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TITLE:

Microinjection of Corn Planthopper, *Peregrinus maidis*, Embryos for CRISPR/Cas9 Genome Editing

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KEYWORDS:

embryonic microinjection, functional genomics, germline transformation, CRISPR/Cas9 genome editing, corn planthopper, Hemiptera

SUMMARY:

Herein are protocols for collecting and microinjecting precellular corn planthopper embryos for the purpose of modifying their genome via CRISPR/Cas9-based genome editing or for the addition of marked transposable elements through germline transformation.

ABSTRACT:

The corn planthopper, *Peregrinus maidis,* is a pest of maize and a vector of several maize viruses. Previously published methods describe the triggering of RNA interference (RNAi) in *P. maidis* through microinjection of double-stranded RNAs (dsRNAs) into nymphs and adults. Despite the power of RNAi, phenotypes generated via this technique are transient and lack long-term Mendelian inheritance. Therefore, the *P. maidis* toolbox needs to be expanded to include functional genomic tools that would enable the production of stable mutant strains, opening the door for researchers to bring new control methods to bear on this economically important pest. However, unlike the dsRNAs used for RNAi, the components used in CRISPR/Cas9-based genome editing and germline transformation do not easily cross cell membranes. As a result, plasmid DNAs, RNAs, and/or proteins must be microinjected into embryos before the embryo cellularizes,

making the timing of injection a critical factor for success. To that end, an agarose-based egg-lay method was developed to allow embryos to be harvested from *P. maidis* females at relatively short intervals. Herein are provided detailed protocols for collecting and microinjecting precellular *P. maidis* embryos with CRISPR components (Cas9 nuclease that has been complexed with guide RNAs), and results of Cas9-based gene knockout of a *P. maidis* eye-color gene, *white*, are presented. Although these protocols describe CRISPR/Cas9-genome editing in *P. maidis*, they can also be used for producing transgenic *P. maidis* via germline transformation by simply changing the composition of the injection solution.

INTRODUCTION:

The corn planthopper, *Peregrinus maidis,* is an economically important pest of maize[1-3]. They cause direct physical damage to the plant, both while feeding with their piercing-sucking mouthparts, and during reproduction when they lay their embryos directly into plant tissue[2,4]. Despite the multiple routes of direct damage to crops, the greatest impact these insects have on crop health is indirect, by acting as the vector of maize mosaic virus (MMV) and maize stripe virus (MSpV)[5,6]. MMV is capable of replicating in the body of its *P. maidis* vector, allowing the virus to persist in individual insects through the entirety of their lives, so they can continue to spread the virus to new host plants[7,8]. The most common methods for controlling *P. maidis*, and thus the viruses it vectors, are insecticides.

Unfortunately, mismanagement of these products has caused development of resistance in the target pest as well as pollution of the environment[9]. Therefore, new strategies are needed to reduce crop losses from this insect/virus-pest combination. Previous work demonstrated that RNA interference (RNAi) could be an effective control method for *P. maidis* because they are susceptible to downregulation in gene expression even when ingesting double-stranded RNA (dsRNA)[10]. However, the most effective way to administer dsRNA in the field would be through the plants the insects feed on; hence, crops could still be susceptible to any viruses the insects are already carrying. With the advent of CRISPR/Cas9 genome editing, new pest control strategies are possible, including Cas9-based gene drive[11,12], which could be used to reduce the size of a pest population, or to replace said population with individuals resistant to the viruses they vector.

However, development and deployment of any type of gene-drive system will require the development of transgenic techniques. Such methods were not necessary for carrying out RNAi experiments in *P. maidis* because dsRNAs and/or siRNAs are presumed to be able to cross cell membranes due to the efficiency of RNAi in *P. maidis*[10,13]. This is not true for the DNAs and/or proteins employed in traditional transgenesis or in Cas9-based gene editing, either of which would be a precursor to creating insects carrying a gene drive. To accomplish gene editing or other forms of germline transformation, these DNAs and proteins are ideally microinjected into embryos during the syncytial blastoderm stage, prior to when the insect embryo cellularizes. Timing is critical, because the syncytial stage is the earliest part of development[14,15]. As *P. maidis* females preferentially lay their eggs in plant tissue, extracting sufficient quantities of precellular embryos for microinjections can be labor-intensive and time-consuming. Therefore, new techniques were developed to quickly collect and microinject *P. maidis* embryos prior to cellularization.

PROTOCOL:

1. Colony-level rearing of *P. maidis* adults

1.1. Plant a minimum of four pots of corn per week per rearing cage, with 3–4 seeds per pot. Grow in an insect-free environment.

1.2. When plants are ~5 weeks old, place inside a 30 cm x 30 cm x 60 cm cage.

1.3. Obtain a sufficient quantity of *P. maidis* adults (~500) from a research laboratory or the wild, and place into an insect-proof cage with 9–12 corn plants (3–4 pots).

1.4. Maintain the colony in an insect-rearing incubator at 25 °C (± 1 °C), with at least 70% humidity and a 14:10 light cycle.

1.5. To generate an age-calibrated colony, remove all initial adults after four days of egg laying, and allow the embryos laid in the cage to hatch and age naturally.

1.6. Move 5-week-old *P. maidis* insects (adults) to fresh corn plants for weekly subculture by collecting with an aspirator (**Figure 1**). Then, release the adults into a clean cage with fresh corn plants. To maintain a steady supply of young adults for experimental purposes, prepare fresh age-calibrated cages every week.

1.7. Water the pots in the cages twice daily. Periodically clip stalks, remove decaying plant material, and replace with fresh corn pots as needed.

NOTE: With proper maintenance, a colony can last ~5 weeks (*i.e.*, long enough for embryos laid in the cage to become adults).

2. Agarose-based egg-lay chamber

2.1. Make egg collection dishes (oviposition medium) by pouring 1% w/v agarose in water into clean 100 mm x 15 mm Petri dishes. Store the oviposition medium at 4 °C after it solidifies.

2.2. Prepare 10% w/v sucrose solution for feeding the adults. Store the sucrose solution at -20 °C for up to a month.

2.3. Make a chamber to hold the adults by cutting a hole in the bottom of a 1 oz cup (see the **Table of Materials**) and gluing a screen over the hole for air exchange (**Figure 2**).

2.4. Cut plastic paraffin wax film into 5 cm x 5 cm squares; set aside 2 squares for each cup.

2.5. Collect ~15 1-week-old adult females from an age-calibrated *P. maidis* colony. To select

females, examine the ventral side of the abdomen, and look for the ovipositor, which is typically darker than the rest of the abdomen (**Figure 3**). Hold adults for up to one hour in a 50 mL conical vial if setting up multiple egg-laying chambers. Chill the insects briefly on ice prior to sexing and transfer to the adult container.

NOTE: This examination can be done without a microscope. Adult females that have had time to feed and mate also typically have larger abdomens than adult males and are more docile; hence, they can more easily be selected from a cage population.

2.6. Transfer the females into an adult container, and seal the cup with 1 layer of plastic paraffin wax film by evenly stretching it 3–4 times its original size (**Figure 4A,B**).

2.7. Apply 400 µL of 10% w/v sucrose solution to the top of the plastic paraffin wax film seal, and add a second layer of plastic paraffin wax film, stretching the plastic paraffin wax film exactly as above (**Figure 4C,D**).

NOTE: The sandwich of stretched plastic paraffin wax film pressurizes the sucrose solution, which is very important for adult feeding, but will not prevent females from piercing their ovipositors all the way through into the oviposition medium.

2.8. Place the adult chamber on an egg collection dish with the plastic paraffin wax film side directly on the oviposition medium, and wrap the entire egg-laying chamber with plastic wrap without covering the air holes as these are required for air exchange (**Figure 5**).

2.9. Incubate each egg-laying chamber at 25 °C with 70% humidity and a 14:10 light cycle.

2.10. Change the sandwich of plastic paraffin wax film and 10% w/v sucrose solution daily, and remove any water that accumulates inside the cup.

3. Embryo collection and alignment in a high-humidity environment

3.1. Set up a stereomicroscope-based microinjection system in a humidified space or hood (humidified hood; **Figure 6**) to ensure the working environment achieves at least 70% humidity throughout the microinjection process.

3.2. Check the oviposition medium for eggs after the desired egg-lay period. Do this in a humidified hood or another humid environment.

NOTE: The egg-lay period typically used was overnight, from 6 PM to 10 AM, lasting ~16 h.

3.3. If any eggs are laid in the agarose, use fine forceps to carefully dig them out, and place them on the surface of the agarose to keep them moist (**Figure 7A**).

3.4. Apply a strip of 1 mm x 15 mm double-sided tape on a 22 mm x 30 mm coverslip (**Figure 7B**).

Place the coverslip tape-side up on the oviposition medium (**Figure 7C**).

3.5. Pick up each individual egg from the agar surface, and move to the double-sided tape using a fine brush. Remove any eggs that are completely white or have black coloration on them. Observe the semi-transparent nature of healthy eggs.

3.6. Place the banana-shaped eggs on their side, with the larger end stuck on the double-sided tape (**Figure 7D**).

NOTE: Always keep the eggs in a high-humidity environment, such as a Petri dish cast with a layer of 1% agar on the bottom.

4. Preparation of CRISPR reagents and injection needles

4.1. Pull quartz needles using a Flaming/Brown type micropipette puller.

4.2. Bevel the quartz needles using a micropipette beveler.

4.3. Use double-sided sticky tape to secure pulled needles in a clear container, such as a Petri dish, until ready to use.

4.4. Prepare the injection solution by combining 0.5 µL of Cas9 protein (5 µg/µL stock solution) and 0.5 µL of sgRNA (4 µg/µL stock solution; see the **Table of Materials**) with 1 µL of phenol red buffer in a final volume of 5 µL. To precipitate particles that could clog the needle, vortex the solution briefly, and centrifuge for 3 min at maximum speed.

4.5. Backfill the injection needle, taking care to leave the injection mix near the tapered end of the needle. Remove bubbles, if any, from the tip of the needle.

4.6. Carefully place the backfilled needle into the needle holder, and tighten the stainless steel collar to hold the needle securely in place during microinjection.

4.7. Generate a reliable flow of injection solution from the needle by gently stroking the beveled tip with a fine, dampened paintbrush, while delivering bursts of air pressure to the needle with the injection system.

NOTE: The needle is ready for injection when the injection mix can leave the tip in small amounts.

5. Microinjection and post-injection care

5.1. Prepare a microinjection platform by filling a clean 100 mm x 15 mm Petri dish with 1% agar to form a level layer of agar that is flush with the top of the dish.

5.2. Place a previously prepared coverslip with ~25 embryos upon the agar platform (**Figure 8A**).

NOTE: All injection steps must be performed inside a humidified hood (~70% humidity).

5.3. Check the injection pressure by placing the needle tip in a drop of water and initiating the injection cycle.

NOTE: A small amount of injection solution should disperse into the water if the pressure setting is correct (**Figure 8B**).

5.4. Insert the needle into the larger end of the embryo, approaching from the left side of the coverslip (**Figure 8C**). Deliver the injection solution into the egg, and pull out the needle quickly.

5.5. After all eggs are injected, place the coverslip on the surface of a new 1% agar dish, and transfer the dish to a humidity chamber (**Figure 9**).

6. Incubating and hatching of embryos

6.1. Put the hatching chamber in a 25 °C incubator for 6 days.

6.2. Transfer any surviving embryos, using clean water and a fine brush, to a 35 mm x 10 mm Petri dish with water-moistened filter paper covering the bottom of the dish. Seal the Petri dish with plastic paraffin wax film, and hold at 25 °C to allow the embryos to hatch. Start checking the embryos 6 days post-injection for survival.

NOTE: First instar nymphs will start hatching around day 8.

6.3. Transfer nymphs, using a fine brush, to a Petri dish containing leaf clippings. Cover the dish, and seal with plastic paraffin wax film.

6.4. Incubate the sealed dish of hatchlings on leaf cuttings for 48 h at 25 °C.

6.5. Transfer all 2-day-old nymphs from a round of injections to a rearing cage with corn plants using a fine brush. If injectees with visible phenotype are recovered in sufficient numbers, rear them separately to maximize the recovery of the target trait in the next generation. Otherwise, perform mass-mating of all the injectees.

NOTE: Place hatchlings gently in the whorl of the corn plant to provide refuge and ensure proper humidity of their immediate environment.

6.6. Rear the insects in the conditions described above, ensuring proper temperature, humidity, and regular transfers to fresh corn plants.

6.7. Screen progeny for expected phenotypes. Place individuals exhibiting the desired phenotype into their own cage to establish homozygous lines.

REPRESENTATIVE RESULTS:

The egg-lay chamber was specifically designed to enable *P. maidis* females to feed while ovipositing in a protective medium from which their eggs could easily be recovered. Using this method, sufficient quantities of precellular embryos were recovered for microinjection with DNA, RNA, and/or proteins. Adult *P. maidis* females usually lay eggs inside the leaf tissue of the corn plants, which makes getting enough eggs in a short amount of time a challenge because it requires a lot of leaf dissection. The artificial egg-laying environment provides a solution to overcome these problems. As shown in **Table 1**, 6,483 eggs were collected from a total of 645 females in 4 weeks. Females usually start laying eggs after day 2 and provide most eggs from day 4 to day 6. Oviposition activity slowed down by day 9. Each oviposition chamber was set up on Friday and checked for eggs from Sunday until the next Sunday. Following this schedule allowed most eggs to be collected for microinjections during the work week.

The first practical application of this egg-laying system was to test the efficacy of Cas9-mediated gene knockout, using the *P. maidis* ortholog of the eye-color gene, *white*, as a target. Mutations in *white* are known to result in substantial loss of eye pigmentation in other insect species, and White is cell-autonomous, allowing mutations to be detected in injected individuals[16,17]. To increase the chance that even a small mutation might result in loss of function, guide RNAs were designed to cut within the region of the ATP-binding cassette which is necessary for White function[16]. *P. maidis* embryos were injected with either 20% phenol red (injection buffer), injection buffer with Cas9 at a final concentration of 800 ng/µL (Cas9 control), or Cas9 in injection buffer along with three guide RNAs added at a concentration of 400 ng/µL each. The combination of three guides within one injection mix was intended to further maximize the chances of generating mutants, both by creating a large deletion, and by compensating for the possibility that any one guide might be ineffective for cutting.

The developmental rates for each treatment were comparable (**Table 2**), with 50–60% of injected individuals showing signs of development. Hatch rates for the buffer and Cas9 controls were also comparable; however, the hatch rates of individuals receiving the three-guide mix was relatively lower. At this time, it is unclear if the reduced survival is the result of the loss of *white* function or the result of unintended consequences of the three-guide mix, such as off-target effects (see the discussion section). However, none of the individuals with complete loss of eye pigmentation (*i.e.*, complete knockout) hatched, and none of the progeny of injected individuals had white eyes. The on-target efficacy of Cas9-based mutagenesis was verified two ways. First, injectees receiving the three-guide mix with Cas9 were screened for loss of eye pigmentation.

Of the 71 guide-injected individuals that developed, 23 showed some degree of pigment loss (**Figure 10**), and 9 of those individuals hatched, resulting in a knockout rate of ≥32%. No eye pigment loss was observed in either control treatment. Second, chromosomal mutations were confirmed via polymerase chain reaction (PCR)[18] and sequencing[19]. Because a mutant line could not be recovered, genomic DNA was analyzed from pools of embryos injected with either the three-guide mix or buffer. The three-guide mix is expected to remove ~180 base pairs from the *white* locus. This can be seen in the PCR products amplified off of genomic DNA isolated from

injected individuals, as well as the associated sequence data generated from those products (**Figure 11**). This combined evidence indicates that embryos were injected before cellularization occurred.

FIGURE AND TABLE LEGENDS:

Figure 1: Aspirator. An effective aspirator can be assembled from attaching a vacuum pump at the intake, via plastic tubing, to a 15 mL plastic conical tube. Approximately 0.5 cm should be carefully removed from the bottom of the conical tube. A cotton ball should be placed in the conical tube, over the opening of the plastic tubing, to catch *P. maidis* adults as they are collected and keep them out of the vacuum pump.

Figure 2: Construction of adult containers. (**A**) The supplies needed (clockwise from top left): screen, hot glue gun, razor blade, 1 oz container. (**B**) A large hole should be cut in the bottom of the 1 oz container, and a square of screen is cut just large enough to cover this hole. (**C**) The screen is then glued over the hole using hot glue. (**D**) Once the glue is set, any excess mesh should be removed.

Figure 3: Sexing *P. maidis* adults. The ventral sides of male (left) and female (right) *P. maidis* adults are shown. The ovipositor, visible over the female abdomen, is the clearest indicator of the sex of an individual.

Figure 4: Sealing adults into containers. (**A**) A 5 cm x 5 cm square of plastic paraffin wax film. (**B**) The film should be stretched evenly to 3–4 times its original size. (**C**) Once adults have been put into the adult container, the stretched film should be placed over the opening to secure the adults. A 400 µL drop of 10% w/v sucrose solution should then be placed on top of the film. (**D**) To provide adequate feeding pressure for the adults, a second 5 cm x 5 cm square of plastic paraffin film should be similarly stretched and placed over the drop of sucrose.

Figure 5: Setting up an oviposition chamber. (**A**) The supplies needed (clockwise from top left): plastic wrap, a completed adult container (with adults), and a Petri dish with 1% agarose (oviposition medium). (**B**) The adult container should be placed onto the agarose with the plastic paraffin film/10% sucrose 'sandwich' placed directly on the oviposition medium. (**C**) Plastic wrap is used to secure the adult container to the oviposition medium. This keeps the medium from drying out too quickly. (**D**) Care should be taken to avoid covering the screen of the adult container, so that air exchange may still continue. (**E**) Diagram of the oviposition chamber.

Figure 6: Humidified hood. A hood outfitted with a humidifier has been set up around the injection scope to minimize air drafts and maintain humidity while the embryos are being handled. Flaps can be folded over the entrance after the worker is in place, to aid in maintaining proper humidity levels.

Figure 7: Collecting embryos in preparation for injections. (**A**) Embryos that have been deposited in the oviposition medium. A pair of fine forceps are used to extract embryos from the medium

and placed on its surface. (**B**) A narrow strip of 1 mm x 15 mm double-sided tape on a 22 mm x 30 mm coverslip. (**C**) The coverslip can be placed on the oviposition medium for ease of transferring embryos from the surface of the medium to the tape on the coverslip. (**D**) *P. maidis* embryos are banana shaped, with one end narrower than the other (narrow end indicated with red arrow head; wider end indicated with yellow arrow head in example embryo). The wide end of the embryo should be placed on the tape.

Figure 8: Injection. (**A**) The injection platform is a Petri dish filled to the brim with 1% agar. The coverslip with a strip of tape holding embryos should be placed directly on the surface of the injection platform. (**B**) The injection pressure should be tested before embryos are injected by 'injecting' a small amount of injection solution into a drop of water. This method can also be used at any time during the injection process to check the needle for clogs. (**C**) Embryos should be injected by inserting the needle into the larger end of the embryo. Injection solution should be visible if the injection was successful.

Figure 9: Post-injection care. (**A**) Once all the embryos on a coverslip have been injected, the coverslip should be placed in a fresh Petri dish containing 1% agarose. (**B**) The Petri dish with the coverslip can then be maintained in a humidity chamber (like the one shown) until embryos hatch.

Figure 10: *Pmw* knockout phenotype. (**A**) Age-matched control and (**B**) *Pmw* knockout embryos, with developing eyes indicated by black arrowheads. The embryo in **B** is mosaic, as a small stripe of pigmentation can be seen. (**C**) Age-matched control and (**D**) *Pmw* knockout hatchlings, with insets showing a different angle on the eyes. The hatchling in **D** is also mosaic. A white arrow points to an area in the main picture showing loss of pigmentation.

Figure 11: *Pmw* knockout sequence. (**A**) To-scale model of *Pmw* mRNA, marked in 500-bp increments, with locations of gRNA binding sites indicated: G1, blue; G2, yellow; G3, pink. Any frame-shift mutations generated at this point will disrupt the majority of the translation product. (**B**) Genomic context of gRNA sites, all in one exon (bold capitalized text). Guide binding sites are highlighted in the same colors as **A**, and PAMs are underlined. Span is ~300 bp. The in-frame translation of the exon is shown above, as single-letter abbreviations in capital text. Two motifs specific to eye pigment transporters are marked. The CDEPT motif of the Walker B functional domain is boxed in purple, and the IHQP motif of the H-loop domain is boxed in green. Both domains are critical to ATP-transporter function. (**C**) The *Pmw* target region was amplified using two rounds of PCR. The second-round product was examined on a gel for evidence of size-shift due to successful removal of the region between the guides. Lanes: L = 100 bp ladder; 1 = PCR water control; 2 = Buffer injected eggs; 3–4 = two separate sets of eggs injected with three-guide mix. Only embryos receiving the three-guide mix produced both the WT band (red arrow) and the band resulting from a complete excision (white arrow). (**D**) To confirm the identity of the lower (white arrow) band, this DNA was purified, cloned, and sequenced. The top line is the wild-type sequence. The other two lines are sequences from two clones. Three additional clones matched the bottom sequence. Blue highlight indicates the binding site of Guide 1, while pink highlight indicates the binding site of Guide 3. In both alleles, the entire region between these

two guide sites has been deleted. Abbreviations: *Pmw* = *Peregrinus maidis white* gene; gRNA = guide RNA; PAM = protospacer adjacent motif; ATP = adenosine triphosphate; PCR = polymerase chain reaction; WT = wild-type; KO = knockout.

Table 1: Representative egg collections from artificial oviposition environment. Data from four sets of egg-collection cups are shown, with egg tallies starting on the second day after setup and running through the ninth day.

Table2: Survival and knockout rates from injections of 3 different injection mixes.

DISCUSSION:

Egg-lay quality and nutrition

Recently, researchers working with a related species, *Nilaparvata lugens*, obtained the eggs they used for microinjections directly from the leaf, keeping the injected eggs in the leaf tissue until they hatched[17]. While this leaf-based method provided a more natural environment for embryonic development, it also increased the chances for infections and for egg damage during the removal process. The artificial oviposition system presented here provides a more uniform environment and reduces the chances of damage to the eggs from handling. By setting up the oviposition cups on Friday, the majority of the oviposited eggs were collected during a typical work week, to the benefit of those doing the microinjection work. One caveat to this method, however, is that the lack of nutrients in the 10% sucrose solution diet will eventually affect the health of the insects, and females in the cups usually start to die off after only 10 days. Egg quality also begins to drop off after 6 days, as evidenced by an increase in dead or unhealthy looking eggs. As a result, it is important to be selective of the eggs used for microinjections and to not keep the females after day 6.

Survival rate and humidity

Two factors seem to be critical for embryonic survival through the microinjection process. The most challenging aspect of handling *P. maidis* embryos is keeping them from desiccating after removal from the oviposition medium and throughout microinjection. As the eggs are typically laid inside plant tissue, they lack an adequate shell to prevent dehydration. Even in the humidified hood whole sets of eggs were lost due to desiccation. However, excessively high humidity could also affect the microinjections if water accumulated on the double-sided tape or on the scope. Unfortunately, egg dehydration was usually not easy to notice during the microinjection process, and they frequently appeared normal until 2 or 3 days later, when they turned completely transparent, showing no signs of development.

Needle quality also appears to play an important role in survival. The needle should be beveled to minimize unnecessary damage to the egg. When the needle is blocked, using the clearing function on the injector while gently stroking the tip of the needle with a dampened paintbrush (see step 4.7) typically returned the needle to a functional state. Regardless, putting only tiny amounts of injection solution (~0.25 µL) into each needle and switching to a new needle every few slides (~50–60 eggs) is recommended to ensure needle quality is maintained throughout the injection process.

Successful generation of a knockout phenotype

To successfully transform the germ cells, embryo microinjections usually have to be done as early as possible before cellularization. Depending on the insect species, the time window for completing the microinjections ranges from only a couple of hours to as long as a full day[14,15,20]. It is still unclear when *P. maidis* embryos undergo cellularization. Cas9-mediated knockout was tested on embryos as young as 4 h post-egg-lay (pel) to as many as 16 h pel, and the expected phenotypes were observed in all experiments, suggesting that all microinjections were performed within the precellurization window.

The *P. maidis* ortholog of the eye-color gene, *white*, was selected because the knockout phenotype was expected to be easy to screen in injectees due to its cell-autonomous nature. Indeed, as expected, both mosaic and total knockouts were clearly identifiable among embryos receiving the injection mixture containing Cas9 and guide RNAs. Unfortunately, no injectees with complete knockout hatched, and a mass mating of surviving injectees failed to generate white-eyed progeny. However, a mutant line was later successfully generated by targeting a different gene (Klobasa et al., in progress). This would suggest that the failure to establish a *white* mutant line is most likely due to either off-target effects (*i.e.*, Cas9 cutting important regions elsewhere in the genome) generating a closely-linked lethal mutation, or to an unpredicted critical role for *white* in *P. maidis*.

Phenotypic and molecular data (**Figure 8** and **Figure 9**) affirm that a significant knockout in the *white* locus was created in a sample of injected embryos, which would result in total loss of gene function. Moreover, while mutations in *white* are viable in some species, there is precedent for reduced *white* activity being detrimental[21,22]. That said, off-target effects cannot be completely ruled out. Predicting likely off-targets requires accurate genome sequence data[23], which the current state of genomic resources in *P. maidis* makes impossible to do at this time. Regardless, with these new methods, testing other target genes can be done confidently, even moving towards more traditional transgenesis in an effort to bring new genetic tools to this pernicious pest.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure_1.pd

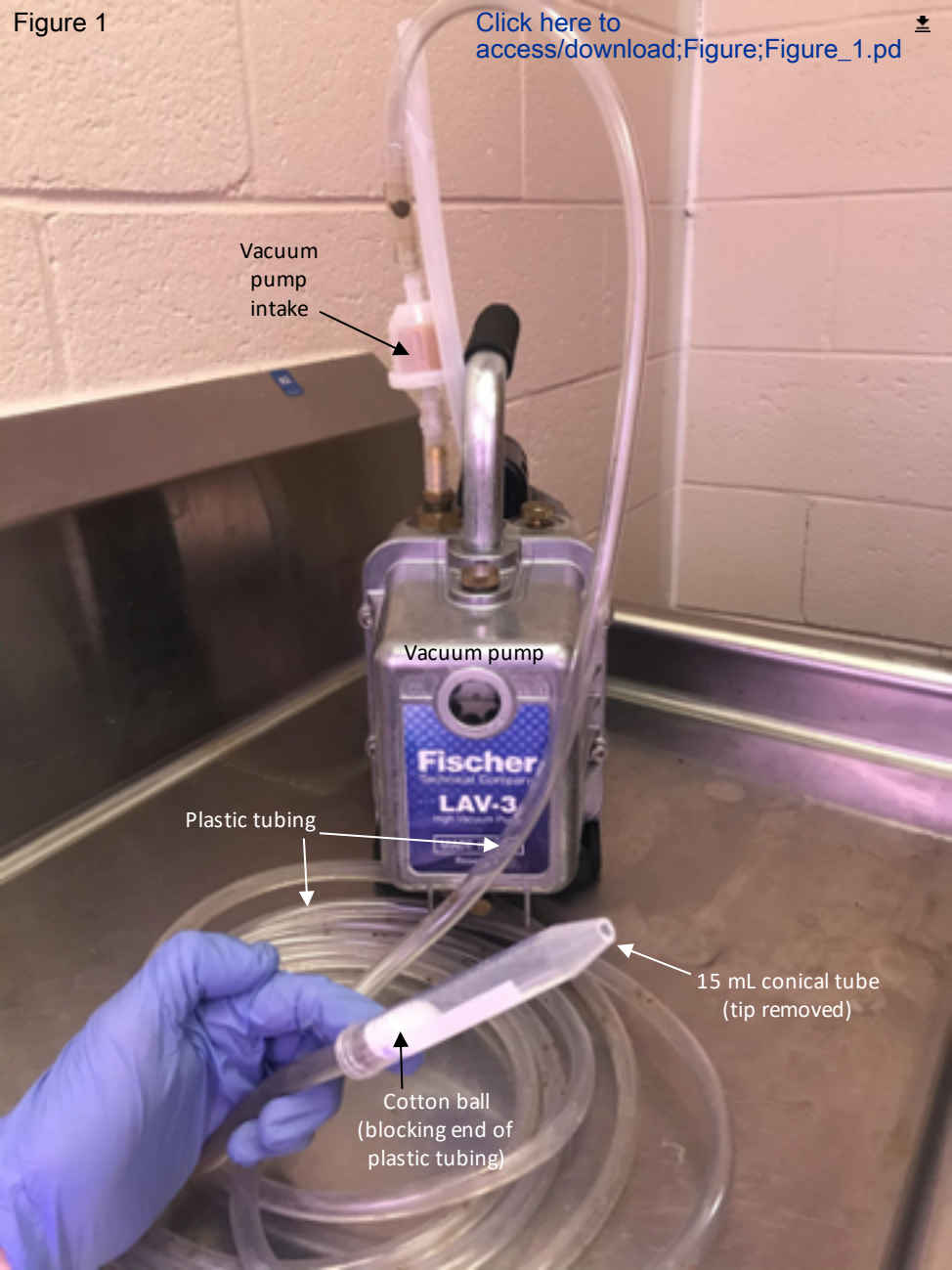

Figure 2

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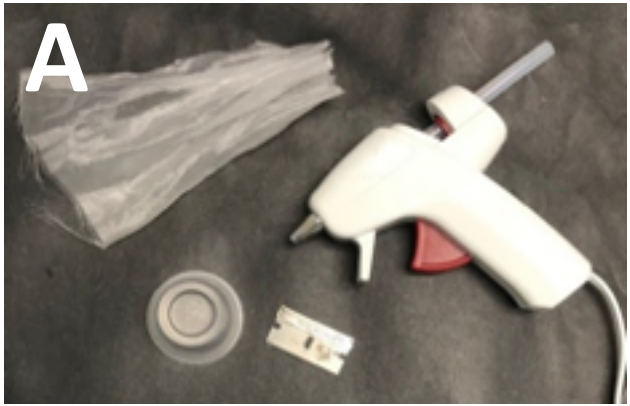

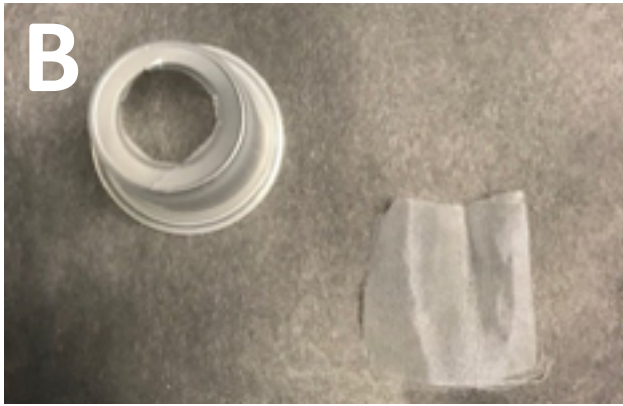

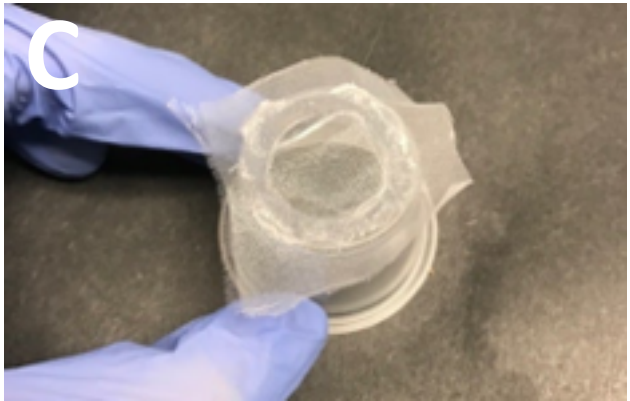

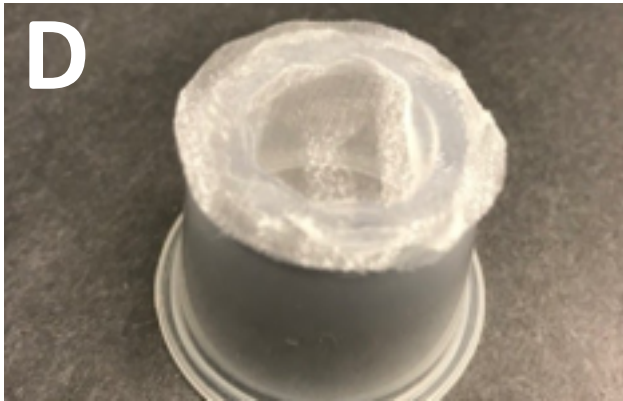

Figure 3

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Figure 4

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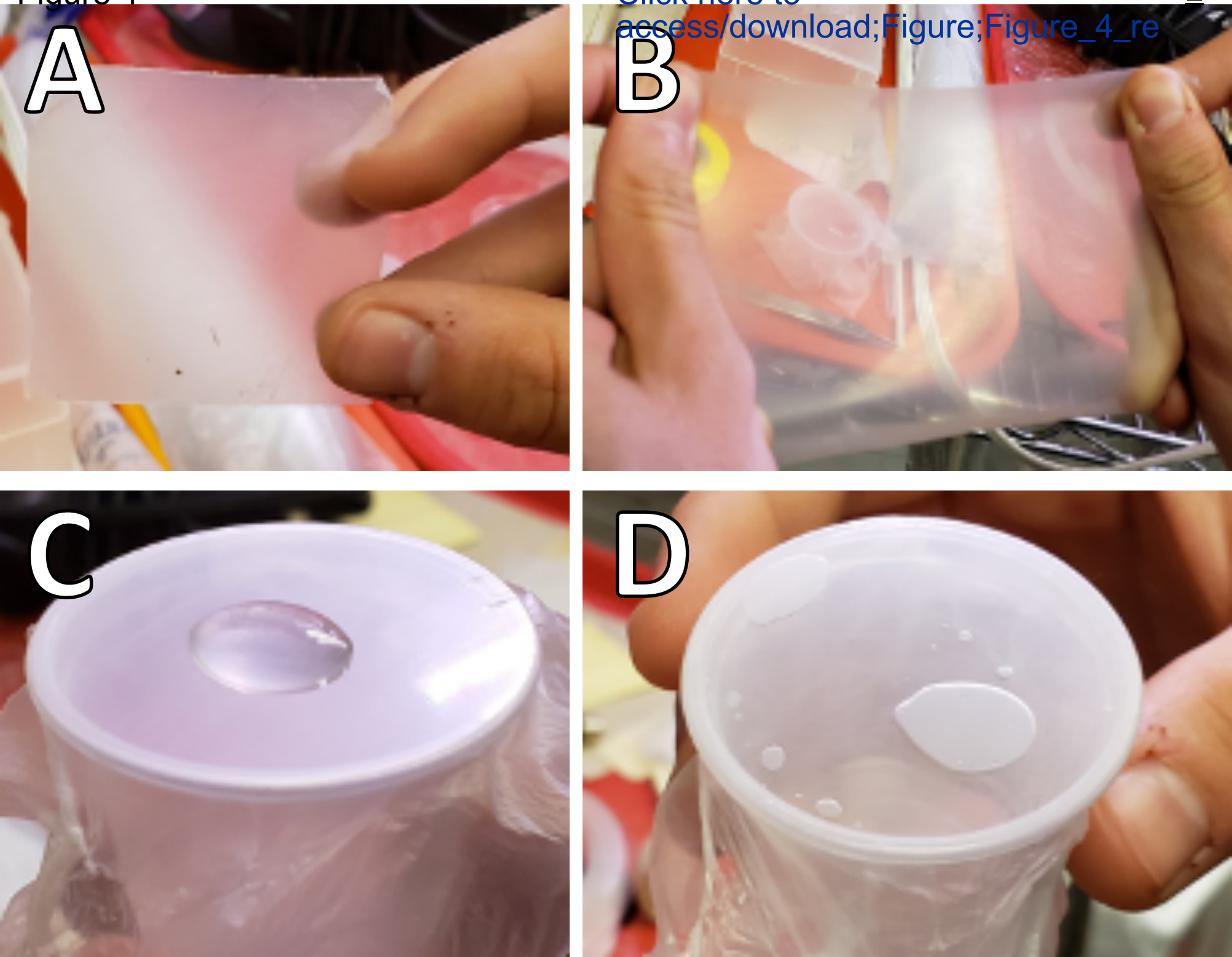
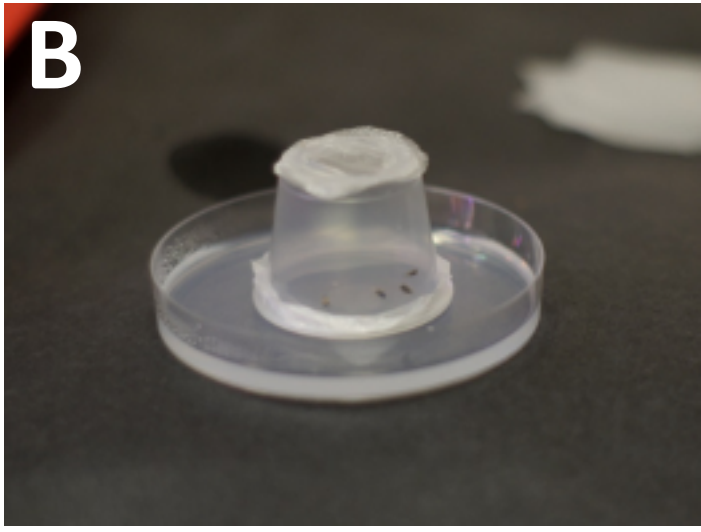
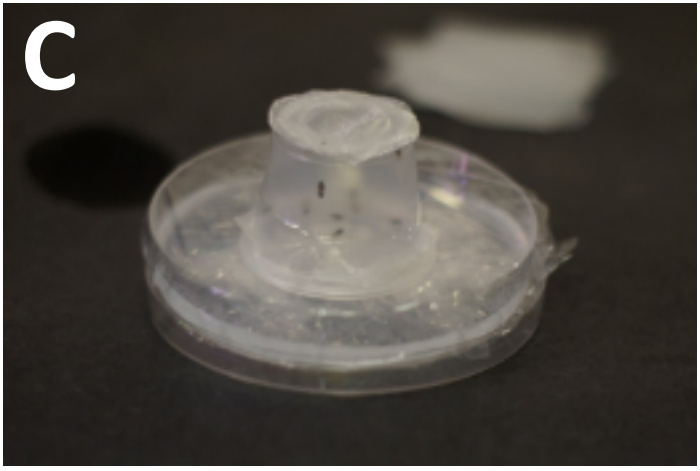
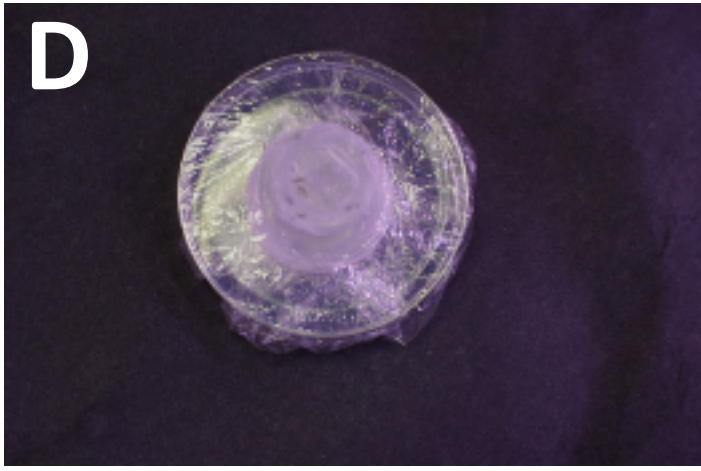


Figure 5







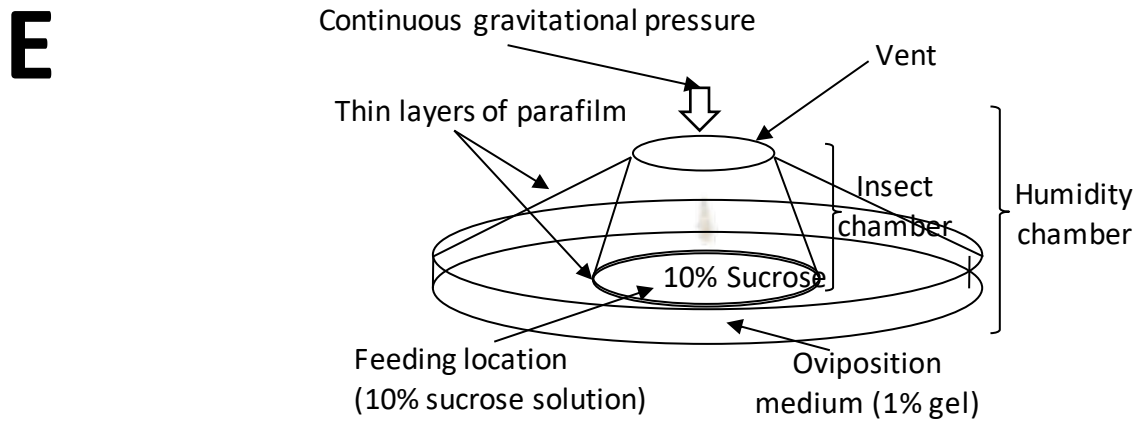


Figure 6

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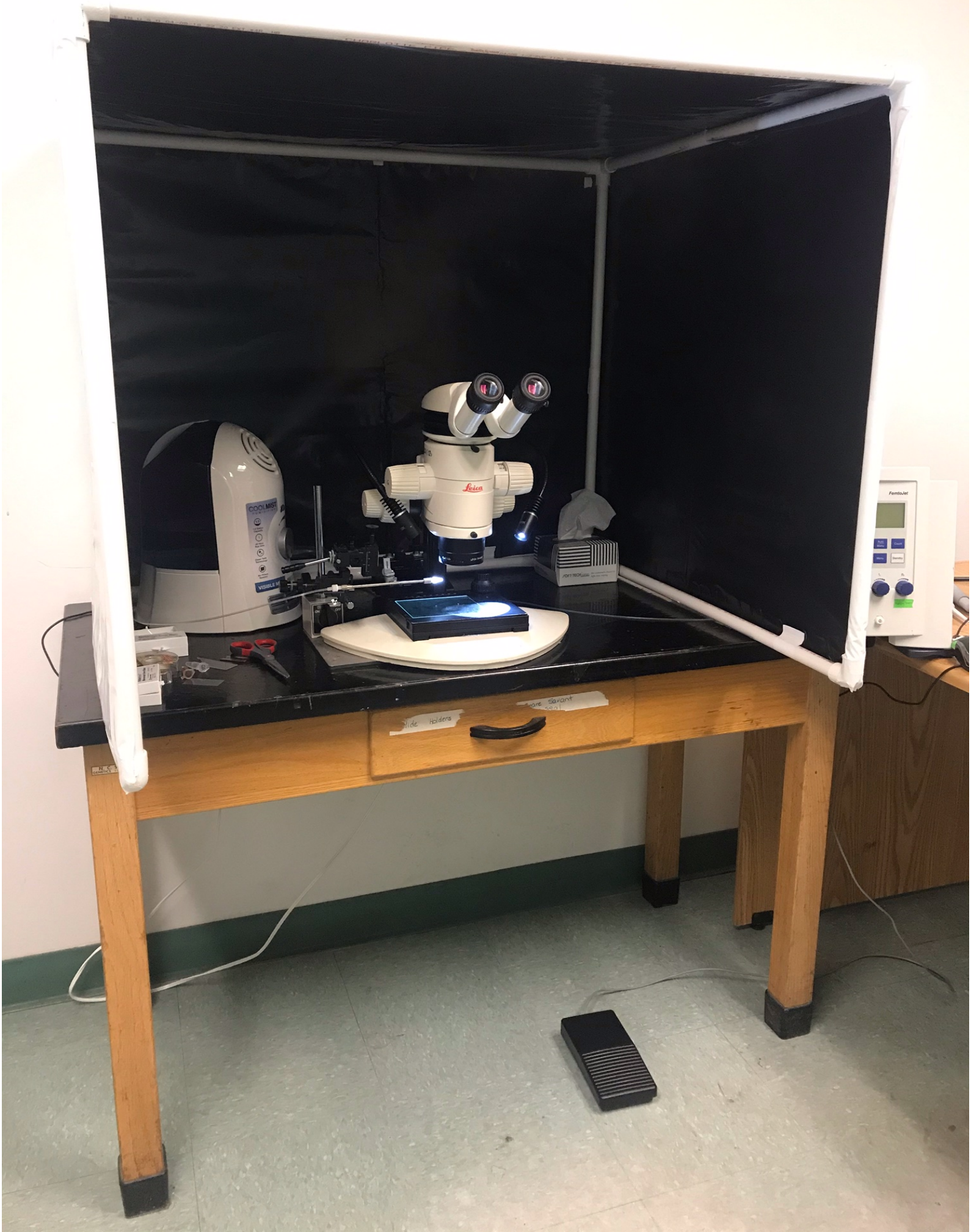

Figure 7

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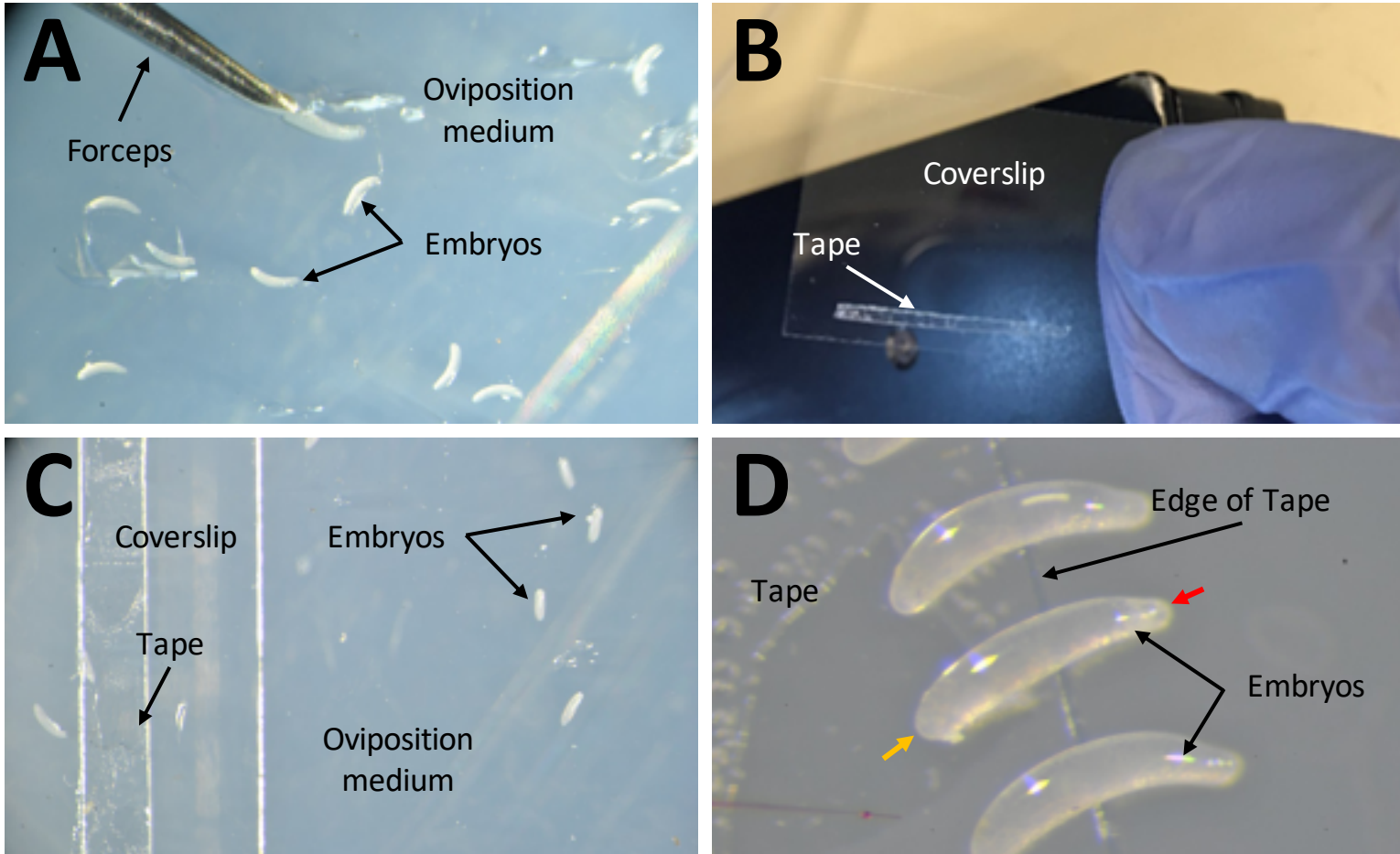

Figure 8

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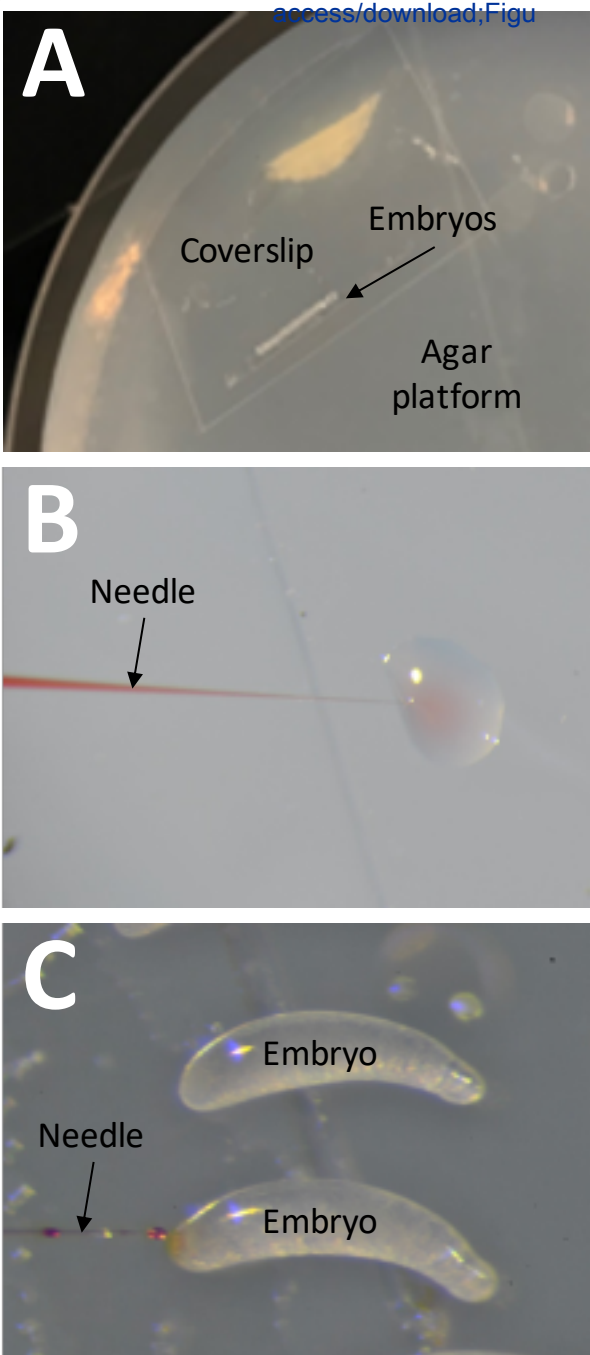
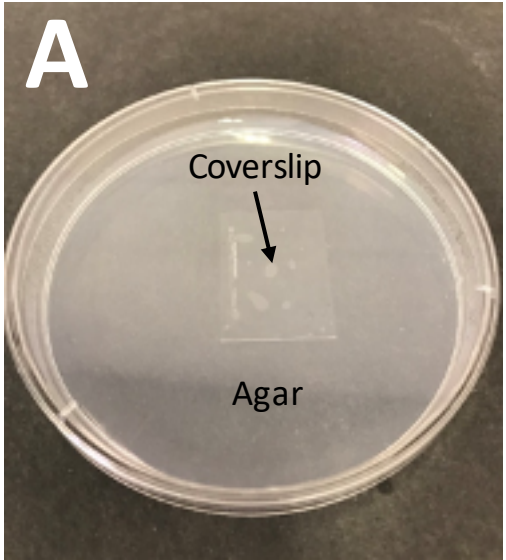


Figure 9



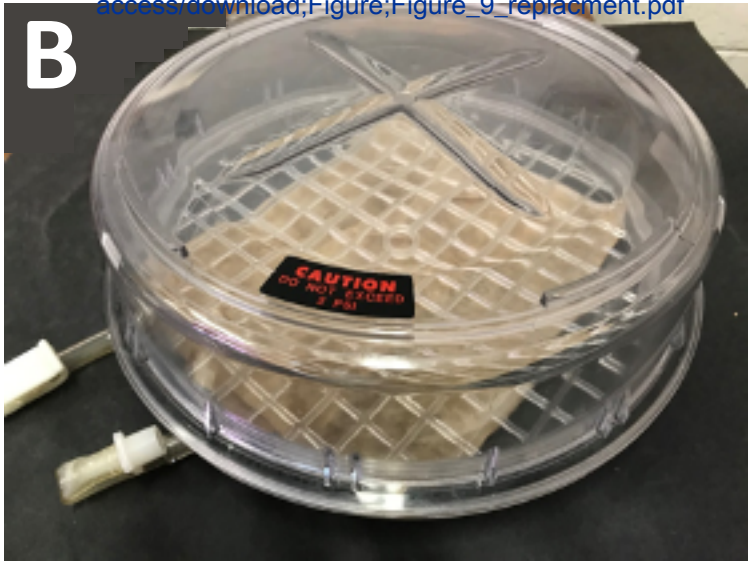


Figure 10

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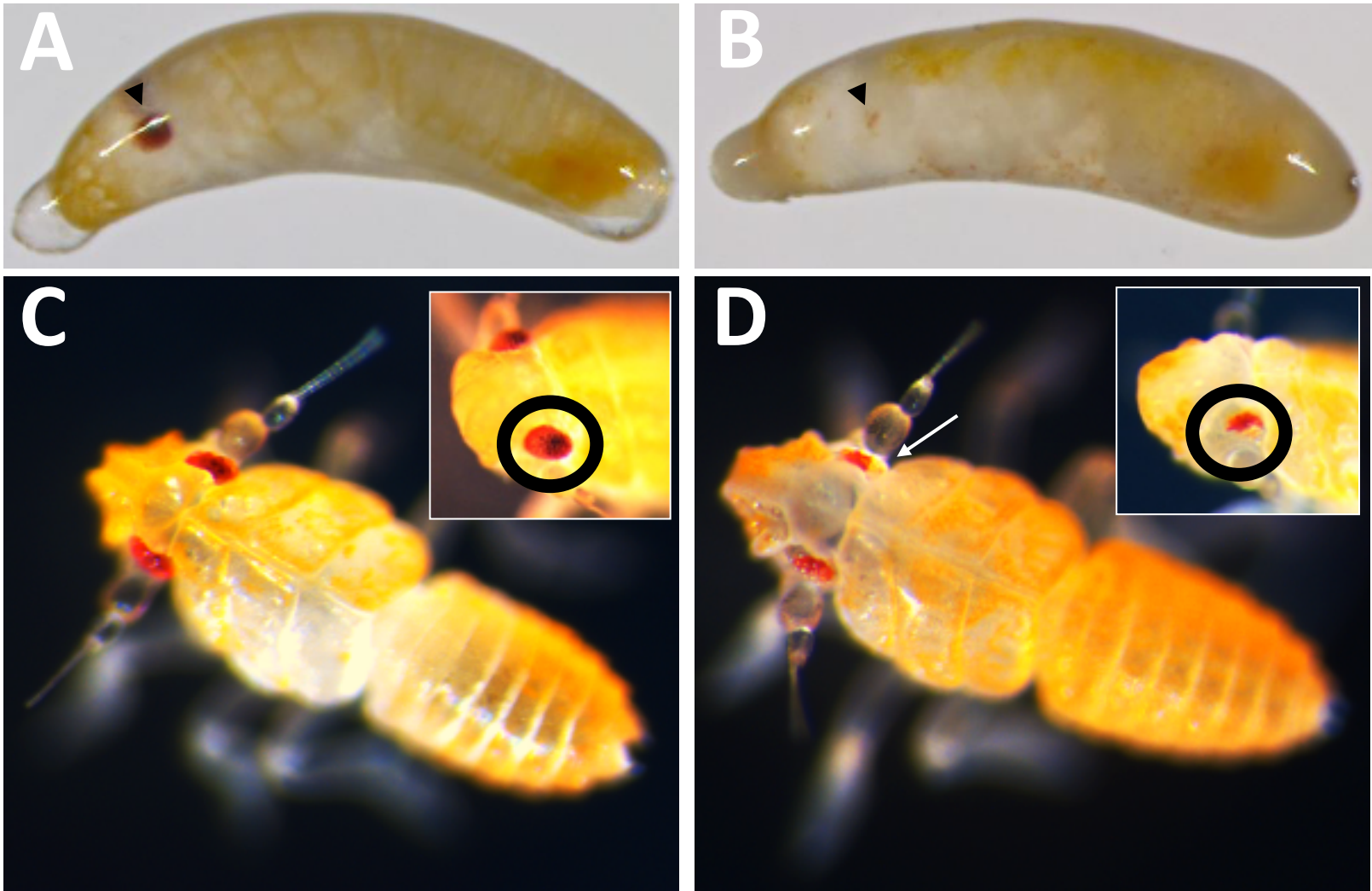

Figure 11

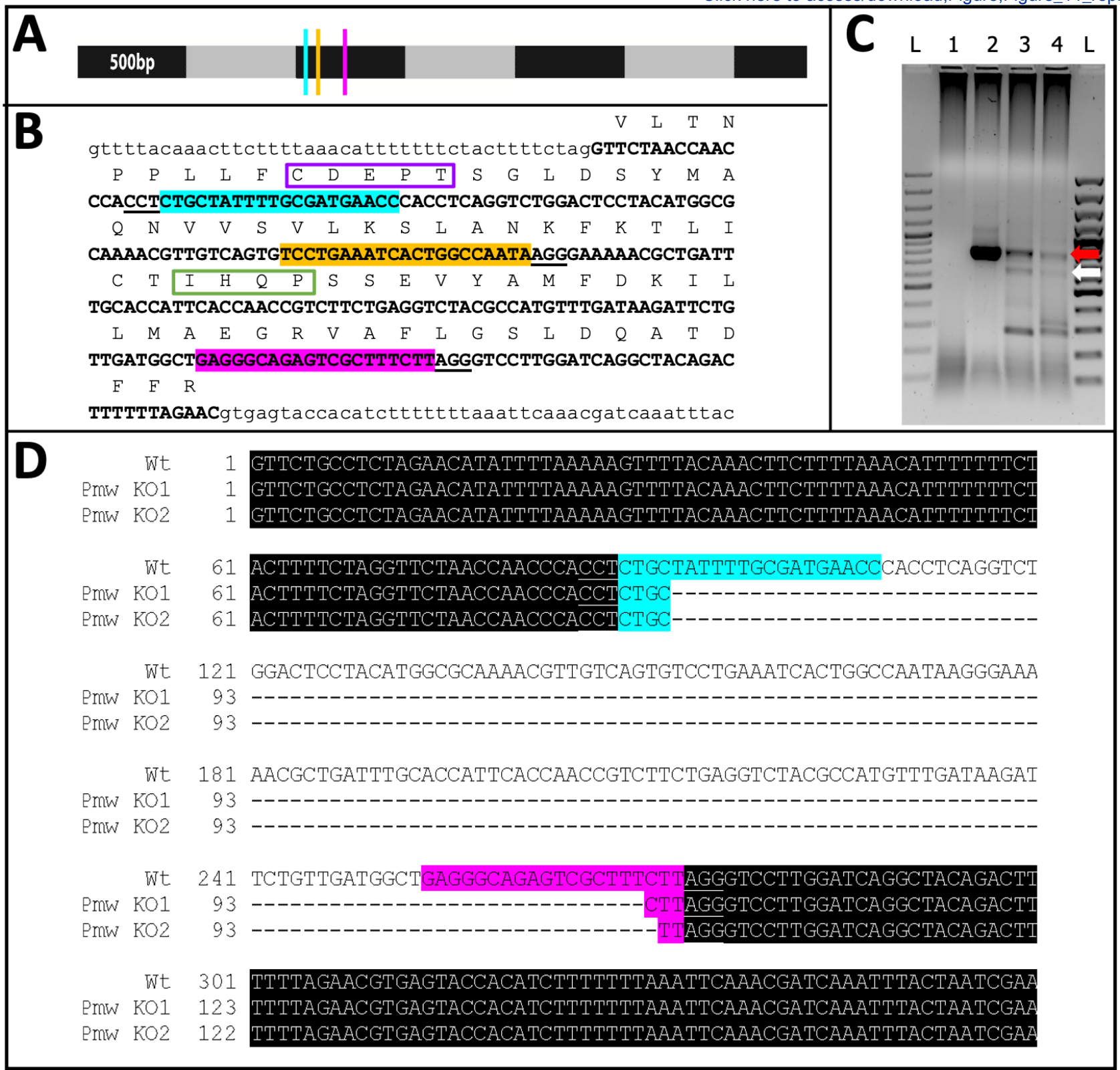

Table 1

Set	# of cups	# of females in each cup	# of eggs								Total # of eggs
			Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	
1	10	15	0	26	166	355	530	193	91	37	1398
2	15	15	22	238	489	699	520	379	203	58	2608
3	8	15	0	57	230	190	116	80	34	1	708
4	10	15	0	226	446	519	301	179	24	15	1710
Total	43	15	23	547	1331	1763	1467	831	352	111	6483

Table 2

Injection Treatment	Total Injected	Total Developed	Total Hatched	Development Rate (%)	Hatch Rate (%)
Buffer	39	20	12	51	31
Cas9	39	24	14	61	36
Cas9 +*Pmw* gRNAs	121	71	28	59	28

Table of Materials

Click here to access/download;Table of Materials;For Resubmission_Pmaidis_Materials_JoVE.xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
1 oz Containers	Dart	P100N	Adult container for egg-laying setup
15 mL Conical Tubes	Olympus	Genesee 28-103	Serves as collection tube on vacuum aspirator setup
50 mL Conical Tubes	Olympus	Genesee 28-106	For making 10% sucorose solution and for holding adults when chilling before screening
Aspirator	Bioquip	1135A	For handling planthoppers
Vacuum Aspirator	Fischer Technical	LAV-3	Vacuum for aspirating larger numbers of insects
Blue Spectrum LED Lights	Home Depot	GLP24FS/19W/LED	Grow lights for potted corn plants hoppers are feeding on
Cas9	TrueCut Cas9 Protein v2	A36498	Endonuclease for cutting planthopper genes
Clear Vinyl Tubing	Home Depot	3/8 in. I.D. x 1/2 in. O.D. x 10 ft.	Connects collection tube to pump on vacuum aspirator setup
Corn planthoppers	North Carolina State University	N/A	Request from Dr. Anna Whitfield's lab
Cotton balls	Genessee	51-101	Serves as a filter/insect catcher in collection tube on vacuum aspirator setup
Double sided tape	Scotch Double Sided Tape	NA	Holding eggs for microinjection
Early Sunglow corn	Park Seed Company	05093-PK-N	Corn for rearing planthoppers
epTIPS Microloader Tips	Eppendorf	C2554691	Backfilling needle loading tips
Femtojet Microinjection System	Eppendorf	5247	Controls injection pressure (12-20 psi, depending on needle bore size)
Nutri-Fly Drosophila Agar	Genessee	66-103	Substrate for everything except egg-laying dish
Fine forceps	Bioquip	4731	Egg handling
General Purpose LE Agarose	Apex	20-102	Substrate inn egg-laying dish (oviposition medium)
Guide RNA 1 - GGUUCAUCGCAAAAUAGCAG	Synthego	CRISPRevolution sgRNA EZ Kit (1.5 nmol)	RNA guides for targeting planthopper *white* gene
Guide RNA 2 - UCUGAAAUCACUGGCCAAUA	Synthego	CRISPRevolution sgRNA EZ Kit (1.5 nmol)	RNA guides for targeting planthopper *white* gene
Guide RNA 3 - GAGGGCAGAGUCGCUUUCUU	Synthego	CRISPRevolution sgRNA EZ Kit (1.5 nmol)	RNA guides for targeting planthopper *white* gene
Humidifyer	Homedics	UHE-CM45	For providing humidity in humidified hood
Humidity chamber	Billups-Rothenberg	MIC-101	For holding injected embryos until hatching
Insect rearing cages	Bioquip (special order)	Close to 1450 L (has plastic front and mesh fabric sides)	Cage for planthoppers on corn
Laser-based Micropipiette Puller	Sutter Instruments	P-2000/G	For making injection needles / Heat = 700, FIL = 4, VEL = 40, DEL = 170, PUL = 160
Leica M165 FC Fluorescence Stereomicroscope	Leica	M165 FC	Planthopper screening
Microinjection Scope	Leica	MZ12-5	Microinjection scope outfited with an XY stage
Micromanipulator	Narishige	MN-151	For positioning microinjection needle
Micropipette beveler	Sutter Instruments	FG-BV10-D	For beveling injection needles / Used 'fine' graded plate at 20° angle
Microscope Stage	AmScope	GT100 X-Y Gliding Table	For positioning and moving embryos under microscope
Miniature Paint Brush	Testor #2 8733	Sold in 3 pack 281206	Fine paintbrushes for embryo handling
Needle Holder	Narishige	HI-7	For holding the microinjection needle
Percival Incubator	Percival	I41VLH3C8	Rearing injectees until hatch
Petri Dishes (100 x 15 mm)	VWR	89038-968	Making agar dish for egg-lay
pGEM-T Easy Vector System I cloning kit	Promega	A1360	Cloning *Pm white* target site
Phenol Red	Sigma	143-78-8	Microinjection buffer
Plain Microscope Slides or coverslip	Fisher Scientific	12-549-3	Hold eggs for microinjection
Plasmid DNA Midi Kit	Zymo	D4200	Purification of injection-ready plasmid DNAs
Plastic paraffin film	Pechiney Plastic Packaging	PM-996	Roll size 4 in. x 125 ft
Plastic wrap	Glad ClingWrap Plastic Wrap	NA	Wrap the entire egg-laying chamber
Primer - PmW CRISPR check F1 - AAGGAATTTCTGGAGGTGAAA	IDT	25 nmole DNA Oligo	First-round Primer for amplifing across target site within the *Pm white* gene
Primer - PmW CRISPR check R1 - GATTCCTCGCTGTTGGGT	IDT	25 nmole DNA Oligo	First-round Primer for amplifing across target site within the *Pm white* gene
Primer - PmW CRISPR check F3 - TCACAGACCCTGGTGCTAATC	IDT	25 nmole DNA Oligo	Second-round Primer for amplifing across target site within the *Pm white* gene
Primer - PmW CRISPR check R3 - GTCCACAATCCACACTTCTGA	IDT	25 nmole DNA Oligo	Second-round Primer for amplifing across target site within the *Pm white* gene
Quartz capillaries	Sutter Instruments	QF100-50-10	For making microinjection needles / O.D. 1 mm, I.D. 0.7 mm, 10 cm length
Screen (White Organza Fabric)	Joann Fabrics	16023889	For covering the adult container
Sparkleen	Fisher Scientific	04-320-4	Wash dishes
Sucrose	Fisher Scientific	BP220-1	To make 10% sucorose solution

We would like to thank you and the reviewers for your advice and guidance on improving this paper. We found the comments helpful in improving a paper we were already proud of. We have copied those comments below, and offered our responses, either by stating that we have done exactly as asked, or explaining why we have deviated from the suggested course of action. We hope this work proves satisfactory for moving our paper forward towards publication.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise the following lines to avoid previously published work: 192, 197-198.

We have done this to the best of our ability. These specific phrases highlighted by the plagiarism checker are referenced back to one of the authors' own papers, and indeed are even from the methods section of that paper, with rote instructions on how to accomplish simple tasks. We hope that we can be forgiven for a little self-imitation in our phrasing when rewriting these steps for the current paper.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: Sutter P-2000, Eppendorf Femtojet microinjection system, etc.

Done

5. Line 131/138/195/196: Please use standard abbreviations for SI units when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2

Done

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

7. Please move the Figure Legends to the end of the Representative Results.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript "Microinjection of corn planthopper, Peregrinus maidis, embryos for CRISPR/Cas9 genome editing" describes a methodology for genetic transformation in the corn planthopper. A step by step methodology for microinjection is described and the CRISPR-Cas9 mediated removal of the white gene is used as a proof of concept.

The article is well written and will no doubt be of use to researchers trying to set up genetic transformation in their labs. I have therefore only included a relatively small set of comments which will hopefully improve the clarity and reproducibility of the methodology. If I have misunderstood anything, please highlight this in the rebuttal.

Major Concerns:

Establishing homozygous lines

It did not seem entirely clear from the manuscript if a homozygous mutant line was established. The sequencing results seemed to show clean deletions, but there was no description of a crossing protocol to isolate these mutants (were they batch crossed?). Also in line 331, there were mentions of "total knockouts". The reviewer suggests that the authors revise the manuscript to make clear whether heritable KOs were achieved and, if so, how this was done.

While hatchlings displaying partial gene KO were recovered, no injectees or G1 offspring have been found that display complete *white* KO. This is not only true for CRISPR-based gene editing of *Pmw*, but also RNAi targeting *Pmw* (Wang et al in progress), suggesting that complete loss of *white* function may be lethal in this species. We have added steps explaining what to do with injectees after they hatch, and have clarified in the results that neither G0 nor G1 white-eyed individuals were recovered.

Egg laying apparatus description

Perhaps I misunderstood the egg laying apparatus, but it was unclear how the eggs were laid on the agar. If paraffin film is used to cover the bottom, wouldn't the eggs be laid on the film rather than the agar plate? This wasn't very clear from the text, so the reviewer recommends some edits to this section.

We thank the reviewer for pointing out this oversight. *P maidis* ovipositors are long enough for them to pierce the plastic paraffin film (once it has been stretched as described) to lay their eggs in the oviposition medium, since they would normally lay

their eggs into the very tissues they feed on. We have added this information to the paper as part of the note under step 2.7 to prevent any further confusion for the readers.

Model Numbers + within text

It might be useful to include the model numbers for equipment within the text in addition to the list at the end. However, this is mostly an editorial decision, so I leave it to the authors and editor.

While we appreciate the Reviewer's concern, the Editor has specifically asked that we not include any commercial language in the text of the paper. As such, the model/catalogue numbers are only in the Materials table. However, all information necessary to identify the items used in our experiments can be found in the table.

Minor Concerns:

Line 105: The wording isn't too clear here and can be improved. Are all adults transferred every week to new plants? Also, it might be useful to reference a more detailed protocol for rearing this species if one exists.

Steps 1.5-1.6 have been rearranged and rewritten to attempt to clarify this question.

Line 111-112: Is there an optimal age of the adults used for embryo production? In the reviewer's experience older individuals can sometimes suffer severe reductions in fecundity similar to those seen when put in less ideal rearing conditions (Lines 298-300).

The age of insects used is addressed in step 2.5. Unfortunately, we have not had the opportunity to test aging adults for a dropoff in fecundity, so we do not know the upper limit for adult usability. However, if readers follow our recommendation to maintain age-calibrated cages, they should have no trouble getting a steady supply of young adults for experiments.

Line 123: "by cutting ..... and gluing"

Done

Line 128-129: Is there an established way to sort males/females in this species? It does not have to be described in detail, but should perhaps be mentioned.

We thank the reviewer for pointing out this oversight. We have added information on how to successfully identify females to step 2.5, and have also included a new image to visually demonstrate this difference to the readers.

Line 195-198: Which method was used for synthesizing sgRNAs. Again a detailed description is not needed, but an established methodology should be cited.Lines 270-273: The authors should provide the sgRNA guide sequences used in this study

Thank you for catching this. Since our gRNAs were purchased, we have added a reference to the Materials table at this step (Step 4.4), which will guide readers to

additional information, including from where the guides were ordered and the individual sequences of the guides we used.

Line 260: "Those" --> "These"

Done

Line 284-285: What exactly was sequenced? A band on a gel, a G1 individual, or a population?

Because we were unable to establish a mutant line, the process of getting any sequence information was rather complicated. Nevertheless, we have altered what is now Figure 11, its legend, and the text discussing this figure to clarify our process.

Lines 332-335: There is another explanation for the lower survival in CRISPR individuals which should be mentioned. As the negative control did not have any sgRNAs, the sgRNAs themselves could be toxic independent of any DNA modification especially at the relatively high concentration of 1200ng/µL of total sgRNA. This could be due to some toxic component that came along during purification or simply overloading the system with RNA.

This is an interesting idea, and one we certainly cannot eliminate in this paper. Our understanding of the literature is that Cas9, rather than the gRNAs, is more likely to be inherently toxic, while lethal effects from Cas9+gRNAs (even at high concentrations) are assumed to be from excessive cutting (including, but not limited to, off targets). In any event, the question we were asking here was the wrong one. We have adjusted the language to indicate that we were concerned with the failure to recover complete KO G0 and G1 individuals, but that we have reason to believe this problem is directly related to the use of these gRNAs to target the *white* gene.

Reviewer #2:

Manuscript Summary:

This topic is about how to use the microinjection system to introduce the CRISPR/Cas9 system into insect embryos.

Minor Concerns:

The research content itself is not novel or new. However, it is well-purposed and well-organized, and it is considered suitable for introducing to researchers as video content.

We look forward to seeing videos created with video content.

Reviewer #3:

Manuscript Summary:

Klobasa et al. presented a detailed protocol for collecting and microinjecting precellular corn planthopper (Peregrinus maidis) embryos for the purpose of gene editing and transgenesis. Whilst similar protocols have been established for other insect pests, this has not been done before for P. maidis, a species in which they had reported that the only precedent of gene expression modulation is RNAi, which is transient. Klobasa et al. highlighted that development of a protocol that will allow production of stable mutant strains will be extremely useful for future research and advancement in control methods. Such a protocol will indeed be very relevant and beneficial to the P. maidis research community as well as provide further valuable information on mutagenesis of non-model organisms.

On the whole, I would like to commend the authors for the very detailed overall description of their methods. The authors have provided a very comprehensive, novel and feasible method for embryo collection by using an artificial egg-laying environment as collection of eggs from a natural environment is quite challenging. They have also shown successful CRISPR/Cas mutagenesis of the white gene, both phenotypically and genetically; however, they have only shown this for the injected individuals but have not looked at the following generation, hence they have not shown that it is a stable germline mutation. There is no reason to think that the mutations will not be stable germline mutations, however it is unusual that they have not included that in the protocol - it would provide more strength and validity to the protocol. With RNAi studies, it is quite common to just look at the injected/treated individuals as the phenotype is transient, but for CRISPR/Cas mutagenesis and transgenesis, the mutant progeny of the injected individuals are what is important for creating the stable mutant strains. In fact, the mutant phenotypes and/or genotypes are often only detected in the following generations. Targeting the white gene in this case allows for detection of successful mutagenesis in the injected individuals as it provides a visible phenotypic output but this is not often the case - if there is no visible phenotype, the mutation can only be confirmed in the progeny, which is where this protocol is lacking.

Overall this protocol is quite suited for publication for JoVE and will be quite useful; the authors just need to address some of those mentioned points in their text.

Major Concerns:

1. It would have been ideal for the authors to have looked at the progeny of the injected individuals and/or subsequent generations to demonstrate that the mutations are germline mutations and that there is complete loss of eye colour and no mosaic phenotype in the mutant progeny. This will also allow the authors to determine the actual mutagenesis success rate as it is possible that some injected individuals may carry the mutation but do not exhibit any visible phenotype - this may depend on when the embryos undergo cellularization and seeing that the

duration of egg collection is quite long (16 hours), it is plausible that there could be successful germline mutagenesis without resulting in visible phenotypes in the injected individuals. Indeed, this has been seen with mutagenesis of white gene in other insects where no mosaic phenotype was observed in the injected individuals, but they were able to pass on a stable white loss-of-function mutation to their progeny. This would involve the authors examining all the 28 hatched individuals and their progeny, not just the 9 that showed loss of pigment. The authors should acknowledge the importance of identifying mutant progeny (not every progeny from the identified injected mutant will carry the germline mutation) and they should at least provide a theoretical description of the steps that would be involved in analysing the progeny to identify successful mutants. This is particularly essential if they are presenting this protocol as one that can be adapted for transgenesis.

While hatchlings displaying partial gene KO were recovered, no injectees or G1 offspring have been found that display complete *white* KO. This is not only true for CRISPR-based gene editing of *Pmw*, but also RNAi targeting *Pmw* (Wang et al in progress). We have clarified in the paper that neither G0 nor G1 white-eyed individuals were recovered. We have also added some steps to the protocol clarifying what should be done with injected individuals after they have hatched and been screened (steps 6.5-6.7).

2. Please include a sentence to comment on why three CRISPR guides were used simultaneously. Whilst it makes sense to design three guides to test them individually, it seems quite unusual to use three guides altogether to target the gene, especially when all the guides are so close to each other. If the aim is to create a large deletion, two guides would have been sufficient, as suggested by the PCR analysis results where the deletion is likely to have been caused by successful targeting of guide 2 and 4. It seems counterintuitive to use more guides than necessary, particularly as addition of components to the injection mix could cause more toxicity to embryos. It would be interesting and useful to know if there is a specific reason for using three guides simultaneously.

We thank the reviewer for this insight. We have added two sentences to the representative Results section to explain our choices of guides, and of the number used.

3. Line 281: Please change knockout rate of ~32% to ≥ 32%. Out of 71 injected and developed individuals, 23 were identified as mutants due to the visible phenotypes but there could be others that are germline mutants but do not exhibit the loss of pigment (as discussed in Point 1).

Done

4. Lines 273-275 and 332-333 - authors mention that it is surprising that the hatch rate of individuals with Cas9/gRNA combo was lower than controls. This is not particularly surprisingly considering that injection mix for those individuals contain 800ng/ul Cas9 + 3 x 400ng/uL of gRNAs; the concentration of Cas9/gRNA components is much higher than the controls (800ng/uL Cas9 or 20% phenol red). Potentially the Cas9/gRNA combo injection mix will be more toxic to the embryos hence contributing to the lower survival/hatch rate. That could be an additional factor to the other two factors (off-target effects + loss of white gene function) already

discussed by the authors, but it does not seem surprising that the Cas9/gRNA combo injected individuals have a lower hatch rate compared to controls. Again, it might be a good reason to not use more guides than necessary.

Our understanding of the literature is that Cas9, rather than the gRNAs are considered to be more likely to be inherently toxic, while lethal effects from Cas9+gRNAs (even at high concentrations) are assumed to be from excessive cutting (including, but not limited to, off targets). Moreover, in papers that test multiple concentrations, there is a tradeoff between survival and KO rate, such that a high death rate is tolerable given the high KO rate. In any event, the question we were asking here was the wrong one. We have adjusted the language in the discussion to indicate that we were concerned with the failure to recover complete KO G0 and G1 individuals, but that we have reason to believe this problem is directly related to the use of these gRNAs to target the *white* gene. In particular, RNAi targeting *Pmw* (Wang et al in progress) suggests that complete loss of *white* function may be lethal in this species.

5. Lines 284-285, Figure 9 - authors mention in the text that PCR analysis was performed for injected individuals, but Figure 9 only shows two mutant sequences. Please clarify in Line 284-285 if the analysis was only performed on 2 individuals or were there only two types of mutations detected from among all 9 individuals. If other mutations have been detected, they should all be shown in Figure 9, otherwise the text should be amended accordingly.

Because we were unable to establish a mutant line, the process of achieving any sequence information was rather complicated. Nevertheless, we have altered this figure (now 11), its legend, and the text discussing this figure to clarify our process.

6. Figure 9A, B and legend - please remove the information regarding the guide 1 (G1) from the figure and legend as it was not used in the study, it does not provide any useful information; especially if the sequence error is due to human error. If the sequence error is due to identification of SNPs within the Peregrinus maidis lab colony after design of guide, then it would be necessary to discuss the importance of SNP genotyping (see point 9).

Done

7. Figure 9A - instead of presenting the mRNA in 500bp increments, it would be more useful to present it by showing the different exons and state which exon the guides are actually targeting.

We also would prefer to do this. Unfortunately, the current state of the *P maidis* genome makes this impossible. We were lucky to find the genomic sequence of the exon we wished to target. Assembling and showing the full exonic makeup of *Pmw* is not currently feasible. However, we have explained the purpose of showing the context of the guides within the full-length mRNA model; namely, to show that most of the coding sequence would be disrupted if a frame-shift mutation were generated within our target site.

8. Figure 9C -it would be useful and will provide a complete story if the authors can include the protein sequence on the figure and show that the deletion results in a premature stop codon/a mutation that result in loss of protein function, corresponding to the phenotype.

We like this idea, but for the purposes of this figure, it was much more practical to add translation sequence to what is now Figure 11B. However, to emphasize the importance of this region, we've indicated critical domains that would have been partially or completely disrupted by deleting regions between our guides. Even if an early termination is not generated, any resulting protein from our mutants should be non-functional. In the mutant alignment (now 11D), however, even if we could easily add translation lines, any resulting stop codons might not occur within the remaining span of the single exon shown in the sequenced region.

9. Authors should mention in the discussion that it would be useful to sequence the target region in their lab colony prior to guide design to determine if there is any population SNPs in the target region - it would enable design of CRISPR guides that have 100% match to the target region and avoid mismatches due to presence of SNPs.

We thank the reviewer for their suggestion, but for many reasons, this idea is not practical for everyone. For example, we struggled to amplify off *P. maidis* genomic DNA, an important step for the kind of individualized sequencing the reviewer is recommending. Due to time constraints from our funding agency, we had to move forward with our CRISPR experiments before we could resolve our amplification issues. Our standard method to overcome possible issues from genetic diversity in wild-derived populations, like our *P. maidis*, is to design 3-4 guides within critical functional domains and co-inject them. While sequencing for SNPs would certainly be useful for identifying highly conserved genomic regions for gRNA design, it is not necessarily the cheapest nor the most efficient way to overcome the limitations presented by SNPs.

10. This is more a comment than a concern - it would be interesting to know if the authors have pre-determined whether injection of phenol red buffer has some level of toxicity to the embryos and contribute to lower development and hatching rate as seen in the buffer control (51% development rate, 31% hatch rate). On one hand the phenol red buffer is useful in providing visible confirmation that the injection mix has gotten into the embryo, but on the other hand, removal of the usage of the buffer could improve the hatch rate. This is among the many factors that researchers often have to weigh up when performing such protocol and experiments, depending on what they hope to achieve and the % of hatch rate that is feasible to work with for their current study; hence it could be an interesting and important discussion point.

We agree that this would be useful to discuss. Unfortunately, the limitations of this journal make such a discussion impractical for this paper. We will, however, keep the Reviewer's comment in mind for future papers on our *P maidis* work.

11. Another comment rather than concern - the authors have established a very successful method of egg collection where they collected 6483 eggs within 9 days and although they mentioned that they did not use eggs after Day 6, even the number collected on Day 5 itself is

very high (n=1763). The number of eggs they required for their injection experiments was 199 and that was sufficient to produce a reasonable level of successful mutagenesis. I'm aware that they probably used more than 199 eggs during the injections but lost some due to desiccation, handling and issues during microinjections which is expected. Since they have a reasonable hatch rate and do not need to inject so many eggs compared to what they are able to collect within a 16 hr egg collection window, the authors could consider narrowing down the egg collection period which will allow them to collect only embryos in the earlier pre-cellularization stages if necessary.

The egg-laying experiments mentioned early in the Representative Results section were just that - tests to see how many healthy eggs we could get. We did not use quite so many cups for actual injection lays. As the reviewer can no doubt tell, the females used for egg lays are essentially sacrificed to the cause. So it is to our benefit to limit the number of cups created every week, lest we decimate our colonies. Moreover, as the Reviewer points out, there is also egg loss. Egg counts of the type we reported don't necessarily examine the eggs carefully enough to identify inviable eggs, while our methods for injections advise readers to carefully examine eggs while they are being laid out, so that the reader does not waste time injecting embryos that they can already tell are inviable. Specifically regarding the egg laying window, there are many factors to consider besides number of eggs. In a small lab like ours, the personnel responsible for injections are also responsible for maintaining lab stocks. As such, the time available for injections is limited. Since our results indicate that we are accomplishing injections during the syncytial stage (otherwise, we would see no knockouts) with the 16-hr window, those doing injections have opted to continue using that time period, as it best fits with their schedule while yielding a sufficient egg load to justify injections. Hopefully, we have made it clear to our readers that they can and should adapt the time frame to meet the needs of their own labs.

Minor Concerns:

1. Line 256: change knockout to knock out

This sentence was completely rewritten to address other concerns, so this term/phrase is no longer used in the same context. However, should this reviewer read our resubmission, we would appreciate their input on any new instances of knock out/knockout.

2. Table of Materials - please fit the comments/description on to the same page as the rest of the table.

We share the reviewer's dismay that our table was not supplied in a readable arrangement. We, the authors, submitted it as an Excel file, so had no control over how it was converted to PDF for the reviewer. Hopefully, when this paper is published, the copy editors will ensure the table is properly formatted for reader convenience.