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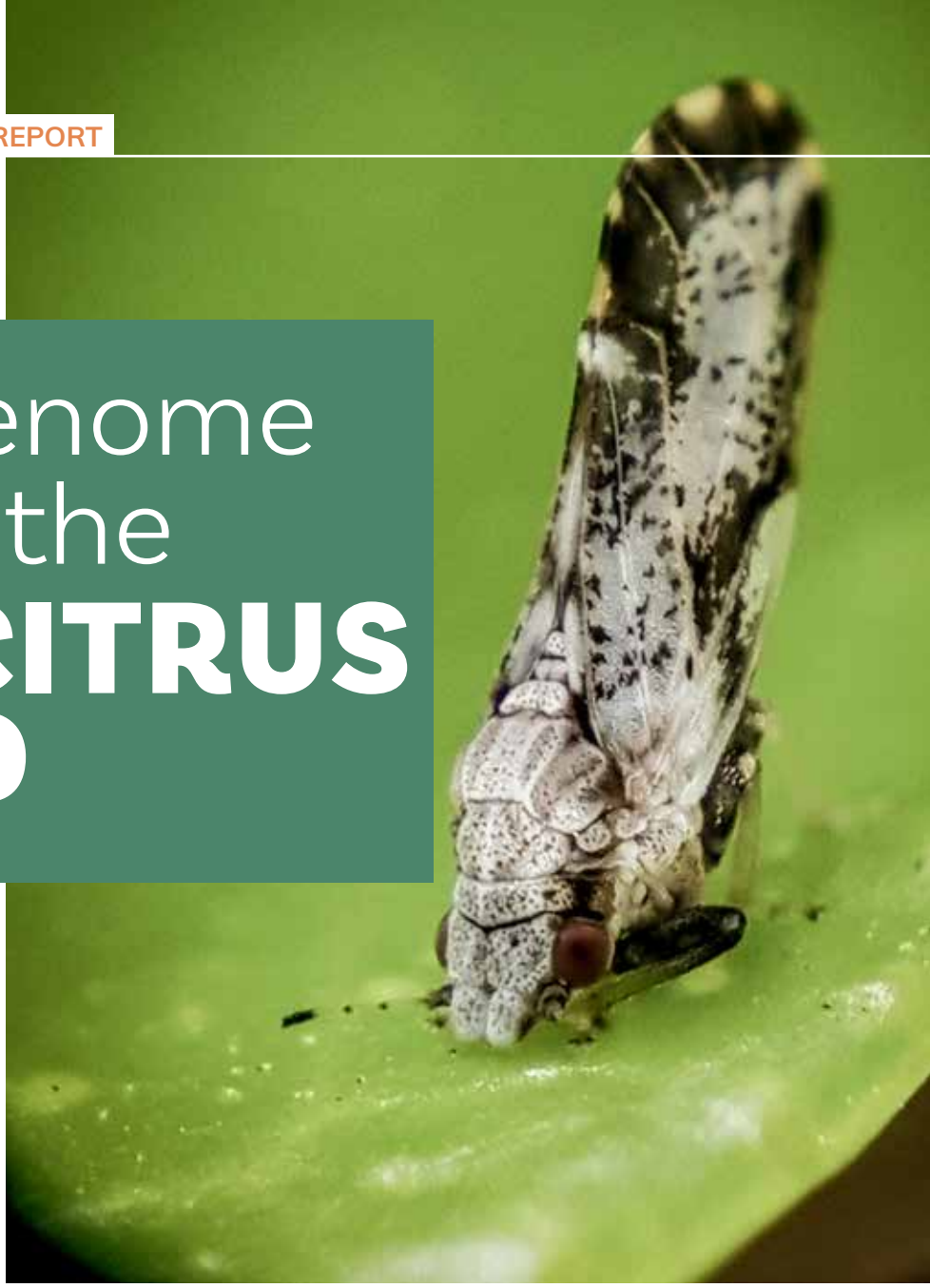

Precise Genome Editing of the ASIAN CITRUS PSYLLID

Michelle Bui, Robyn Raban and Omar S. Akbari

Project Summary

'Candidatus *Liberibacter asiaticus*' (CLas), the presumptive causal agent of huanglongbing (HLB), through its insect vector[1], the Asian citrus psyllid (ACP), has negatively impacted the citrus industry. Chemical insecticides, biocontrol agent releases, the removal of infected trees and establishment of quarantine zones with HLB-affected trees are the current control methods for this disease in California. Although these methods have thus far prevented the spread of HLB into commercial citrus in California, it remains uncertain how long they may be effective. Other insect pests and pathogen vectors, such as mosquitoes and fruit flies, have had their available control tools expanded through the development of gene-based vector control tools. These include a wide range of tools from the use of sterile insects to the development of population suppression or modification-based gene drives[2]. The goal of our work is to lay the foundation for building such tools for effective ACP control. Until now, genetic engineering has remained elusive and difficult in ACP. We are the first to demonstrate CRISPR/Cas9[3] mutagenesis of the ACP genome through embryo microinjections and have developed ACP-specific genome editing methods that can support the expansion of gene-based technologies in ACP. This advancement could help stimulate the future development of gene-based methods to control the spread of ACP and increased incidence of HLB.

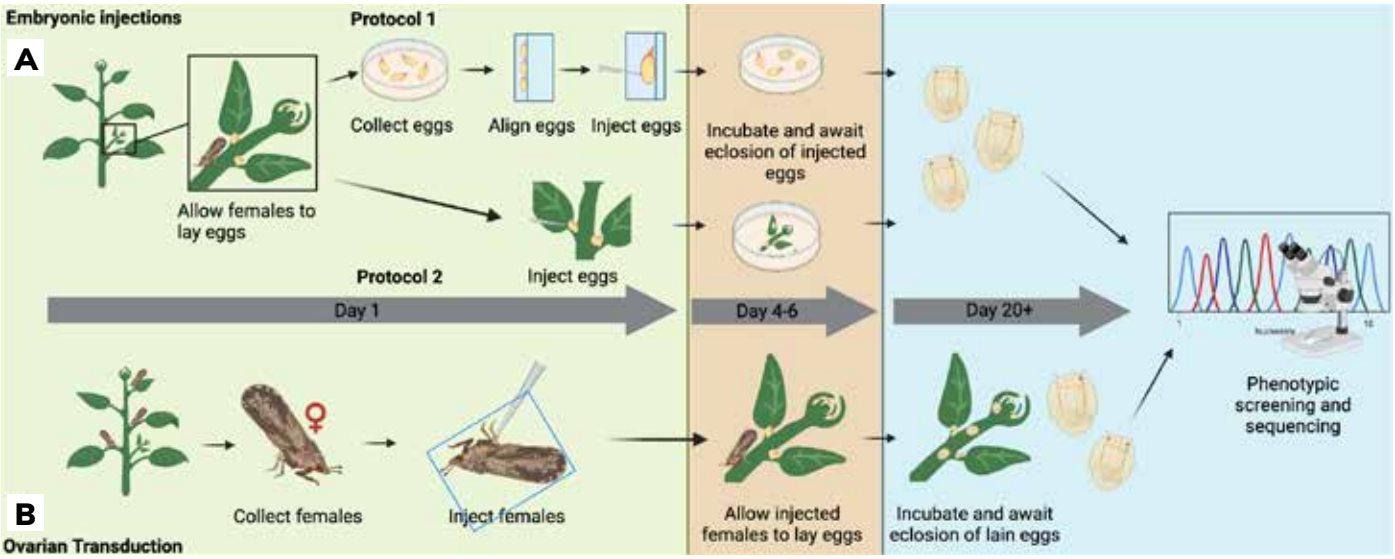

Figure 1. Methods developed for delivering Cas9 protein and guide (gRNAs) to Asian citrus psyllid eggs. For embryonic injections, freshly laid eggs were collected by either complete removal from curry leaf flush or collected still attached to the plant. Eggs then were injected and allowed to grow and emerge to the nymphal stage (known as eclosion). The resulting individuals were observed for potential phenotypes and collected for genome sequencing. Adult female injection methods were performed by injecting Cas9 protein and gRNAs directly into the female abdomen near the ovaries. These females then were mated to males and allowed to lay their eggs on curry leaf flush. The resulting offspring were observed, and their genomes were sequenced for potential mutations. Credit: Chaverra-Rodriguez, D.; Bui, M.; Gilleland, C.L.; et al. 2023. CRISPR-Cas9-mediated mutagenesis of the Asian citrus psyllid, *Diaphorina citri. GEN Biotechnology*. 3(4):317-329, by permission of Mary Ann Liebert, Inc. Publishers. Created with BioRender.com.

Huanglongbing is a major disease with a tremendous cost to the citrus industry. HLB is spread through the transmission of the bacterium CLas through ACP. Current methods for HLB management in California, including chemical insecticides, biocontrol agent releases, removal of infected plants and establishment of quarantine zones, have been moderately successful. However, these tactics are insufficient at preventing the increased incidence of the disease, while also being costly to growers. Thus, the aim of this project was to develop additional genetic methods and tools for ACP control.

In recent years, there have been significant advancements in gene-based control tools in many insect species, such as mosquitoes and fruit flies. Gene drives, for example, which can rapidly spread desired genes throughout wild insect populations at higher than normal rates of inheritance, have been at the forefront of current research due to their potential to rapidly control vectored diseases (Wang et al. 2021) and pest species (Legros et al. 2021). Other genetic tools also are being developed to produce sterile males, such as precision guided sterile insect technique (pgSIT). The pgSIT and other genetic-modification technologies for sterility can be released in large numbers in the field to reduce pest populations (Kandul et al. 2019; Li et al. 2021). To produce similar genetic modification-based control tools in ACP, robust genetic modification methods are required. However, stable germline (heritable) genetic modification of the ACP has remained elusive.

Below, we describe the first ACP genomic modification methods. These methods set the groundwork for further development of gene-based control tools in ACP.

Embryonic Injections

Precise genetic modification in insects is commonly achieved by injection of molecular gene editing components, such as CRISPR/Cas9 and guide RNAs (gRNAs), directly into freshly laid insect eggs. Cas9 protein and gRNAs are key components of the CRISPR/Cas9 gene editing system. Cas9 protein functions as "molecular scissors" to cut DNA at a target location in the genome. To inform Cas9 protein where to cut, specific target gRNAs, designed to match the target DNA sequence, are injected along with Cas9 protein.

Our first goal was to develop a method for embryonic injections of CRISPR/Cas9 to mutate the genes that can give rise to an easily visible phenotype, including *white(w)* and *kynurenine monooxygenase(kh)*, that affect insect eye color in many insects. Normal insect eyes are black or dark red, depending on the species; but when these genes are mutated, the pigmentation is lost, resulting in eye shades lighter than normal or even completely white. Selecting these as target genes, therefore, can serve as an easy marker for successful mutation of the genome.

To perform embryonic injections, ACP eggs were collected within 30 minutes to three hours after they were laid.

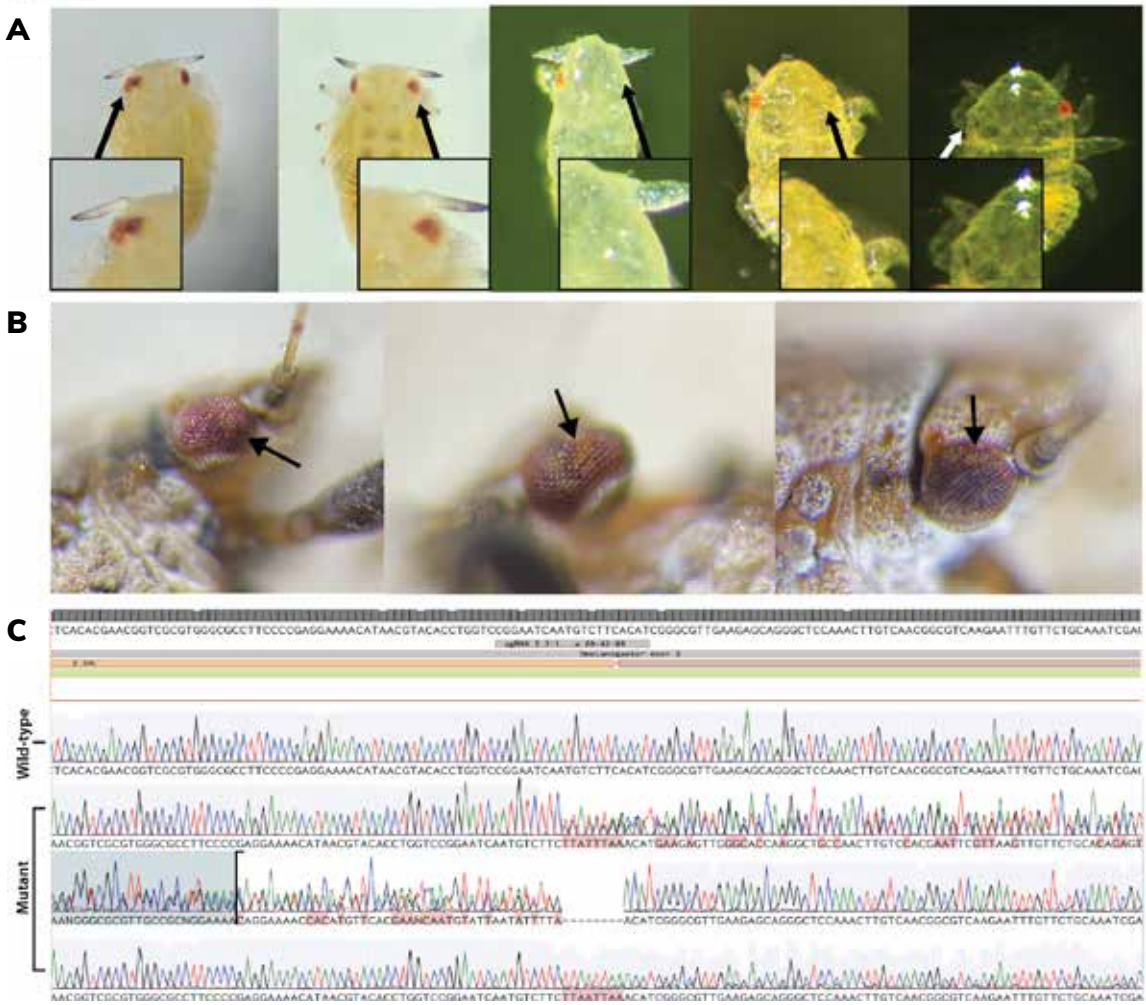

Figure 2. Non-heritable mutagenesis of the Asian citrus psyllid (ACP). A) ACP nymphs with non-heritable mosaic mutations were commonly identified by loss of pigmentation in one eye. These mutants were generated through both embryo and adult female injections. B) ACP adults also demonstrated mosaic mutant phenotypes. Shown are some eye cells that show reduced pigment in some regions adjacent to fully pigmented regions. C) Genomic sequencing data confirmed mutations within individuals with mutant phenotypes.

Because ACP lay their eggs attached to the flush of their host plant, we cut the flush from the plant and, to minimize egg handling, separated the egg-containing fragments (**Figure 1**). After collection and sterilization, we used a customized automated embryonic microinjection system to simultaneously deliver Cas9 protein and gRNAs into the eggs.

In our experiments, we have designed our gRNAs to target either the w or *kh* gene. Eggs injected with Cas9 protein and gRNAs, along with fragments of plant tissue the eggs were laid on, were kept on 5.6 percent gelatin plates containing sugar and nutritional yeast until they hatched into nymphs. Based on our experiments, we generated ACP with some of their cells mutated, but these mutations were only in somatic cells (nonreproductive cells) and could not be transmitted to the next generation (**Figure 2**). However, this is a very important step in the development of genetic technologies and the first example of mutagenesis in the ACP via embryonic injections. These exciting results merit future research to continue to develop methods so that genome edits are heritable.

Adult Female Injections

In addition to direct embryo injections, we also injected Cas9 protein and gRNAs into adult female abdomens, whereby the Cas9 protein and gRNAs could be absorbed into the ovaries and developing oocytes[4]. We evaluated three methods of Cas9 protein and gRNA delivery into oocytes without the need for direct injections, including Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control), which consists of a modified Cas9-protein with a bound peptide, Branched Amphiphilic Peptide Capsules (BAPC) and Direct Paternal (DIPA)-CRISPR that use large concentrations of commercially available Cas9 protein to encourage oocyte uptake (Chaverra-Rodriguez et al. 2018; Huang et al. 2021; Hunter et al. 2021; Shirai et al. 2022). Adult female injections are easier to perform than embryo injections since adult females are significantly larger and less delicate than embryos.

In addition, the equipment required for adult injections is more affordable and accessible than what is needed for embryo injections. Using this injection method along with

the Cas9 delivery methods outlined above, we were able to partially change eye color in some of the psyllid offspring (**Figure 2**).

ACP Transgenesis

To develop tools for pest control, foreign DNA needs to be inserted into the ACP genome[5]. This foreign DNA could encode genes that make ACP incapable of transmitting CLas or encode Cas9 protein and gRNAs that can kill ACP by mutating genes required for their survival or fertility. We used CRISPR/Cas9 and foreign DNA injections to insert a foreign genetic sequence for a red fluorescent protein (RFP) and the components needed to express this protein. ACP do not naturally express RFP; but when properly mutated to contain our foreign RFP sequence in their genome and excited at the correct wavelength, they will glow red and allow us to identify genetically modified individuals.

Conclusions

Gene modification-based technologies could revolutionize the control of ACP. Basic genetic tools are limited in the ACP; however, we have developed multiple methods that improve genome engineering capabilities in the ACP. More notably, we were the first to generate visible, non-heritable mutations in the ACP using embryonic and adult female injections of CRISPR/Cas9 (Chaverra-Rodriguez et al. 2023). These accomplishments demonstrate that we are close to developing the necessary tools for genetic control technologies. Our next steps in this work are to confirm the ability to generate heritable mutations in the ACP genome as well as the integration of foreign DNA. The same methods used to perform these tasks can then be used to generate a functional genetic-based control tool for ACP such as gene drives and pgSIT.

CRB Research Project #5500-217

Glossary

[1]Vector: A living organism that can harbor and transmit infectious agents from one infected organism to another.

[2]Gene Drive: A system that biases the frequency of a gene above rates derived from normal inheritance. Gene drives may be used, for example, to spread genes detrimental to survival, reproduction or disease agent transmission into insect populations.

[3]CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats is a genomic editing system with the capability of making precise genomic modifications by removing, adding or altering sections of DNA.

[4]Oocyte: Egg cell.

[5]Genome: A cell or organism's complete set of genes.

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