellet was washed with cold 70% ethanol, dried, and resuspended in 20 μL of water. Serial dilutions of a purified and spectrophotometrically quantified *E. coli* 5S rRNA were used as concentration standards.

2.4 Production of circular dsRNA in *E. coli*

Circular dsRNAs were synthesized in *E. coli* under the control of the constitutive lipoprotein (lpp) promoter using the double-intron system (Fig. 1(B)). Production was first performed at a low scale in 5 mL culture media for 24 h and later scaled-up in larger flasks with 250 mL culture media. An RNase III-deficient *E. coli* BL21(DE3) derivative [BL21(DE3)Δrnc], generated according to previously described,[51] was electroporated (Eporator; Eppendorf, Hamburg, Germany) with pLPIE-derivatives and transformed clones were selected in plates with ampicillin. For low-scale production of circular dsRNA, individual recombinant *E. coli* clones were grown in 50-mL tubes containing 5 mL of Terrific Broth (TB) medium [12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol, 0.17 M potassium dihydrogen phosphate (KH_2PO_4), and 0.72 M dipotassium phosphate (K_2HPO_4)] with ampicillin (50 μg/mL), at 37 °C with vigorous shaking (225 rpm). In some cases, induction of dsRNA expression under the T7 promoter was carried out by growing *E. coli* cells to an optical density at 600 nm (OD_{600}) of 0.6 and then adding isopropyl thiogalactopyranoside (IPTG) to 0.4 mM. At the required time, bacteria in 2 mL of the cultures were sedimented by centrifugation, and resuspended in 50 μL of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). 50 μL of a 1:1 (*v/v*) phenol/chloroform was added and vigorously mixed by vortexing to break cells. After centrifugation, the aqueous phase containing total bacterial RNA was recovered and frozen. Aliquots of the RNA preparations were analyzed by PAGE as previously explained. For large-scale production of circular dsRNA, 2-L baffled Erlenmeyer flasks with 250 mL of TB buffer containing ampicillin were used. After 24 h culture, cells were sedimented (8000 rpm for 15 min) and resuspended in 50 mL of water. After the second sedimentation under the same conditions, cells were resuspended in 10 mL buffer 50 mM Tris–HCl, pH 6.5, 0.15 M NaCl, and 0.2 mM EDTA. The cells were mixed with one volume of a 1:1 (*v/v*) phenol/chloroform and broken by vigorous vortexing. Total bacterial RNA contained in the aqueous phase was recovered after centrifugation (8000 rpm for 15 min) and re-extracted with one volume of chloroform. The RNA was then precipitated, adding 0.1 volume of 3 M sodium acetate pH 5.5 and 2.5 volumes of ethanol, and resuspended in water. The RNA was re-precipitated with one volume of isopropanol. Aliquots of the RNA preparations were analyzed by electrophoresis, as previously explained.

2.5 Injection of dsRNAs and feeding assays in *Ceratitis capitata* adults

For the study of the effect of dsRNA by injection, in each knockdown experiment medfly adults less than 24-h-old were cold anesthetized and injected intrathoracically with 0.5 μg of dsRNA using a Drummond Nanoject II microinjector and a micromanipulator (Drummond Scientific Company, Broomall, PA, USA) as previously described.[52] To manipulate adults, groups of about ten individuals, either males or females, were placed in Petri dishes of 140 mm in diameter and were cold anesthetized by placing them 1 min in a −15 °C freezer. Once removed from the freezer and until use, they were kept anesthetized by placing the Petri dish on ice. This process was repeated until the necessary number of repetitions was achieved. Each dsRNA was injected into 30 males and 30 females. Both males and females were grouped into three biological replicates of ten individuals each and placed in a plastic cage (15 cm × 7 cm × 10 cm), with a hermetic lid having a mesh area of 12 cm × 8 cm for ventilation.[4] Medfly adults were fed with sugar placed on a 1.5-mL Eppendorf tube lid fixed to the bottom of the cage with plasticine. In all experiments, the mortality of medfly adults was evaluated daily until the 30th day after adults' introduction. Additionally, 72 h after the adult injection, three live individuals were removed from each cage, placed in 2-mL Eppendorf tubes and immediately frozen by immersion

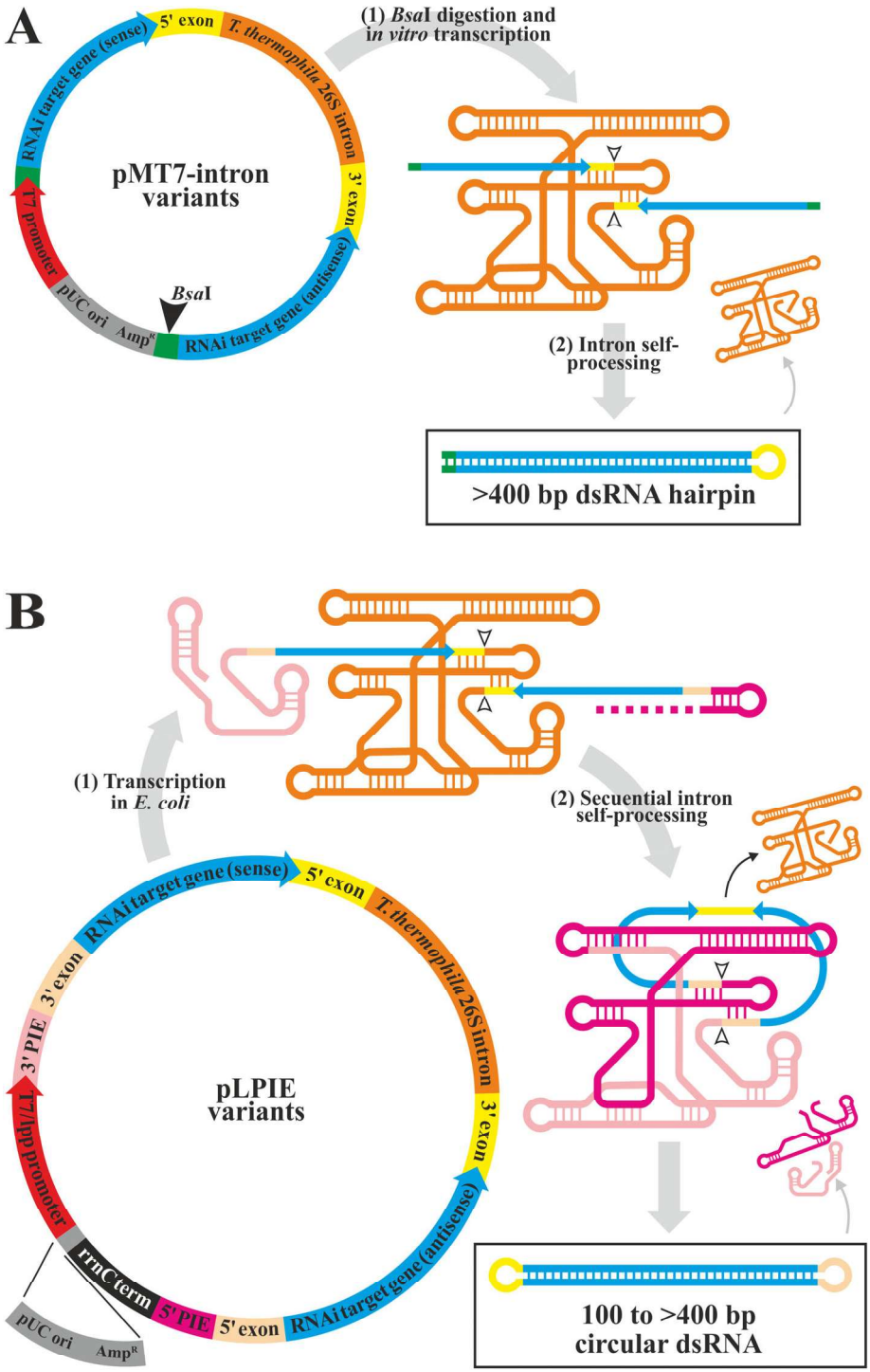

Figure 1. Schematic representation of the self-splicing-intron-mediated strategies for the production of (A) hairpin dsRNAs *in vitro* and (B) circular dsRNAs in *Escherichia coli*. (A,B) A *Tetrahymena thermophila* autocatalytic intron (plus 10-nt flanking exons) stabilizes the plasmids containing inverted repeats; the intron is entirely removed from the final transcript, generating a hairpin with a short 20-nt loop containing both exon sequences. (B) For circular dsRNA generation, the intron-separated inverted repeats are placed between two halves of a second autocatalytic intron with intron-exon permutation. The central regular intron is likely processed prior to the permuted one, possibly while the antisense direction of the inverted repeat is still being transcribed, hence bringing both halves of the introns closer to each other. Then, the second intron is self-spliced, releasing the dsRNA as a circular molecule, capped at both ends by loops arising from the exon fragments. (A,B) Schemes are not at scale.

in liquid nitrogen. The removed flies appeared to be healthy in every way, with no visible deleterious effects on any individual at the time of removal. On these adults, expression levels of the seven targeted genes at 72 h post-injection were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) relative to alpha tubulin. These three individuals removed per biological replicate were not included in the survival analysis.

Following microinjection assays, the three dsRNAs that resulted in higher mortality (*ATPsynbeta*, *V-ATPase*, and *RPS13*) were

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selected and administered orally to adult medflies. Equal amounts of circular dsRNAs produced in *E. coli* with homology to the three endogenous genes or GFP, as a control, were dissolved in 30% sucrose and used as a sole nutritional source for adult medflies. Following the similar methodology described earlier, each dsRNA was orally offered to 30 males and 30 females. As indicated earlier, males and females were grouped into three biological replicates of ten individuals each and placed in a plastic cage. Adult medflies were fed for 3 days with the 30% sucrose solutions containing circular dsRNAs at 1 μg/μL. With the help of a micropipette, a 10 μL droplet was placed on a 1.5 mL Eppendorf tube lid fixed to the bottom of the cage with plasticine. Diet solutions were replaced daily. Transcript levels 72 h post-treatment were analyzed in six randomly picked flies of each sex as previously detailed.

Following the same methodology for oral dsRNA delivery, an additional experiment was conducted to assess the impact of oral dsRNA on the mortality, fecundity, and fertility of *Ceratitis capitata*. Briefly, 30 males and 30 females were grouped into three biological replicates, each consisting of ten couples. Following the previously described procedure, they were placed in identical plastic boxes and provided with dsRNAs dissolved in a sugar solution. The mortality of individuals in each replicate was evaluated over a period of 14 days. After this duration, ten surviving females and ten surviving males from each treatment were collected and placed in oviposition boxes measuring 20 cm × 20 cm × 20 cm. These boxes were made of methacrylate, with one side covered by muslin fabric to allow the females to lay their eggs. Underneath the muslin fabric, a plastic collector measuring 25 cm × 6 cm × 4 cm and filled with water was placed to collect the eggs laid by the females, which would naturally fall into the collector due to gravity. Additionally, each oviposition box was equipped with a water dispenser consisting of a 50 mL bottle with an opening fitted with an absorbent wick and with sugar placed on a 40 mm diameter Petri dish. Three counts of the number of eggs laid were taken at 3 day intervals, using the values from each day as pseudoreplicates for subsequent data analysis.

In parallel, random cohorts of approximately 80 eggs laid by medflies from each treatment were placed in 40 cm Petri dishes lined with dark and moist filter paper at the bottom. The experiment was monitored daily for 5 days to record the number of hatched larvae and calculate the hatching rates. Three repetitions corresponding to each pseudoreplicate in time were considered. For each evaluated variable (mortality, fecundity, and fertility), the survival probability was calculated.[53,54] We defined this variable as:

$$\text{Survival probability} = \text{Mortality} \times \text{Fecundity} \times \text{Fertility}$$

Each term represents the percentage reduction relative to the control for the different parameters considered.

2.6 Analysis of gene silencing by RT-qPCR

Total RNA from *Ceratitis capitata* adults treated with gene-specific or control dsRNA was purified from frozen flies 72 h post-injection or feeding start using silica-gel columns (Zymo Research). Aliquots (500 ng) of each purified RNA were used to remove genomic DNA contamination and first-strand cDNA synthesis (iScript gDNA Clear cDNA Synthesis; Bio-Rad, Hercules, CA, USA). The cDNAs were used in qPCR amplification with target-specific primers (D4077–D4090) in a 96-well plate using a CFX96 thermal cycler (BioRad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). All primers used for these analyzes are shown in Table S2. PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. In all cases, a melting analysis was performed to confirm the specificity of the qPCR amplicons (from 65 to 95 °C in 0.5 °C increments with a hold time of 5 s for each read). Alpha-tubulin (amplified with specific primers D3007 and D3008) was used as a reference gene for data normalization.[55] The qPCR analysis was based on three biological replicates (individual flies) for each treatment and three technical replicates for each biological replicate. The data were analyzed with the Bio-Rad CFX manager software using the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of target genes compared to the control. The amplification efficiency for each gene was evaluated with a standard curve.

2.7 Data analysis

The survival of the adult medflies in both RNAi experiments (microinjection and oral feeding) was evaluated using Kaplan–Meier survival analysis. Adult survival curves of the time to death were plotted to reflect the mortality due to the corresponding RNAi treatment. Comparisons of the survival curves of each gene and control treatment were tested for statistical significance (Gehan–Breslow–Wilcoxon tests; $P < 0.05$). Gene expression data were analyzed using a two-way analysis of variance (ANOVA) ($P < 0.05$) to compare expression levels between the two study factors (dsRNA treatments and sexes), and to examine the potential interaction between them. Statistical analyses were performed using GraphPad Prism Software (San Diego, CA, USA). All data are reported as means ± standard error of the mean (SEM).

3 RESULTS

3.1 Selection of *Ceratitis capitata* candidate target genes and *in vitro* transcription of homologous RNA hairpins

Since no previous studies were available on *Ceratitis capitata*, we first selected seven candidate genes to identify suitable targets for robust RNAi-mediated control of this insect. The selection was based on previous successful reports of increased mortality in other dipteran and insect species: *Snf7*,[44,56] *RyR*,[57,58] *Nep4*,[59,60] *IAP2*,[58] *ATPsynbeta*,[61,62] *V-ATPase*,[63–65] and *RPS13*.[45,62,66] Target regions between 400 and 500 nt in the coding sequences of these genes were selected, taking into account their essentiality and specificity. In addition, we selected a GFP cDNA fragment of similar size as a negative control. We produced hairpin RNAs that included dsRNAs regions homologous to the selected sequences in the mentioned target genes and the control by *in vitro* transcription. Aliquots of the *in vitro* transcripts were analyzed by non-denaturing PAGE. Analysis showed prominent bands corresponding to RNA species that migrated between the 500 and 600 bp DNA markers and that likely correspond to the properly processed RNA hairpins (Supporting Information Fig. S1).

3.2 Injection of long dsRNAs in *Ceratitis capitata* adults triggers knockdown of target genes and increased mortality

Aliquots (0.5 μg) of the purified hairpin RNAs were injected into the thorax of adult medflies of both sexes. Three live flies of each sex were randomly selected and frozen 72 h after injection to analyze the RNAi response to the injected dsRNAs by RT-qPCR (Fig. 2, column charts). Six of the seven tested dsRNAs significantly downregulated their target messenger RNAs (mRNAs) in male flies (*Snf7*, *RyR*, *Nep4*, *ATPsynbeta*, *V-ATPase*, and *RPS13*) compared to

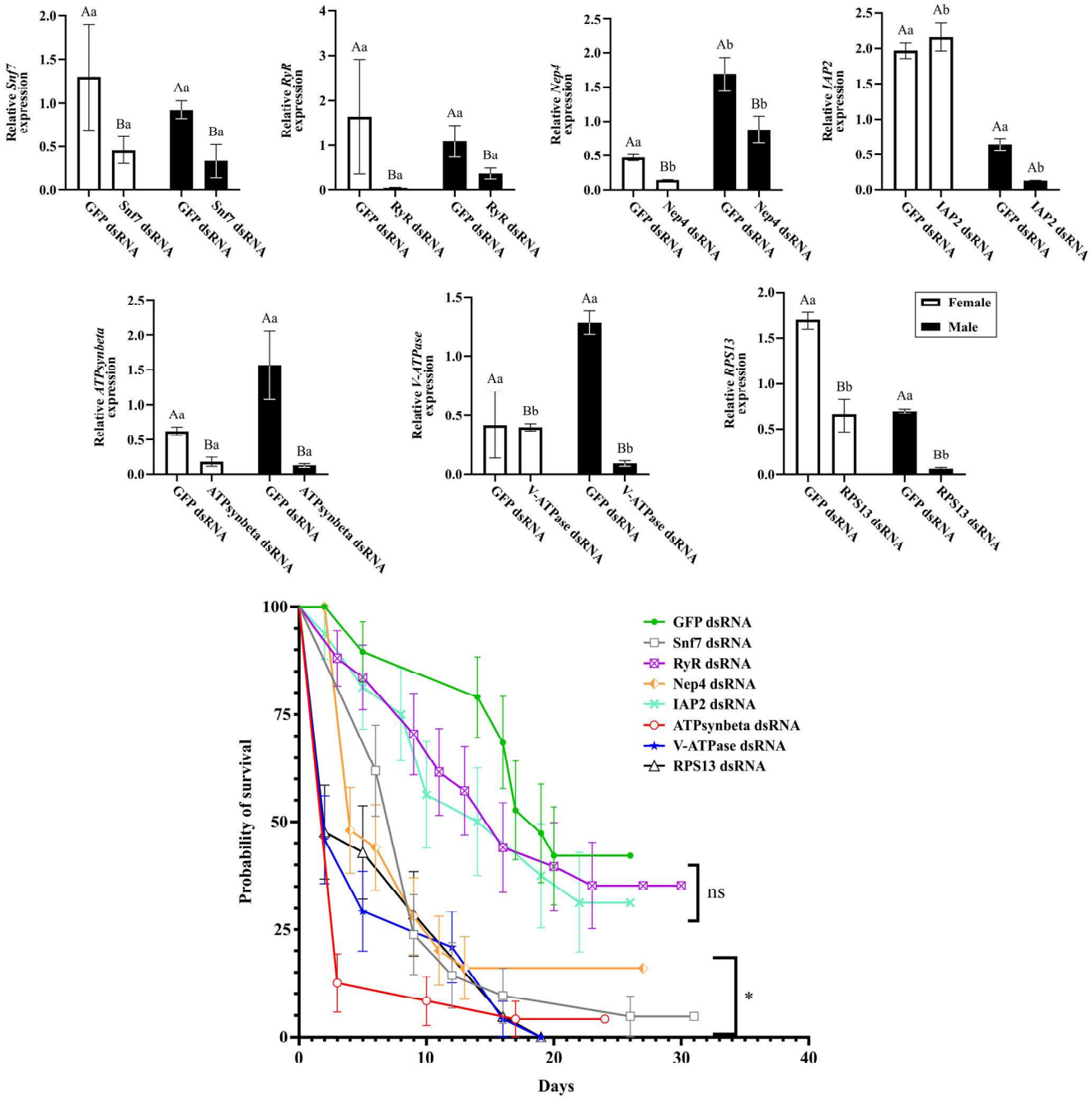

Figure 2. The dsRNA injection-based target gene screening for RNAi-mediated control of *Ceratitis capitata*. Expression levels of seven targeted genes at 72 h post-injection were determined by RT-qPCR relative to alpha tubulin (column charts). All expression data are reported as mean ± SEM of three independent technical replicates of three biological replicates (for each sex and treatment) and normalized against the mean of the controls of both sexes. Male flies black bars; females white bars. Uppercase letters indicate significant differences between dsRNA treatments, while lowercase letters denote differences between sexes (two-way ANOVA; $P < 0.05$). Kaplan–Meier survival curves for *Ceratitis capitata* adults of both sexes ($n = 42$) following the microinjection of 0.5 µg of seven different dsRNA homologous to genes of the medfly (*Snf7*, *RyR*, *Nep4*, *IAP2*, *ATPsynbeta*, *V-ATPase*, and *RPS13*) compared with flies treated with control dsRNA. Error bars show SEM. ns: not significant, *$P < 0.05$ in Gehan–Breslow–Wilcoxon tests (line charts).

male flies treated with the control dsRNA (GFP) ($F_{1,7} = 8.223$, $P = 0.0241$; $F_{1,7} = 6.074$, $P = 0.0432$; $F_{1,7} = 10.16$, $P = 0.0153$; $F_{1,7} = 11.16$, $P = 0.0124$; $F_{1,7} = 30.32$, $P = 0.0009$ and $F_{1,7} = 72.81$, $P < 0.0001$, respectively). The expression was substantially reduced in three of these genes, *ATPsynbeta*, *V-ATPase*, and *RPS13*. We also found differences between both sexes in four out of the seven studied genes (*Nep4*, *IAP2*, *V-ATPase*, and *RPS13*) ($F_{1,7} = 30.50$, $P = 0.0009$; $F_{1,7} = 173.6$, $P < 0.0001$; $F_{1,7} = 6.367$, $P = 0.0396$; $F_{1,7} = 66.02$, $P < 0.0001$, respectively) (Fig. 2, column charts). Only one dsRNA (*V-ATPase*) showed significant interaction between both factors, and this was because its target mRNA was down-regulated more strongly in males ($F_{1,7} = 28.45$, $P = 0.0011$).

We also recorded the fly mortality over time in the first 30 days after injection (Fig. 2, line chart). A Gehan–Breslow–Wilcoxon test indicated that mortality was significantly increased in five (*Snf7*, *Nep4*, *ATPsynbeta*, *V-ATPase*, and *RPS13*) of the seven tested genes

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in comparison with GFP control ($\chi^2 = 14.24$, $P = 0.0002$; $\chi^2 = 15.98$, $P < 0.0001$; $\chi^2 = 31.73$, $P < 0.0001$; $\chi^2 = 26.36$, $P < 0.0001$; $\chi^2 = 22.40$, $P < 0.0001$, respectively). In these cases, tested dsRNAs achieved mortalities between 95 and 100% except in the case of *Nep4*, with a survival probability of 15%. These differences were strongly significant in three genes, namely *ATPsynbeta*, *V-ATPase*, and *RPS13*, with mortality rates higher than 50% after 48 h post-injection (90, 60, and 55%, respectively). Only two genes (*RyR* and *IAP2*) did not affect insect mortality in our assay conditions. Strikingly different to gene expression, there were no significant differences in mortality between both sexes. Taken together, these results show that exogenous dsRNAs homologous to some medfly coding sequences can effectively knockdown the expression of essential genes and also induce the mortality of adult flies when directly injected into the insect bodies.

3.3 Large amounts of recombinant circular dsRNAs can be produced in *E. coli* using a two type-I self-splicing intron system

Next, we selected the three dsRNAs with the best death-inducing properties (*ATPsynbeta*, *V-ATPase*, and *RPS13*), along with the GFP control, to assess the effectiveness of orally-delivered RNAi. Since dsRNA stability and concentration are essential to reach the insect cells and ensure a substantial RNAi induction, we decided to produce circular versions of the selected dsRNAs using *E. coli* as a biofactory. For this purpose, we employed our recent method that uses two autocatalytic introns, the second in a permuted fashion, to produce large amounts of circular dsRNA in *E. coli*.[47] The cDNA of the first self-splicing intron stabilizes the inverted repeats that will produce both strands of the dsRNA. The second permuted intron flanking the inverted repeats promotes RNA self-circularization (Fig. 1(B)). Prominent bands likely corresponding to the circular dsRNAs were detected for all species in both large and low-scale cultures (Figs. S2 and S3). Taking *ATPsynbeta* as a model test, we analyzed the effect of the length of the dsRNA stretch on the recombinant RNA accumulation in *E. coli*. A series of plasmids with deletions was built to produce dsRNAs corresponding to *Ceratitis capitata ATPsynbeta* of 100, 200, 300, 400, and 464 bp. Analysis of RNA preparations from *E. coli* by non-denaturing PAGE showed little variation in length-dependent dsRNA accumulation in this size range, and even a better accumulation of the full-length dsRNA concerning the smaller versions (100 and 200 bp) (Fig. S4). We also assayed the effect of the promoter that drives the expression of the dsRNA precursor. We built a new plasmid to produce *RPS13* circular dsRNA in which the constitutive lpp promoter was replaced with an inducible T7 bacteriophage RNA polymerase promoter. The same *E. coli* strain was transformed with both plasmids. Selected recombinant clones were grown to OD_{600} 0.6 in liquid cultures, and 0.4 mM ITPG was incorporated to that corresponding to the inducible T7 promoter to induce T7-driven expression. A time-course analysis of total RNA showed no substantial difference in the level of circular dsRNA accumulation with the two promoters (Fig. S5).

Thus, we produced the molecules to be orally tested under the best experimental conditions. The non-denaturing PAGE analysis of aliquots of the total RNA showed that the bacteria produced the recombinant circular dsRNA in high quantities. Gel quantification of the produced RNAs via dilution analysis and comparison to standards of known concentration showed a production of up to 50 mg of dsRNA in 250 mL culture in 24 h (Fig. S6).

3.4 Feeding *Ceratitis capitata* adults with bacteria-produced dsRNA strongly down-regulate target genes, which affects biological parameters

Ceratitis capitata adult flies were fed for 3 days with the 30% sucrose solutions that contained the circular dsRNAs at 1 μg/μL. Diet solutions were replaced daily. Transcript levels down-regulation 72 h post-treatment were analyzed in six randomly picked flies of both sexes as previously detailed. The oral delivery of dsRNA induced a significant reduction of transcript levels for *V-ATPase* and *RPS13* (with a reduction in the expression to 48% and 6%, respectively) after 3 days of feeding ($F_{1,17} = 31.13$, $P < 0.0001$ and $F_{1,20} = 37.78$, $P < 0.0001$, respectively), while *ATPsynbeta* mRNA was not significantly affected ($F_{1,20} = 0.537$, $P = 0.4735$) (Fig. 3(A)). Significant differences were observed between sexes in the case of *RPS13* ($F_{1,20} = 4.510$, $P = 0.0464$) (Fig. 3(B)). These differences were not observed in the *V-ATPase* treatment ($F_{1,17} = 0.4094$, $P = 0.5308$). No interaction was observed between the two studied factors in any of the three experiments.

When adult *Ceratitis capitata* were offered oral dsRNA, the Gehan–Breslow–Wilcoxon test did not indicate significant differences in mortality between treatments, neither in females nor males (Fig. 4(A),(B)) ($\chi^2 = 4.870$, $P = 0.1816$ and $\chi^2 = 4.257$, $P = 0.2350$, respectively). However, among the surviving females, those treated with *V-ATPase* dsRNA showed a significant reduction in fecundity compared to the control ($F_{3,8} = 10.15$, $P = 0.0042$) (Fig. 4(C)), while the fertility of the eggs was lower only in the eggs laid by females fed with *RPS13* dsRNA ($F_{3,8} = 36.09$, $P < 0.0001$) (Fig. 4(D)). When combining the different evaluations in this experiment, the probability of survival was reduced to 74%, 22%, and 29% for the populations treated with *ATPsynbeta*, *V-ATPase*, and *RPS13* dsRNAs, respectively ($F_{2,6} = 30.90$, $P = 0.0007$) (Fig. 4(E)).

These results highlight the feasibility of using dsRNA molecules as an orally-supplied insecticide to control *Ceratitis capitata*. The down-regulation of crucial medfly mRNAs was achieved in this work using circular dsRNAs easily obtained in *E. coli* using the two-self-splicing-intron system.

4 DISCUSSION

Since its discovery in the model organism *Caenorhabditis elegans*,[67] RNAi has become a basic strategy in the toolset used for the study of gene function and the starting point of potential biotechnological applications. Pest control using RNAi is a topic of current interest,[68,69] with hundreds of studies in various stages of development in many species, including one treatment approved for its use in the field.[70–72] Here we show the feasibility of this strategy for controlling the worldwide distributed pest *Ceratitis capitata*, as orally-supplied dsRNA can significantly reduce the expression of insect-targeted genes.

In this work, we used two different methodologies to produce dsRNA based on our recently described self-splicing-intron-based system.[47] On the one hand, the straightforward assembly of transcription plasmid derivatives with a single self-splicing intron allows the construction of libraries for producing hairpins containing a dsRNA stretch under standard reaction conditions for high-throughput RNAi screening assays. The resulting dsRNAs are constituted of a single molecule and not hybridized single-stranded RNAs (ssRNAs), thus reducing unnecessary labor and

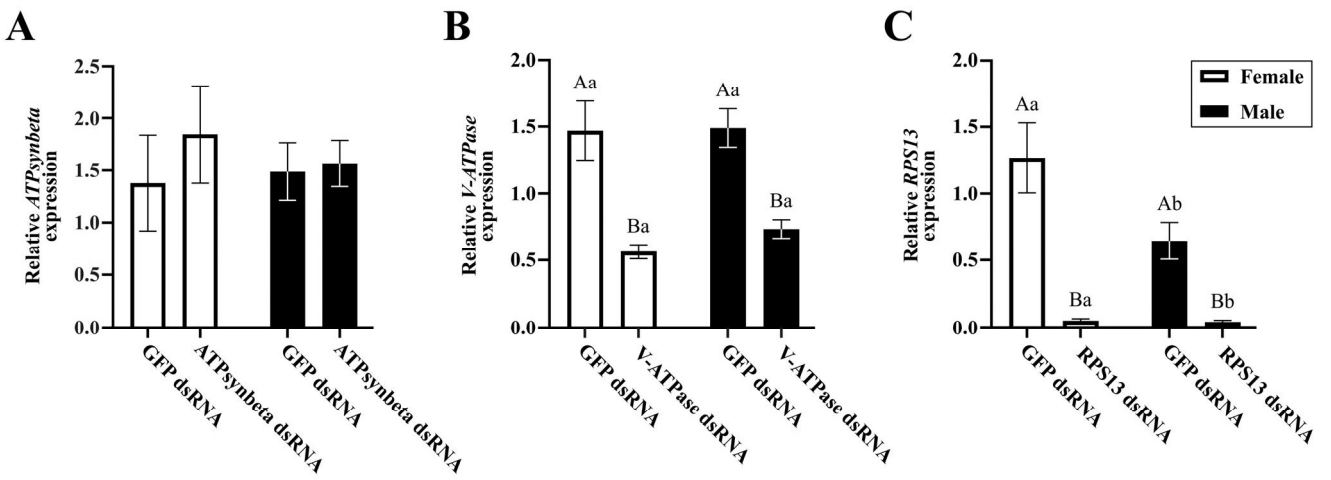

Figure 3. Effect of orally-supplied, gene-specific dsRNAs on the accumulation levels of males and females of *Ceratitis capitata* (A) *ATPsynbeta*, (B) *V-ATPase*, and (C) *RPS13* genes. Equal amounts of circular dsRNAs with homology to three endogenous genes, or GFP as a control, produced in *Escherichia coli* were dissolved in 30% sucrose and used as a sole nutritional source for adult medflies. Furthermore, 72 h after the end of the treatment, six individuals of each sex were collected for RNAi-mediated knockdown analysis. The relative expression levels of the three genes were determined by RT-qPCR. All data are reported as mean ± SEM of six biological replicates (for each sex and treatment) and normalized against the mean of the controls of both sexes. Uppercase letters indicate significant differences between dsRNA treatments, while lowercase letters denote differences between sexes (two-way ANOVA; $P < 0.05$).

variability resulting from hybridization. From our view, this is an exciting alternative to classical *in vitro* synthesis using convergent T7 promoters. On the other hand, the *E. coli* production system, including a second permuted intron, results in circular dsRNA and seems very promising for producing the large quantities required for pest control. Using multicopy plasmids (with pUC replication origin) containing a strong constitutive *E. coli* promoter (lpp) seems to ensure high and constant transcription levels. In fact, our effort to further optimize production showed that accumulation of the circular dsRNA is not improved using a strong inducible promoter such as that from bacteriophage T7. In addition to transcription, our *in vivo* system relies on the activity of two self-splicing type-I introns. Although this type of introns only requires magnesium ion (Mg^{2+}) and guanosine to self-cleave, certain protein factors have been shown to accelerate the functional folding of the structure, thus resulting in saturable reactions.[73–76] This could be especially relevant given the presence of two intron copies in the RNA precursors, one of which is permuted, hindering even more its correct folding. This maximum rate can also explain size-dependent expression differences in the benefit of longer RNAs, as the shortest dsRNA precursors are transcribed faster, surpassing those of the self-processing activity and resulting in unprocessed molecules likely prone to degradation. Therefore, the dsRNAs size might be further increased, if necessary, without expected reductions in production. In addition, the system maintains a large yield of the RNAs of interest when the culture is upscaled 50 times. Thus, it is expected that the production should increase proportionally in larger culture volumes. These properties, in combination with transcription from the constitutive promoter and accumulation at the stationary phase, support indisputable industrial applicability.

Gene target screening assay showed increased silencing and mortality when affecting a broad spectrum of functions, such as energy metabolism, membrane transporters, detoxification, and translation factors. Intriguing differences in the basal levels of expression and its silencing between males and females were observed without implying variation in mortality. Experimental parameters or differences in treatment responses may have caused this variation, such as using only three biological replicates per sex and treatment. Therefore, future studies would be necessary to clarify the possible differences in response between sexes to these treatments. Regardless, the robust mortality effect achieved with three of these RNAs (*ATPsynbeta*, *V-ATPase*, and *RPS13*) stimulated us to take a step forward and further analyze if some of those dsRNAs were suitable for oral RNAi-derived medfly control. All tested dsRNAs lengthened 400–500 bp; this is a key parameter as RNA length is correlated positively with traversing through plasma membranes[15,77] and greater diversity of siRNAs generated, but also negatively with inter-species off-target and resistance development.[15] The RNAi feeding assay yielded two genes whose silencing was significantly reduced with only 3 days of treatment compared with the GFP control. The first gene encodes a subunit of a vacuolar ATPase (*V-ATPase*), ubiquitous membrane protein complexes present in both plasmatic and vesicular membranes and functioning as ATP-consuming proton pumps with multiple cellular functions.[78] Multiple genes usually encode vacuolar ATPase subunits with splicing variants, some with specific spatio-temporal expression patterns.[79] We here targeted subunit A, which belongs to the complex hydrophilic ATP-hydrolyzing (V1) region. *Ceratitis capitata* has three different A subunits; our target here (homologous to *Drosophila melanogaster Vha68-2*) exhibits the highest expression among them.[79] This subunit also has three splicing variants that encode the same polypeptide. The second gene, *RPS13*, encodes the ribosomal protein S13, one of the many proteins associated with rRNAs, to form ribosomal subunits. Specifically, RPS13 is an essential structural element of the 30S ribosomal subunit since it seems responsible for establishing a communication network not only for the correct assembly of the ribosome but also for its proper translocation.[80,81] Several dozens of genes encode this type of small proteins, but unlike vacuolar ATPases, most are single-copy genes, such is the case of our *RPS13* target.

Under our experimental conditions, we achieved both silencing and mortality by microinjection but despite achieving targeted

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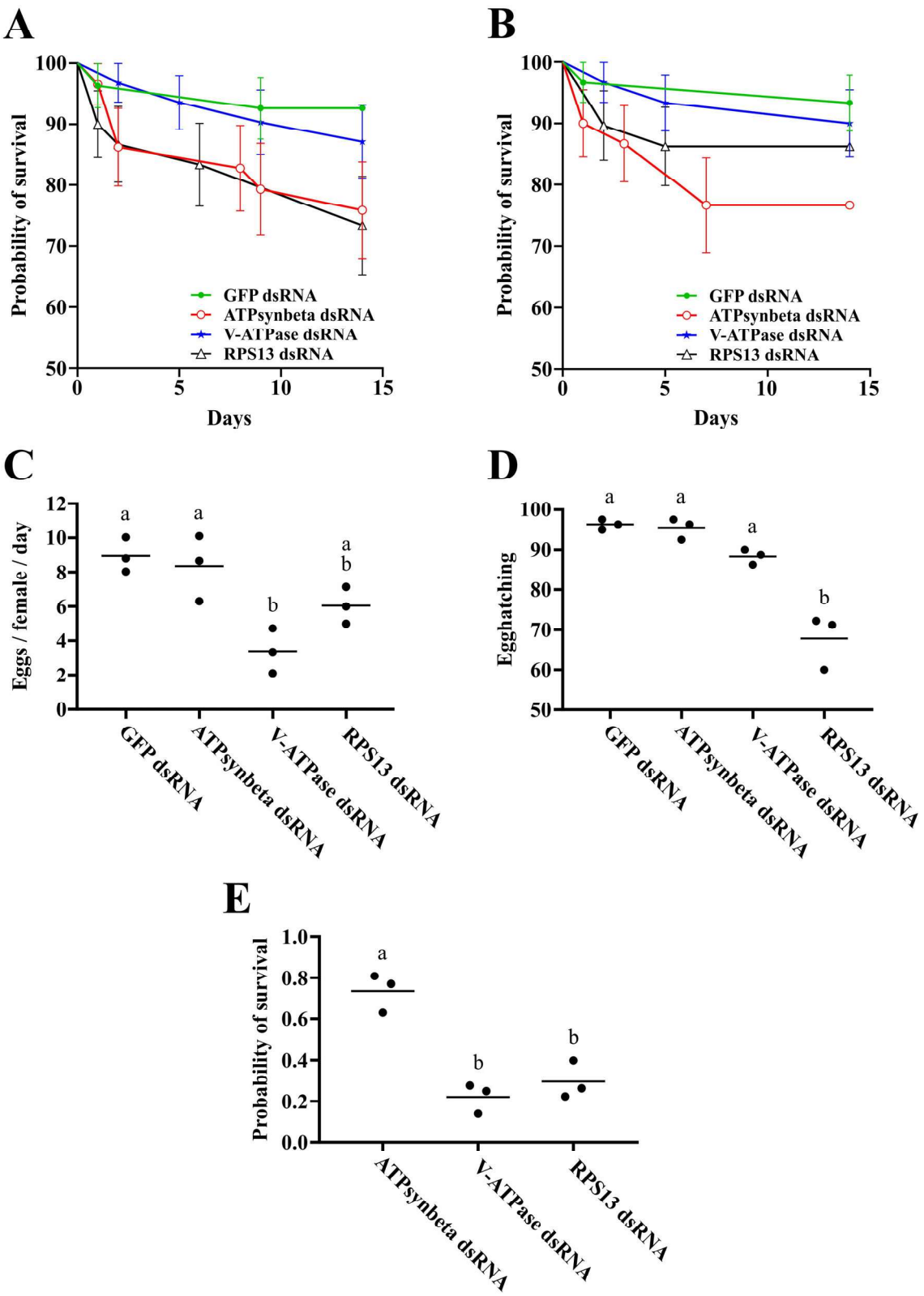

Figure 4. Effect of orally-supplied, gene-specific *ATPsynbeta*, *V-ATPase*, and *RPS13* dsRNAs on the survival of (A) females and (B) males during their first 14 days of life after emergence (Kaplan–Meier survival curves). (C) Fecundity was measured from ten surviving females and ten surviving males from each treatment between day 15 and day 24 of adulthood. (D) Fertility was assessed in three cohorts of 80 eggs for each experimental treatment. (E) The probability of survival was calculated as the product of the percentage reduction relative to the control for the three different parameters considered: mortality × fecundity × fertility. Lowercase letters indicate statistical differences among treatments (ANOVA, Tukey test; $P < 0.05$).

knockdown in feeding bioassays, no significant mortality was observed in these assays. However, the levels of gene silencing achieved with these assays suggest a strong systemic response to introduced dsRNAs in this species. This was confirmed by the fertility and fecundity assays, where it was observed that when both parameters were combined with mortality, the probability of survival could theoretically be reduced by 74%, 22%, and 29% for the *ATPsynbeta*, *V-ATPase*, and *RPS13* dsRNA-treated populations, respectively. While differences in the down-regulation of the vacuolar ATPase subunit-A (~90% by injection *versus* ~50% orally) could partly explain the difference in mortality, the *RPS13* results are certainly intriguing, given the silencing similarity between both methods (*c.* 90%) (Figs. 2 and 3). Experimental factors such as side-effects of microinjection, and the functional supplementation with other isoforms or related proteins due to the slower development of the silencing in the oral experiment, may cause a lack of mortality. Nevertheless, it should be noted that sublethal exposure to both dsRNA (as confirmed by the fecundity and fertility assays) might still reduce crop damage in sustainable crop protection approaches.

Vacuolar ATPase complexes and ribosomal proteins such as RPS13 have been implicated in developing and maintaining female reproductive organs and with embryonic and juvenile development. The dsRNA-mediated silencing of various vacuolar ATPase subunits in immature and adult females resulted in deformed ovaries and oocytes,[82,83] leading to increased embryonic necrosis, reduced egg laying and hatching, and a higher rate of egg deformity.[84] Similar phenotypes are generated by silencing ribosomal proteins, such as reduced egg production and hatching thus highly affecting fecundity.[85–87] These observations are consistent with our experimental results in which females fed with circular dsRNAs with homology to *V-ATPase* and *RPS13* genes had significantly reduced egg laying and hatching, respectively (Fig. 4). Further effects on the larval development could be expected since, in *D. melanogaster*, defects in protein biosynthesis as a result of reduction of the expression of ribosomal proteins such as RPS13 leads to the appearance of characteristic phenotypes as larval development delay, poor viability, altered body size, and reduced fertility.[88,89] Vacuolar ATPase silencing in nymphs and larvae reduced their food intake, decreased larval growth, adult development, and emergence, and even increased mortality,[90] probably by affecting the integrity and function of the larval midgut and Malpighian tubules.[91–93] However, the detrimental effect of silencing these genes on *Ceratitis capitata* larvae remain to be assessed.

5 CONCLUSION

To sum up, this work shows that orally delivered circular dsRNAs induce strong silencing of crucial genes in the devastating pest *Ceratitis capitata*. This kind of RNAi-inducing molecules can be reliably produced in *E. coli* bio-factories using a two-self-splicing-intron system for sustainable crop protection programs. Nonetheless, many factors remain to be still analyzed, such as the viability of deriving direct mortality from silencing other genes or the potential benefits of dsRNA encapsulation.

AUTHOR CONTRIBUTIONS

All authors participated in work conception and design. BO, ME, and MP-H performed the experiments. All authors carried out data analysis and interpretation of the results. BO, MP-H, and J-AD wrote the manuscript with input from the rest of the authors. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

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

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