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Preparation and Use of a Yeast shRNA Delivery System for Gene Silencing in Mosquito Larvae

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Abstract

The mosquito genome projects facilitated research in new facets of mosquito biology, including functional genetic studies in the dengue and Zika virus vector *Aedes aegypti* and the primary African malaria vector *Anopheles gambiae*. RNA interference (RNAi) has facilitated gene silencing experiments in both of these disease vector mosquito species and could one day be applied as a new method of vector control. Here, we describe a procedure for the genetic engineering of *Saccharomyces cerevisiae* (baker's yeast) that express short hairpin RNA (shRNA) corresponding to mosquito target genes of interest. Following cultivation, which facilitates inexpensive propagation of shRNA, the yeast is inactivated and prepared in a ready-to-use dry tablet formulation that is fed to mosquito larvae. Ingestion of the yeast tablets results in effective larval target gene silencing. This technically straightforward and affordable technique may be applicable to a wide variety of mosquito species and potentially to other arthropods that feed on yeast.

Keywords

Aedes aegypti; *Anopheles gambiae*; *Saccharomyces cerevisiae*; RNAi; dengue; Zika; malaria; vector; development; insect

1. Introduction

Blood feeding *Aedes* and *Anopheles* vector mosquitoes transmit disease-causing pathogens that result in hundreds of thousands of human deaths each year. Dengue, a leading cause of morbidity in the tropics, Zika, a public health emergency of international concern, as well as yellow fever and chikungunya, result from infections with arboviruses transmitted through the bites of *Aedes* mosquitoes [1]. The global incidence of dengue has increased

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dramatically, with over 400 million estimated cases occurring each year [2]. Cases of Zika, which has been linked to severe birth defects and neurological disorders, are currently occurring in many countries in the Americas, and Zika has rapidly spread to previously unaffected geographic areas [3]. Malaria results from infection with *Plasmodium spp.* parasites, which are transmitted to people through the bites of infected *Anopheles* mosquitoes, including the primary African vector *Anopheles gambiae* [4]. Despite the devastating global impact of mosquito-borne illnesses on human health, effective means of preventing and treating these diseases are lacking, and mosquito control is presently the best method of disease prevention.

In recent years, advances in the genetic engineering of mosquitoes have made the potential for using transgenic vector control strategies a reality [5–7], challenging researchers to identify novel gene targets for vector control and additional methods of manipulating mosquito gene function. Altering gene expression during development, which proved useful for generation of the female-flightless control intervention [5], may promote the elucidation of novel mosquito control strategies. However, to date, the functions of very few genes have been characterized during disease vector mosquito development. RNA interference (RNAi), initially discovered in *C. elegans* [8], has facilitated characterization of gene function in a wide variety of organisms, including insects [9, 10]. The RNAi pathway is initiated by Dicer, which cleaves long dsRNA into short 21–25 nucleotide-long small interfering RNAs (siRNAs) that function as sequence-specific interfering RNA molecules. siRNAs silence genes that are complementary in sequence by promoting transcript turnover, cleavage, and disruption of translation [10]. Although most mosquito researchers use longer (300–400 bp) dsRNA molecules for RNAi experiments, the short length of custom siRNAs and their short hairpin RNA (shRNA) counterparts facilitates design of interfering RNA with less potential for off-site targeting. It is also possible to confirm gene silencing phenotypes by performing experiments with multiple siRNAs that recognize different target sites within a gene of interest. Moreover, if siRNAs were to one day be used as insecticides, the development of multiple siRNA insecticides to silence the targeted gene will be useful for combating resistance resulting from a point mutation in any single target site. Additionally, the use of short sequences facilitates the design of interfering RNA molecules that recognize target sites that are not found in non-target organisms, but which are conserved in multiple mosquito species.

Although RNAi does not generate heritable germline mutations, it offers several advantages that may be of utility. First, through management of the timing of siRNA/shRNA delivery, researchers can control the time at which gene silencing initiates. This advantage can be used to overcome challenges such as developmental lethality or sterility, issues which can hinder both the production and maintenance of strains bearing heritable mutations. Moreover, genetic engineering of non-model insects is still a relatively expensive and labor-intensive process. Thus, although the degree of gene silencing by RNAi can vary depending on the gene targeted, the tissue type, and also from subject to subject, RNAi is still frequently used for functional genetics studies in mosquitoes and other insects [9, 10]. Several different interfering RNA delivery strategies have been implemented in developing mosquitoes. For example, we have successfully used microinjection to deliver siRNAs for silencing of developmental genes in *A. aegypti* embryos, larvae, and pupae [11–18].

However, this labor-intensive delivery strategy, which requires both technical skill and a microinjection setup, cannot be extended to the field. Although ingestion-based strategies do not work in all insect species, notably *Drosophila melanogaster*, RNAi through oral delivery promotes gene silencing in a variety of different insects, including mosquitoes [9, 19, 20, 21, 22, 23, 24]. For example, soaking mosquitoes in interfering RNA solution, which they ingest, can induce gene silencing [19, 20, 22, 23, 24], and this convenient technique requires little equipment or labor. Chitosan nanoparticle-mediated interfering RNA delivery also effectively silences genes in *A. gambiae* [22] and *A. aegypti* larvae [23, 24]. However, while soaking and chitosan/siRNA methodology facilitate relatively affordable laboratory studies and require little equipment or labor [15], the present costs of RNA synthesis may still be a concern in large-scale laboratory and field applications.

The use of microbes facilitates affordable RNA propagation through microbe cultivation. For example, *Escherichia coli* strains engineered to produce double stranded (dsRNA) molecules targeting several genes of interest have generated successful gene silencing when fed to *A. aegypti* [25, 26] and *A. gambiae* [27] larvae. We have also engineered *Saccharomyces cerevisiae* (baker's yeast), a model organism that is genetically tractable and inexpensive to culture, to produce shRNA corresponding to target sequences in several mosquito genes of interest [26, 27]. Yeast, which is added to both chitosan nanoparticles [15] and *E. coli* pellets [25] for larval feeding assays, is a strong odorant attractant and a source of nutrition for laboratory-bred mosquito larvae. Therefore, *S. cerevisiae,* unlike *E. coli* or chitosan, can serve both as the RNA delivery system and directly as the bait. Moreover, if RNAi were to one day be introduced as a mechanism for mosquito control, it seems likely that yeast, a natural product that is used in food and beverage production across the globe, could be a microbe that is readily accepted by users and stakeholders. Our laboratory studies demonstrate that yeast interfering RNA strains, which can function as larvicides, effectively silence genes in both *Aedes* [26] and *Anopheles* [27] larvae. Moreover, we have developed a procedure for production of ready-to-use dried and inactivated yeast interfering RNA tablets, a formulation which facilitates laboratory studies and that could one day promote the seamless integration of these larvicides into mosquito control programs. Importantly, laboratory trials demonstrate that the larvicidal capacity of this yeast is maintained even when the microbes are heat-killed [26, 27,], an important advantage given that additional regulatory approvals are often required for the feeding of living recombinant organisms to live animals in the laboratory, as well as in the field. *S. cerevisiae* has been cultivated worldwide for thousands of years, and this technology, which is readily scaled, can be adapted in most laboratories using the methodology for recombinant yeast production (Steps 3.1 and 3.2 below), yeast culturing (Step 3.3) and tablet preparation (Step 3.4), larval feeding assays (Step 3.5), and confirmation of gene silencing (Steps 3.6 and 3.7) described herein.

2. Materials

2.1 Equipment:

1. Horizontal gel electrophoresis system.
2. Microwave oven.

3. Gel imaging system.
4. Nanodrop or comparable spectrophotometer.
5. Shaker incubator.
6. Microcentrifuge (i.e. Eppendorf 5415D or a comparable model).
7. High speed refrigerated centrifuge (i.e. Eppendorf 5810R plus or a comparable model).
8. Water bath (a shaking bath is preferred, though not required for Step 3.7).
9. Heat block.
10. Vortex.
11. Incubator with good outward ventilation.
12. Micropipetters.
13. Ice bucket.
14. Racks for holding microfuge and centrifuge tubes.
15. Dissection microscope.
16. qRT-PCR machine (if detecting silencing via qRT-PCR, Step 3.6).
17. Rocking platform shaker or nutator (if detecting silencing via *in situ* hybridization, Step 3.7).
18. Cell spreader.
19. Bunsen burner.

2.2 Construction of shRNA expression vector:

Molecular grade reagents and ultrapure water should be used to prepare sterile solutions and media in sections 2.2 – 2.6.

1. Shuttle vector: Use of the *pRS426 GPD* yeast shuttle vector permits constitutive expression of inserts cloned into the multiple cloning site downstream of a *GPD* promoter and upstream of a *cyc1* terminator [28]. *pRS426* has an ampicillin resistance marker for selection in *E. coli*, in which all cloning experiments are performed. The *pRS426 URA3* marker facilitates selection of *S. cerevisiae* that have been successfully transformed with the construct.
2. Custom oligonucleotides encoding the shRNA transcript; store annealed oligonucleotides at −20° C.
3. T4 DNA ligase and buffer stored at −20° C.
4. LB media: 10 g tryptone, 5 g yeast extract, 10 g NaCl (add 15 g of bacto-agar for LB plates). Bring the volume to 1000 ml with ultrapure water, pH to 7.5, then autoclave and store at 4° C. *E. coli* DH5α competent cells transformed with the

pRS426 shRNA expression plasmid are grown at 37° C on standard LB media containing 100 μg/mL ampicillin.

5. Plastics: Sterile petri dishes, 1.5 mL microfuge tubes, 10 ml culture tubes.
6. Plasmid miniprep kit.
7. Restriction enzymes corresponding to the *pRS426 GPD* multiple cloning site (stored at −20° C); we typically use the BamHI (5') and XhoI (3') sites.
8. Agarose (stored at room temperature).
9. 50x TAE buffer: 242.2g Tris base, 57.1 ml glacial acetic acid, 18.6g EDTA, and ultrapure water to volume of 1 L with ultrapure water. Stored at room temperature and diluted 1:50 with ultrapure water for 1x working concentration.
10. Ethidium bromide (10 mg/ml in ultrapure water). Stored at 4° C and dilute 1:20 for gel electrophoresis.
11. Sterile 500 mL Erlenmeyer flask.
12. Sequencing primers M13F 5'GTAAAACGACGGCCAGT3′ and M13R 5′CACACAGGAAACAGCTATGACCAT3′; store at −20° C.

2.3 Yeast transformation, culturing, and preparation of dried inactivated tablets:

1. *S. cerevisiae* strain *BY4742 (genotype MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) [29].
2. 10x Lithium acetate (1 M in ultrapure water). Filter sterilized and stored at 4° C.
3. 50% PEG 3350 in ultrapure water. Filter sterilized, aliquoted, wrapped in parafilm, and stored at 4° C.
4. Salmon sperm DNA (2 mg/mL in ultrapure water); should be phenol chloroform extracted, sheared, and stored at - 20° C. Heat-denature prior to use in transformation.
5. Standard YPD media: 20 g bacto peptone, 10 g yeast extract, and water to 900 mL volume. The solution should be autoclaved, then cooled to the touch prior to the addition of 100 mL sterile 20% glucose solution and storage at 4° C.
6. Petri dishes (sterile).
7. Synthetic complete media with dextrose lacking uracil (SCD-ura): 1.7g of yeast nitrogen base, 5 g ammonium sulfate, and 0.77g complete supplement mixture lacking uracil (CSM-ura). For plates, add 20 g of agar. Bring the volume to 900mL with ultrapure water and autoclave. Once the media has cooled to the touch, add 100mL of sterile 20% glucose solution to the cooled media. Store media plates at 4° C for up to one month.
8. Tubes: Sterile 1.5 ml and 2 ml microfuge tubes, 10 ml culture tubes, and 50 ml centrifuge tubes.
9. Sterile 1 L Erlenmeyer flasks.

2.4 Mosquito strains, rearing, and recombinant yeast feeding assays.

1. Maintain *A. gambiae* M strain or other strains of interest according to standard lab practice or as described [30, 31].
2. Maintain *A. aegypti* Liverpool IB12 or other strains of interest according to standard lab practice or as previously described [31].
3. Membrane Feeding System (i.e. Hemotek Ltd.; replaces vertebrate animals for blood feeding mosquitoes) and commercially purchased blood (i.e. defibrinated sheep's blood; store at 4° C).
4. 500 mL plastic cups and tray in which to place the cups.
5. Petri dish lid or mesh to cover cups (holes should be small enough to prevent adult escape).
6. Rubber bands to secure mesh to cups.

2.5 Detection of target gene silencing by qRT-PCR:

1. Trizol reagent (Invitrogen); stored at 4° C.
2. High capacity RNA to cDNA Kit (i.e. Applied Biosystems kit); stored at −20° C.
3. SYBR green I PCR kit (i.e. Applied Biosystems kit); stored at 4° C.
4. Sterile PCR tubes.

2.6 Detection of target gene silencing by whole mount *in situ* hybridization:

1. PEM: 34.63 g of PIPES disodium salt, 10 ml of 0.2 M EGTA, and 1 mL of 1M $MgSO_4$. Add ultrapure water and mix for 20 min, then adjust pH to 7.0 with HCl. Bring to 1L volume and filter through a 0.22 μm filter. Store this solution at 4° C for up to 1 year. To prepare PEM-F, dilute 37% formaldehyde 1:10 in PEM just prior to use.
2. DIG-labeled riboprobe prepared with DIG Labeling Kit (SP6/T7, Roche Life Science); stored at −20° C.
3. Hybridization solution (Hyb): 25 mL deionoized formamide, 12.5 ml 20x SSC, 2.5 mg heparin, 50 μL Tween-20. Adjust the pH to 5.0 using HCl. Bring the final volume to 50 mL with sterile ultrapure water and store at −20° C.
4. Hyb-DNA-SDS: 14.85 ml Hyb solution with 150 μl of 10 mg/ml sonicated salmon sperm DNA. Store at −20° C; just prior to use, warm the Hyb-DNA solution and add SDS to a final concentration of 0.3%.
5. 10x PBS: 11.9 g Na_2HPO_4, 2.23 g NaH_2PO_4 (anhydrous), and 102.2 g NaCl. Bring to 1 L total volume with ultrapure water and the pH adjusted to 7.4. Autoclave and store at room temperature. Dilute to 1x working concentration prior to use.
6. PT: PBS and 0.1% Triton X-100 stored at room temperature.

7. PTw: PBS and 0.1% Tween-20 stored at room temperature.
8. Detergent solution: 5 ml 10% SDS, 250 μl Tween-20, 5 mL 0.5 M Tris-HCl (pH 7.5), 100 μl 0.5 M EDTA (pH 8), and 7.5 ml 1 M NaCl. Add ultrapure water to 50 mL and store at room temperature.
9. AP Buffer and color reaction solution: 250 μl 1M $MgCl_2$, 5 mL 1 M NaCl, 5 mL 1M Tris (pH 9.5), 50 μl Tween-20, and ultrapure water to 50 mL. Prepare fresh at room temperature just prior to use. For the color reaction, add 20 μl NBT-BCIP solution (Roche) to 1 mL of AP buffer and use immediately.
10. Glycerol solution: 50% and 70% glycerol solutions are prepared with ultrapure glycerol in PBS. Store at room temperature.

3. Methods

3.1. Preparation of yeast transformation vector

1. Select shRNA target sites and design shRNAs according to standard lab practice. Scramble the sequence of a knockdown shRNA target site to design negative control shRNA that does not correspond to any mosquito gene. The shRNA sequence designer tool [32] will facilitate design of DNA oligonucleotides corresponding to each target sequence and allows the user to add restriction enzyme overhangs to facilitate downstream cloning (*see* Note 1). DNA oligonucleotides corresponding to the custom shRNA sequences can be purchased through a number of reputable vendors.
2. DNA oligonucleotides corresponding to the shRNA sequence are annealed and cloned into the multiple cloning site of the *pRS426* shuttle vector [28] (*see* Note 2) using standard double digestion and cloning techniques (*see* Notes 3 and 4). Confirm positive colonies by harvesting and double digestion of the plasmid DNA followed by agarose gel electrophoresis to confirm inserts. The correct sequences of positive inserts should be verified through sequencing with the M13F or M13R primer (*see* Note 5).

3. 2 Yeast transformation:

A timeline outlining the sequencing of steps 3.2 through 3.7 is presented in Fig. 1.

1. Inoculate 2–5 mL of liquid YPD with *S. cerevisiae* strain *BY4742* and incubate with shaking overnight at 30°C and 250 rpm.
2. Use 600 μl of the pre-culture (which should be at an OD_{600} of ~0.2) to inoculate 25 ml of fresh YPD. Shake at 250 rpm and 30°C until the OD_{600} reaches ~0.8 (approximately 3–5 hours).
3. Harvest the culture in a sterile 50 ml centrifuge tube spun at 1000 x g for 5 min.
4. Pour off the medium and resuspend the cells in 25 ml of sterile water. Centrifuge again at 1000 x g for 5 min.

5. Pour off the water and resuspend the cells in 1.0 ml of 0.1M (1x) LiAc. Transfer the cell suspension to a 1.5 ml microfuge tube.
6. Pellet the cells at 8000 rpm for 30 sec and remove the LiAc with a micropipette.
7. Resuspend the cells to a final volume of ~250 μl by adding 200 μl of 0.1 M (1X) LiAc (2×10^9 cells/ml).
8. Boil the salmon sperm DNA for 5 min and quickly chill it on ice (*see* Note 6).
9. Gently mix the cell suspension and pipette 50 μl samples into microfuge tubes. Pellet the cells and remove the LiAc with a micropipette.
10. Prepare the transformation mixture, which consists of 240 μl PEG (50% w/v) (*see* Note 7), 36 μl 1.0 M LiAc, 50 μl heat-denatured salmon sperm DNA (2.0 mg/ml), ~3.0 μg of plasmid DNA, and sterile ultrapure water to a total volume of 360 μl.
11. Add the transformation mixture to the cell pellet. Vortex each tube briefly until the cells are resuspended.
12. Heat shock the cells in a water bath at 42°C for 15–20 min.
13. Microfuge at 6,000–8,000 rpm for 30 sec and remove the supernatant with a micropipette.
14. Pipette 600 μl of sterile water into the tube and resuspend the pellet by pipetting it up and down gently.
15. Plate the cells on SCD-ura media plates.
16. Incubate the plates for 2 – 4 days at 30° C to recover transformants. Re-streak yeast from an individual clone onto a new SCD-ura media plate (*see* Note 8).

3.3 Yeast culturing and preparation of dried inactivated yeast tablets:

1. To prepare yeast from each desired strain (experimental or control), inoculate a 2 ml culture of yeast SCD-ura media placed in a sterile 10 ml culture tube (*see* Notes 9 and 10). Allow the culture to grow for 24 hrs in a shaker incubator set at 30° C and 250 rpm. The culture tube should be placed at a 45° angle.
2. Transfer the 2 ml culture into a 1 L flask with 250 ml SCD-ura media and allow it to grow at 30° C and 250 rpm in a shaker incubator until the OD^{600} of the culture is between 2.5 – 3.0 (*see* Notes 11 and 12).

3.4 Preparation of dried inactivated yeast tablets:

1. Transfer the 250 ml culture into five 50 ml centrifuge tubes, each with 50 ml of culture. Spin the balanced tubes for 20 min at 4000 rpm (*see* Note 13). Decant the supernatant.
2. Heat kill the yeast by incubating the tubes containing the pellets in a water bath set to 70° C for 5 min; once heat killed, transfer (*see* Note 14) the pellets into 2 ml Eppendorf tubes (*see* Note 15).

3. Spin the 2 ml tubes at ~13000 rpm for 2 min and remove the supernatant entirely by pipetting. Place these pellets in a 30° C incubator that has good outward ventilation (i.e. it must constantly suck moist air from the chamber). Alternatively, a food desiccator can be used for the drying process. Dry the pellets for approximately 48 hrs, when a gap between the pellet and the side of the tube is noted and the pellet can be dislodged from the tube (*see* Note 16). The final weight of each yeast tablet (shown in Fig. 2A) averages ~85 mg (~1.6 × 10^10 cells). For best results, proceed immediately to the feeding assays (section 3.5). If necessary, the pellets can be stored at −80° or −20° C for 1–2 weeks.

3.5 Feeding mosquito larvae with recombinant yeast tablets:

1. Prepare 16 oz plastic cups with 50 ml of sterile ultrapure water and label each cup appropriately. For each biological replicate experiment, prepare at least three cups per strain of yeast used (*see* Note 17).
2. Place one control or experimental 85 mg yeast pellet in each cup.
3. Add 20 freshly hatched age-synchronized mosquito larvae (*see* Note 18) to each of these cups (Fig. 3). Cover each cup with mesh secured with a rubber band or with the lid of a petri dish. Put the cups in a plastic tray to catch any spills and place the tray in an insectary maintained at 26.5° C with relative humidity of ~85% and a light-dark cycle of 12h. If an insectary is not available, then an incubator can be used.
4. Examine the transcript levels as discussed below and evaluate other phenotypic changes at the desired developmental time points. For example, when evaluating yeast interfering RNA larvicides, larval death can be monitored (Figs. 2B, 3). For analysis of some phenotypes, it may be useful to supplement the fourth instar (L4) larval diet with an additional source of nutrition, which will allow the larvae to progress to the pupal stage more quickly (*see* Note 19). If adult phenotypes are to be assessed, be sure to move the cups into an adult cage.
5. It is recommended that at least three biological replicate feeding experiments are performed for gene silencing quantification and for analysis of each phenotype to be assessed.

3.6 Confirmation of gene silencing by qRT-PCR:

1. Gene silencing can be confirmed with qRT-PCR, which can be performed according to your standard laboratory procedure or using the method summarized below, which has worked in our hands [11, 22, 23]. Perform and analyze qRT-PCR assays with at least three yeast feeding biological replicates, each with at least 10 pooled control vs. experimental larvae (we typically assess L4 animals) as described [11, 22, 23]. For analysis of a particular tissue type/body part (i.e. the brain), perform qRT-PCR following dissection to recover the tissue of interest (see Mysore et al. [24] for example and *see* Note 20).
2. Extract total RNA with Trizol reagent according to the manufacturer's instructions. Use the RNA to prepare cDNA with the High Capacity RNA to

cDNA Kit (Applied Biosystems) following directions supplied by the manufacturer.

3. Perform qRT-PCR using the SYBR green I PCR kit according to the manufacturer's instructions in conjunction with an Applied BioSystems Step One Plus Real-Time PCR System or a comparable model. For each biological replicate experiment, all PCR assays should be run in triplicate. Quantification of results is performed through comparison to reference standard transcript levels [11]. Transcript quantification data can be statistically analyzed using a t-test.

3.7 Confirmation of gene silencing by whole mount *in situ* hybridization:

1. *In situ* hybridization permits spatial confirmation of silencing in specific tissue or cell types. Representative results are shown in Fig. 4A1, A2, B1, B2, and C1, C2). Assess tissues prepared from 20 animals (we typically use L4 animals) in at least three biological replicate yeast feeding experiments. Execute *in situ* hybridization according to standard lab practice or in accordance with the Haugen *et al.* [33] protocol, which is summarized below.

2. Synthesize digoxygenin-labeled antisense and sense control riboprobes according to standard lab practice or using the DIG RNA Labeling and detection kit according to the manufacturer's directions, which are explained in further detail by Patel [34] (*see* Note 21).

3. A detailed protocol for tissue fixation and preparation is included in Clemons et al. [31]. In summary, larval tissues of interest are dissected and then fixed for 20 min in PEM-F (*see* Note 22).

4. Fixed tissues are then processed for detection of mRNA transcripts according to the method below, which was described previously in greater detail [33]. The fixed tissues can remain in eppendorf tubes throughout the procedure. Proceed with a 5 min rinse in PBS and 2× 10 min rinses in PTw. Use 1 ml wash volumes unless otherwise indicated.

5. After removing the PTw, add 1 ml of detergent solution (*see* Note 23) and incubate the tissues for 30 min with gentle rocking. After 30 min, remove the detergent solution and rinse the tissues 2× 10 min in PTw.

6. After removing the PTw, rinse the tissues 1× 5 min with 500 μl of 50% PTw-50% Hyb solution. Then replace the 50% PTw-50% Hyb with 500 μl of Hyb solution and incubate for 10 min. While the tissues are incubating in Hyb, boil an aliquot of the Hyb-DNA-SDS solution for 10 min in a heat block or boiling bath to denature the DNA; store this solution on ice while initiating step 7.

7. Following removal of the Hyb solution from the tissues, add 100 μl of denatured Hyb-DNA-SDS solution to the tissues and place the tubes in a floating rack located in a 60° C water bath for 60 min. A shaking bath is preferred, though not absolutely required, for blocking, overnight hybridization, and post-hybridization washes. Toward the end of the 60 min incubation, heat denature the riboprobe

suspended in Hyb-DNA-SDS solution by boiling it for 5 min in a heat block or boiling bath; store briefly on ice while initiating the next step.

8. Remove the Hyb-DNA-SDS blocking solution from the tissues and replace it with the riboprobe/Hyb-DNA-SDS solution. Gently stir the mixture with a pipette tip, and then place the tubes back in the 60° C water bath and continue with hybridization overnight.

9. In the morning, perform the following wash steps in the 60° C water bath using 1 ml volumes of solutions prewarmed in the 60° C bath: 1× 30 min with Hyb solution; 5× 30 min with PTw. After the last PTw wash, bring the tubes to room temperature and wash for an additional 30 min with PT.

10. Remove the PT wash solution and add 300 μl of AP-conjugated anti-DIG antibody diluted 1:2000 in PT. Gently stir the mixture with your pipette tip and then incubate overnight at 4° C.

11. The next day, rinse the tissue 4× 30 min with PT at room temperature.

12. After removing the PT, rinse the tissue 3× 5 min in AP buffer (*see* Note 24).

13. Remove the AP wash buffer and add 300 μl of AP-NBT-BCIP solution. The color reaction should be performed in the dark, monitoring the progress of the reaction by occasional visualization of the tissues with a dissection microscope. The reaction can take anywhere from several minutes to several hours to complete.

14. Stop the progress of the color reaction by removing the AP-NBT-BCIP and rinsing the tissues 4× 15 min with PT.

15. Remove the PT wash solution and rinse the tissues 1× 5 min with PBS. Following removal of the PBS solution, add 500 μl of 50% glycerol, which can be replaced after 60 min with 70% glycerol. Clear the tissues at room temperature overnight prior to mounting and analyzing the tissues in 70% glycerol. Silencing can be evaluated through transcript quantification analyses, which are performed through analysis of mean gray values (average signal intensity over the selected area) calculated for digoxigenin-labeled transcript signal in control or experimental brains [26, 27] (Fig. 4A3, B3, C3). These analyses can be performed with Adobe Photoshop or comparable image processing software. Results should be combined from at least three biological replicate experiments, each with tissues prepared from 10–20 animals. Transcript quantification data can be statistically analyzed using a t-test.

4. Notes:

1. We use the 5’ BamHI and 3’ XhoI sites in the in the *pRS426* vector and include sticky ends for cloning into these sites. Before using these sites, be sure to check that there are no internal cut sites for these enzymes in your shRNA expression insert.

2. If longer dsRNA molecules (200–400 bp) are preferred, then it is likely that the dsRNA expression system described by Murphy et al. [35] would be compatible with the yeast cultivation and mosquito oral feeding protocols described herein. However, for reasons outlined in the Introduction, we have opted to use shRNA in our own studies.

3. Although not necessary for most laboratory applications, one can also insert shRNA expression cassettes into vectors that are compatible with integration of the cassettes into the yeast genome. We have pursued this for strains that will be used in field trials [26], as it dispenses with the use of plasmids with antibiotic resistance markers and reduces the risk of horizontal transfer to other species.

4. Likewise, although not necessary for most laboratory applications, shRNA expression constructs can be placed under the control of an inducible promoter (i.e. *Gal1*), which allows for high levels of shRNA expression after integration of the expression cassettes into the yeast genome [26].

5. We have found it challenging to clone shRNA expression cassettes, presumably because a DNA oligonucleotide may bind to itself rather than to the complementary strand when the oligos are annealed prior to cloning. Likewise, although sequencing of the shRNA expression cassette clones is critical, it can also be challenging to sequence through hairpin-encoding sequence due to complications with secondary structure. We have combatted both these issues by testing multiple clones (sometimes 20 or more are necessary).

6. Although *E. coli* is readily transformed with double stranded plasmid DNA, yeast requires single stranded carrier DNA to enhance plasmid uptake.

7. PEG is prone to evaporation, which can alter the PEG:water ratio and lead to poor transformation efficiency. It may be helpful to make the PEG solution fresh for each transformation or to make it in small aliquots that are sealed tightly with parafilm.

8. Frozen glycerol stocks should be prepared from each transformant yeast strain. If you will be working with the same clones over an extended period, then it is helpful to prepare two frozen samples from each strain, one that researchers will open more frequently, and one that is set aside for long-term permanent storage. It is also useful to store the DNA plasmid in case the yeast needs to be re-transformed.

9. For the best results, use yeast that has been plated from a frozen stock on SCD-ura media plates (poured generously so that the media doesn’t dry and crack) within the past week.

10. If you initiate the pre-culture in the late afternoon, it will be ready at approximately the same time the following day.

11. The culture will likely need to be diluted 1:5 using SCD-ura medium prior to taking the OD_{600} reading. The culture will likely need to grow for 42–48 hrs to reach an appropriate OD_{600} reading.

12. If the OD_{600} reading is less than 2.5, then let the culture grow longer. Do not harvest the culture too early.

13. If you are using a centrifuge that can spin at a higher speed, then it may be possible to reduce the length of this step.

14. It is important to use a 2 mL eppendorf tube rather than a 1.5 mL tube at this step. The 2 mL tube has a larger conical surface area that permits better drying of the yeast.

15. It is useful to perform this step in several batches, as it is easiest to transfer a warmed pellet.

16. It is important to dry the yeast thoroughly. When the yeast pellet is under-dried, it can become sticky, which makes it hard to remove from the tube and more difficult to use in feeding assays. Failure to dry the pellet thoroughly may also decrease the shelf life of the yeast interfering RNA.

17. You may wish to plan your experiments so that the larvae do not pupariate over the weekend. In our insectary, this means that we would begin experiments on a Tuesday or a Wednesday.

18. For best results, hatch *Aedes* eggs in deoxygenated water, which facilitates synchronized hatching of diapaused eggs. To deoxygenate the water, it can be autoclaved, or the eggs can be hatched in water placed under vacuum pressure. *A. aegypti* eggs that are less than one month old typically hatch within 30 min at 25 psi.

19. For *Aedes*, during L4, we typically add 100 μl of a 60 g/L suspension of bovine liver powder in sterile ultrapure water (store at 4° C). For *A. gambiae,* during L4, we have sprinkled ~10 mg of a 1:1 mixture of liver powder and nutritional yeast at the water surface. Other larval foods can likely be substituted depending on the preferred laboratory rearing protocol.

20. Examination of gene expression can be complicated because larvae will grow at different rates depending on how much food is eaten by each individual animal, and expression of many developmental genes is very dynamic. For some developmental genes, we find that expression levels differ widely even in individual control or untreated animals that were age-synchronized at the beginning of the experiment. Preparation of a specific tissue rather than use of whole animals may improve results and can be worth the effort. For some larval transcripts, we have found that *in situ* hybridization experiments can detect significant levels of silencing which correspond to strong and penetrant phenotypes, but that qRT-PCR experiments have at times failed to detect.

21. If multiple transcripts are expressed, be sure to synthesize a probe that corresponds to the transcript(s) of interest.

22. For some tissues, modification of the fixation solution and an overnight fixation procedure will yield better results. For example, we fix larval brains overnight at 4° C in 1x PBS containing 1% Triton-X and 7.4 % formaldehyde.

23. This SDS detergent step substitutes for the proteinase K treatment steps found in many *in situ* hybridization protocols and gives more consistent results than enzymatic treatment of tissues for permeabilization.

24. It is important to perform this step as described. We have noted that rinsing the tissues too quickly can significantly lengthen color reaction times.

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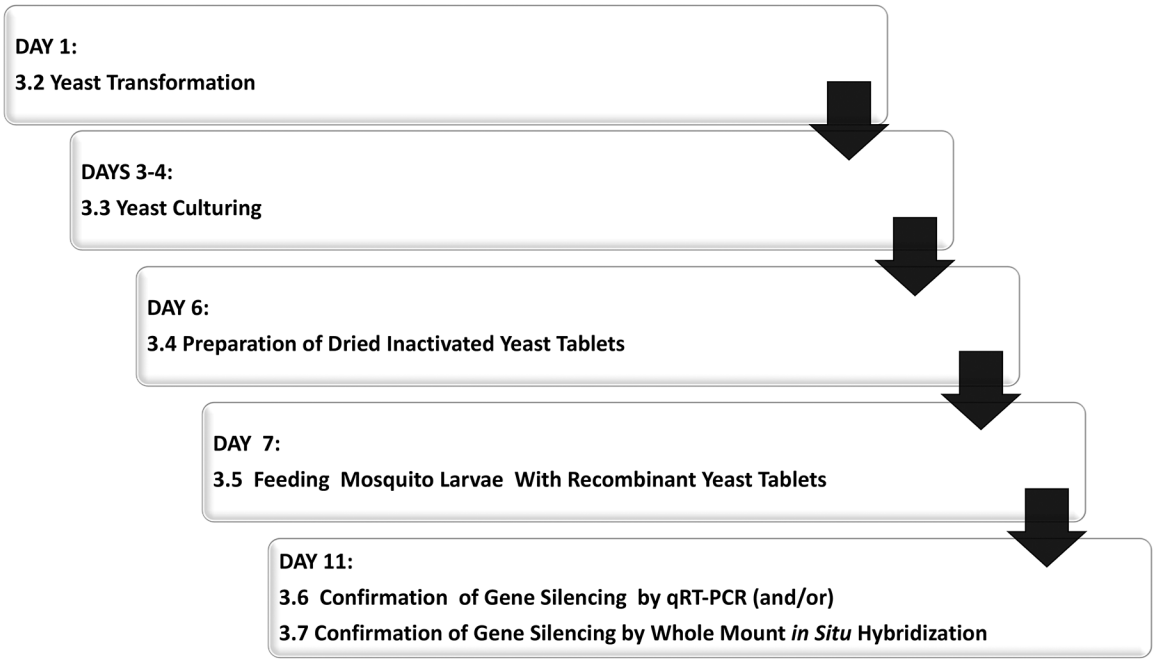

Fig. 1. Experimental Timeline.

The sequence of experimental events over an ~11 day experimental timeline is presented for Steps 3.2 through 3.7, which initiate following preparation of the shRNA expression construct (Step. 3.1) and conclude with analysis of silencing in fourth instar larvae.

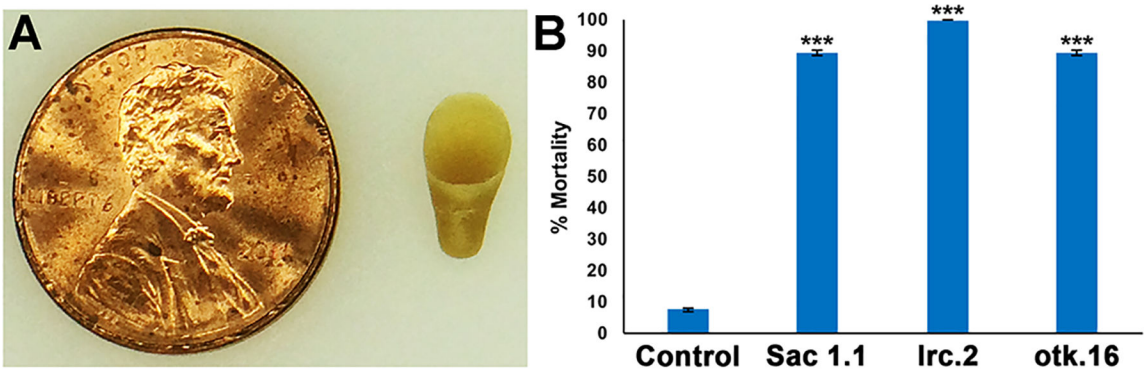

Fig. 2. Yeast interfering RNA tablets induce significant *A. gambiae* larval death.

Dried inactivated yeast interfering RNA tablets (A; penny shown for scale) were prepared and fed to 20 *A. gambiae* larvae. Significant death was observed in larvae fed with yeast expressing shRNA hairpins corresponding to the *Sac1, Irc,* and *otk* genes as compared to larvae fed control yeast interfering RNA tablets. These data were compiled from three biological replicate experiments (n = 240 larvae total/condition) and analyzed by ANOVA with Tukey's multiple comparison test. ***=$p<0.001$ as compared to control-fed larvae; error bars denote standard error of the mean (SEM). Reproduced through open access from reference [27].

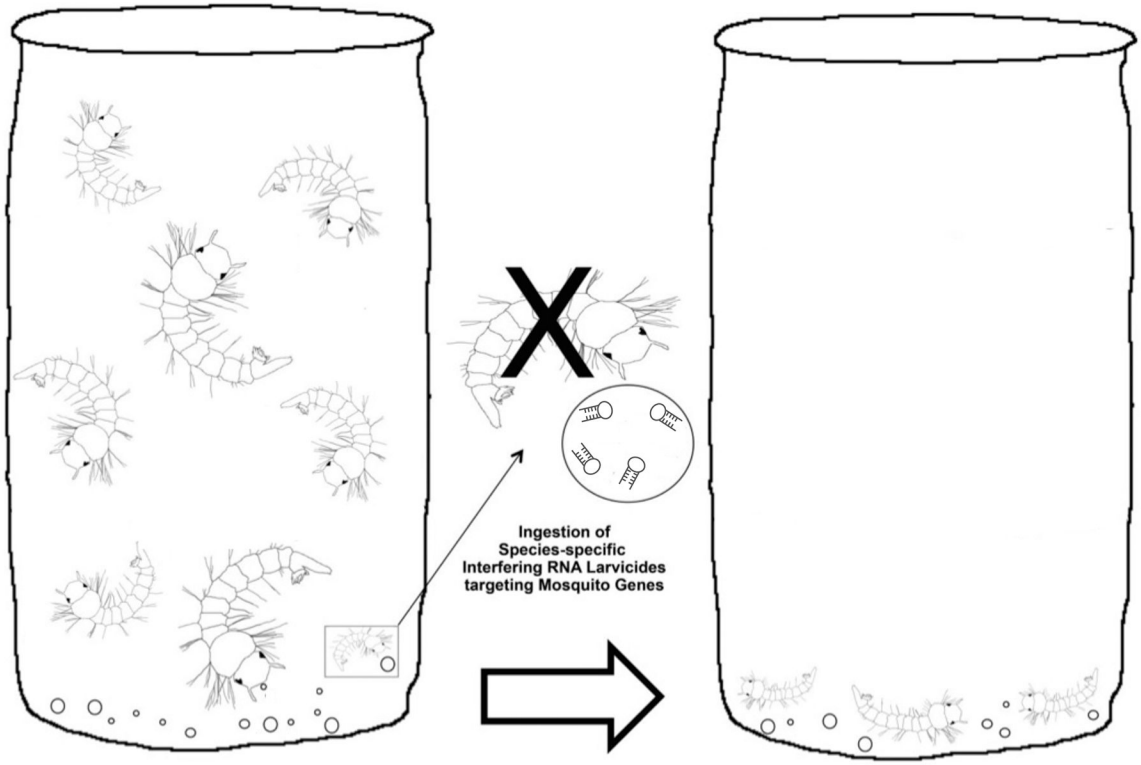

Fig. 3. Mosquito larval oral feeding assays.

Larvae placed in a beaker consume yeast interfering RNA tablets. This procedure can be used to assay the impact of gene silencing on various larval phenotypes, including larval death.

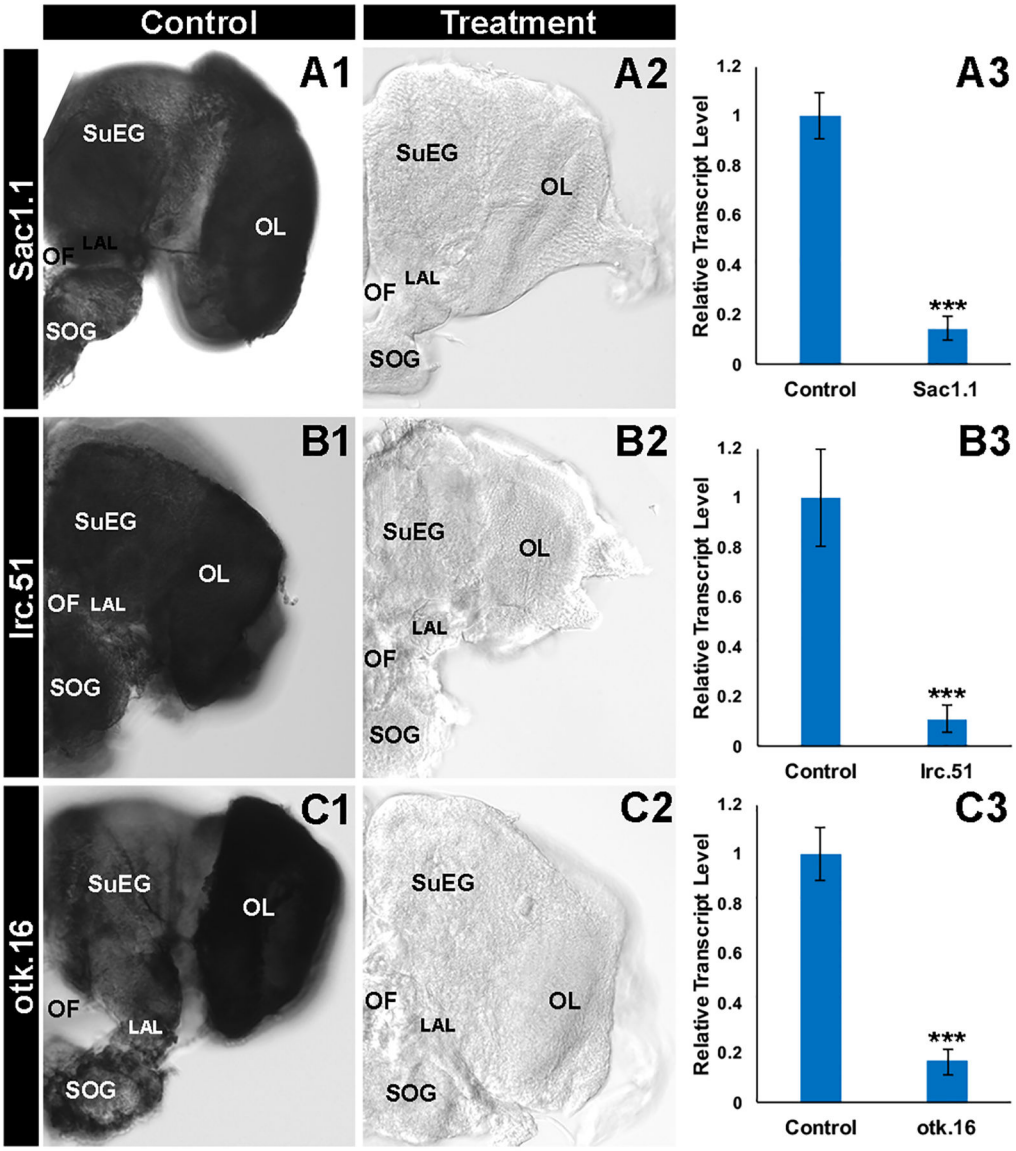

Fig. 4. Confirmed silencing of the *Sac1*, *lrc*, and *otk* genes in the *A. gambiae* larval brain by dried inactivated yeast interfering RNA tablets.

Significantly lower *Sac1* (A1–A3), *lrc* (B1–B3), and *otk* (C1–C3) transcript levels were detected through *in situ* hybridization in the L4 brains of larvae fed dried inactivated yeast interfering RNA tablets corresponding to the *Sac1* (A1), *lrc* (B1), and *otk* (C1) genes vs. animals fed with control yeast interfering RNA tablets (A2, B2, C2). For each probe, results from three biological replicate experiments were compiled (n=85 total brains from larvae treated with the *Sac1* interfering RNA tablet, n=80 total brains from larvae treated with the *lrc* interfering RNA tablet, and n=80 brains from larvae treated with the *otk* interfering RNA tablets; n=40 brains from control-treated larvae/per experiment). Data were evaluated by the Student's t-test. All brains are oriented dorsal upward in this figure. **LAL:** Larval antennal lobe; **OF:** Olfactory foramen; **OL:** Optic lobe; **SOG:** Sub-oesophageal ganglion; **SuEG:** Supra-oesophageal ganglion. Reproduced through open access from reference [27].