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New Faces at the CRB's Helm

Developing Genetic Tools to Control ACP

Duverney Chaverra-Rodriguez, Michelle Bui, Ming Li, Robyn Raban and Omar S. Akbari

Project Summary

Control of the Asian citrus psyllid (ACP) is a priority for the viability of the California citrus industry, and it must be addressed immediately. Genetic engineering to control wild populations has been developed in other insects, including mosquitoes and fruit flies. While these technologies also may prove promising for ACP, currently genetic engineering of the psyllid remains elusive. To overcome this limitation, which could enable the engineering of potent genetic control systems for ACP, we aim to first develop a "toolkit" to reliably engineer ACP that can be exploited to develop population control technologies to combat this pest in California.

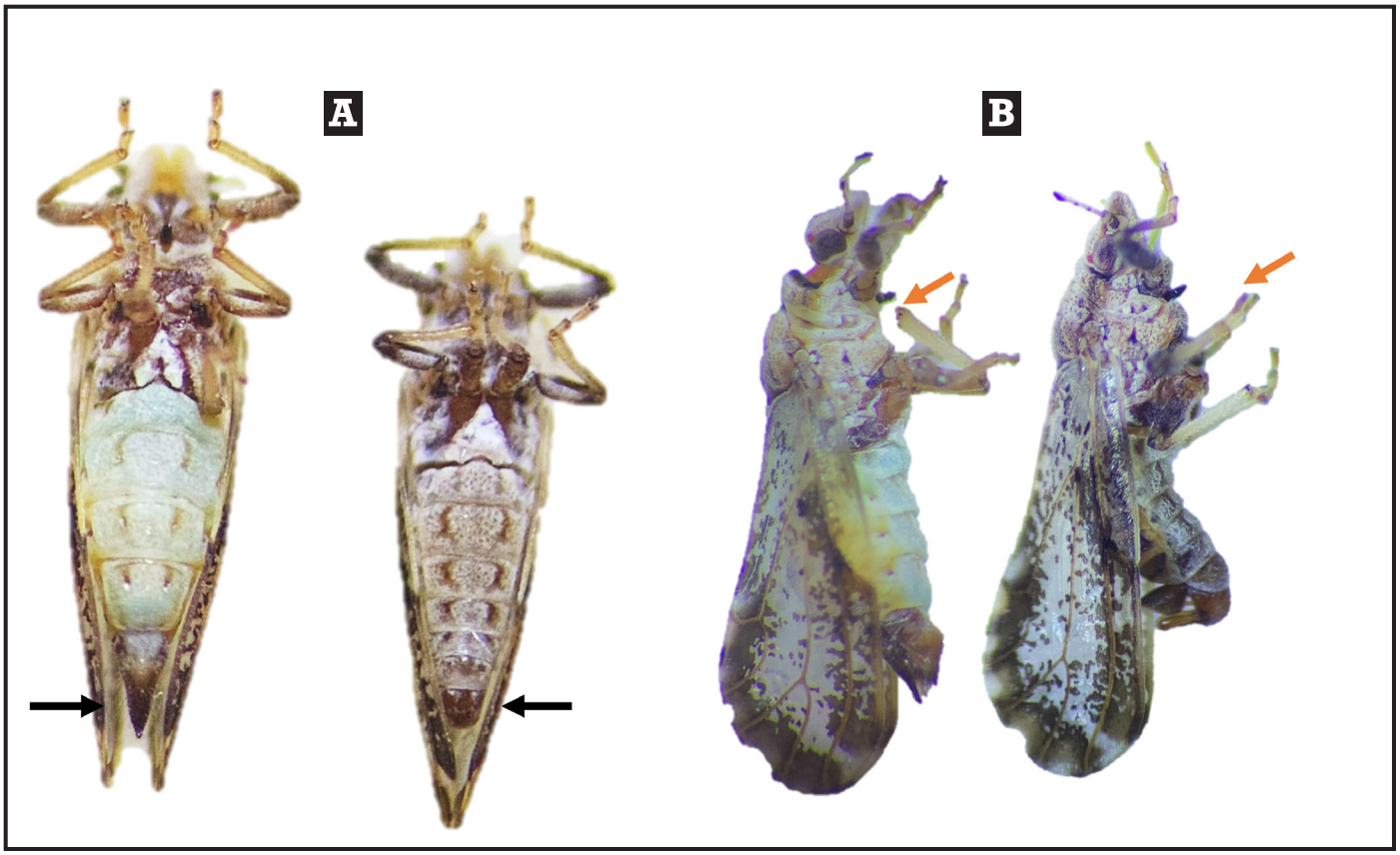

Figure 1. A) Identification of reproductive structures of the Asian citrus psyllid female (left) and male (right); the black arrows point to the differences in the abdomen of a female (pointed) and a male (rounded). B) Side view of the male and female showing the differences in reproductive structures and the mouth pieces used to pierce plants and suck plant fluids (orange arrows). **Source: The Akbari lab. Photo credit: D. Chaverra-Rodriguez**

'Candidatus Liberibacter asiaticus' (CLas) is a bacterium associated with huanglongbing (HLB) disease. It is transmitted by the ACP (*Diaphorina citri*) (**Figure 1**). Current HLB management practices are insufficient to completely control this disease. Thus, there is a significant need for novel tools and techniques to control HLB. Genetic methods can be used to eliminate the ACP, which will affect this species only and not require insecticides. For instance, our goal is to produce sterile males through genetic engineering so, if released into the environment, it will reduce wild populations. As more of these genetically engineered sterile males are released, the population will decline until it is eliminated. These technologies work exclusively against ACP populations, so beneficial insects, like pollinators, will not be affected. Hence, this non-insecticidal, targeted method to control ACP could mitigate costs, technical obstacles and other negatives associated with insecticide use, making it a more sustainable approach for pest control.

Certainly, the advancement of genetic control technologies for ACP will provide new options to halt the spread of HLB. However, the genetic transformation of ACP has been thus far challenging and elusive. Currently, few laboratories have been able to achieve reliable genetic changes in this insect successfully; and none has been able to introduce foreign DNA (Hunter et al. 2018) into the ACP, a required step to generate genetic control technologies.

Our research focuses on developing the tools required to create genetic control technologies for ACP that can be applied as HLB control strategies in California. Specifically, we are designing CRISPR-Cas9[1]-mediated genetic transformation tools for ACP. We have successfully created CRISPR-Cas9 engineered lines for multiple mosquito species (Li et al. 2017a; 2017c; 2019), wasps (Li et al. 2017b) and pests such as *Drosophila suzukii* (Buchman et al. 2018b).

The biggest challenge for a real-world application of these strategies for pest control is to guarantee the spreading of the engineered gene in the field population. For instance, to suppress a wild ACP population, it would be possible to release genetically-engineered ACP (geACP) that are able to produce sterile female offspring and fertile males. Thus the number of ACP in the field would be reduced, and geACP males would be available to transmit engineered genes. However, to spread their engineered genes, these males will have to compete for wild females with wild-type ACP (wACP) males. In the field, the encounters of geACP males with wACP females would be very low compared to encounters between wACP females and males. Also, each time that a geACP male mates with a wACP female, only 50 percent of his offspring will have the engineered genes. Consequently, in each generation, the proportion of geACP is reduced (**Figure 2A**).

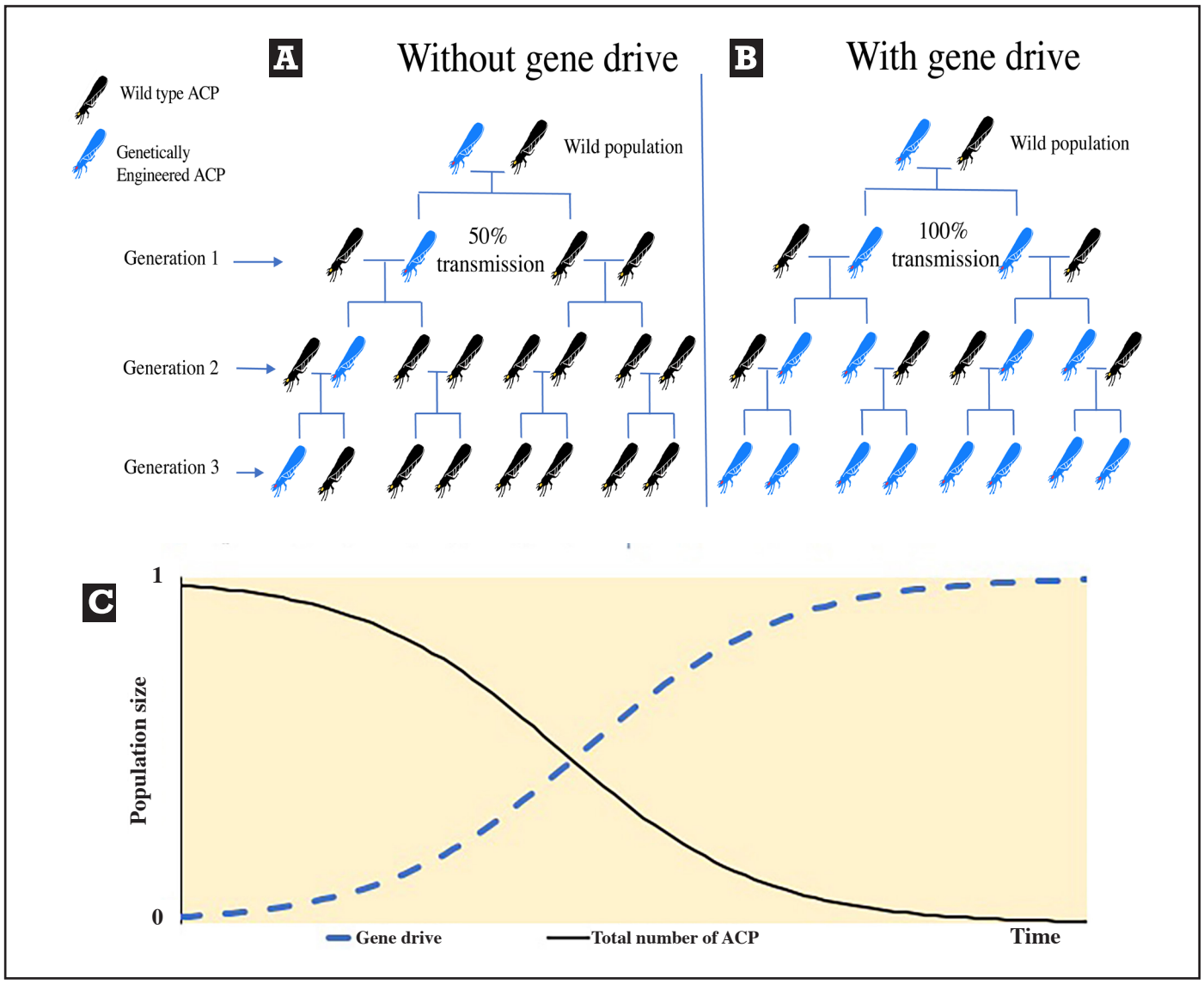

Figure 2. Expected spread of an engineered gene that suppresses Asian citrus psyllid (ACP) reproduction into a wild population. A. Without gene drive, the chances to pass the engineered gene are only 50 percent. The engineered and wild type offspring ACP will mate with wild type. Since the wild type cross will lead to 100 percent wild type, the engineered gene would get diluted each generation. B. With gene drive, the chances to pass the engineered gene are more than 50 percent and close to 100 percent. Then the ACP will produce offspring that have the engineered gene, which favors its own spread in the next generations until it gets fixed in the population. C. Expected reduction of an ACP population (black line) after the introduction and subsequent increase in numbers of genetically engineered ACP with a gene drive (blue dashed line).

One way to circumvent this problem is to incorporate a genetic element that increases the inheritance of the engineered gene. Such elements are present in nature and are called gene drives[2]. An ideal gene drive element introduced in a geACP mated to a wACP would increase the transmission of the engineered gene to roughly 100 percent of the offspring instead of 50 percent. As a consequence, over time the wACP will be more likely to encounter geACP to mate, and the frequency of the gene increases with each generation (**Figure 2B**). CRISPR-Cas9 can be used as a tool to create the geACP and can also be used as a gene drive, facilitating the spread of modified genes into the wild population.

Our goals are to:

1. generate a protocol to create genetic modifications in the ACP, and then
2. generate a protocol to use CRISPR-Cas9 as a gene drive and evaluate its potential application to drive such genetic changes in the field and reduce the ACP population (**Figure 2C**).

To achieve the first goal, we must surpass the major obstacle in ACP genetic transformation, which is the successful delivery of the Cas9-guideRNA[3] (gRNA) molecules into the eggs and the survival of the eggs injected with these

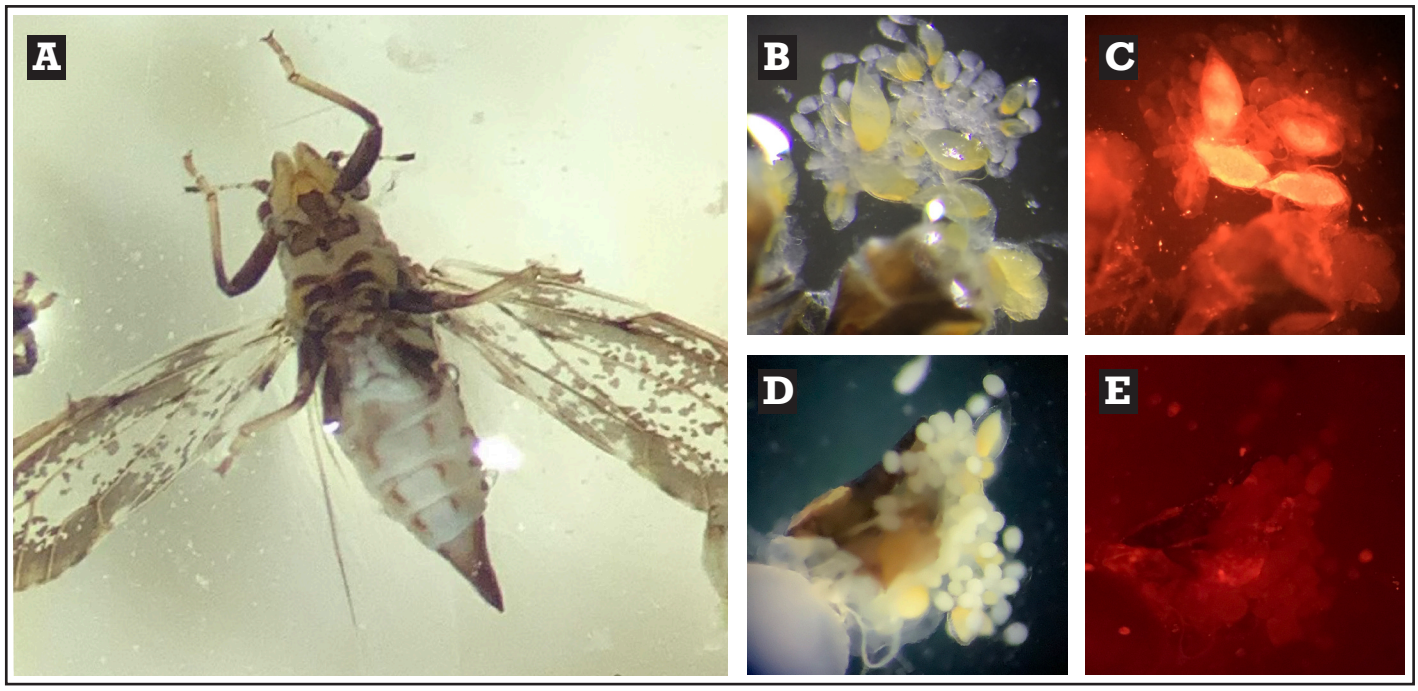

Figure 3. A) Female prepared for injection. B-C) Ovaries of female injected with a red fluorescent protein that targets the developing eggs. D-E) Ovaries of female not injected with the protein.

molecules. Eggs must be collected early after they have been laid, then aligned and injected with a Cas9-gRNA mixture. After injections, the eggs are placed on host plants or other favorable environments, allowing them to hatch into nymphs that are screened to identify the modified individuals. To optimize ACP transformation, we have to carefully choose genes that can be screened to identify the best conditions for injections.

We identified several potential target genes for which mutations can be observed in nymphs or adults (e.g. eye color, body color or short wings). From these, we selected four genes related to eye color and designed gRNAs that we co-injected with Cas9 into the eggs of ACP to test their effect on egg survival. These preliminary egg injections with Cas9-gRNA produced between 10-14 percent survival of embryos. We are now aiming to increase the rate of survival by testing different conditions for egg collection, injection and hatching. Injection protocols may be difficult, but we have successfully adapted this technology to many insect species (Buchman et al. 2018a; Li et al. 2017a; 2017b; 2017c; 2019).

As an alternative to egg injections, we also have evaluated the injection of the Cas9-gRNA into adult females using the technology termed Receptor-mediated Ovary Transduction of Cargo "ReMOT Control" (Chaverra-Rodriguez et al. 2018). This system uses a peptide, called P2C, to deliver the molecules specifically into the developing ovaries of insects. Researchers have not evaluated this system in ACP; hence, we tested the efficiency of P2C to introduce a red fluorescent protein in the ovaries of ACP females. We detected the fluorescent protein in the developing eggs (**Figure 3**). This is encouraging and suggests that P2C can be used to deliver Cas9-single gRNA (sgRNA) into the ovaries. The next step is to inject females with P2C-Cas9-sgRNA to investigate the efficiency of this method.

Conclusions

Genetic transformation traditionally has been difficult for many insect species, including ACP. However, with the advent of CRISPR-based gene editing technologies, genetic engineering is becoming a reality for many organisms. Our preliminary results to generate targeted genetic changes in ACP are encouraging. Successful achievement of this goal will allow us to produce technologies based on genetically-modified ACP that can be used to control CLas transmission. These technologies also will increase research capabilities, facilitating discovery of new methods for ACP control that could benefit California citrus growers. For example, in other pest species, genetic technologies allow researchers to study insecticide resistance mechanisms, pathogen transmission patterns and other functions with direct application to ACP and HLB control.

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Glossary

[1]CRISPR-Cas9: Clustered Interspaced Short Palindromic Repeats refers to DNA sequences present in bacteria that are used to target and degrade foreign DNA. The system has been adapted to cut and paste DNA into other organisms such as bacteria, fungi, plants and animals, in order to change specific sequences within their genomes.

[2]Gene drive: A system that biases the frequency that a gene is inherited from a parent by its offspring.

[3]Cas9-guideRNA: An RNA molecule that guides the Cas9 protein to a specific region of a gene. The gRNA molecule can be designed to target virtually any gene.

Duverney Chaverra-Rodriguez, Ph.D., is a post-doctoral scholar at the University of California, San Diego. Michelle Bui is a Ph.D. student in cell biology and development at the University of California, San Diego. Li Ming, Ph.D., is a post-doctoral scholar at the University of California, San Diego. Robyn Raban, Ph.D., is a research project manager at the University of California, San Diego. Omar S. Akbari, Ph.D., is an associate professor at the University of California, San Diego. For additional information, contact **oakbari@ucsd.edu**