BAPC-assisted CRISPR/Cas9 System:

Targeted Delivery into Adult Ovaries for Heritable Germline Gene Editing (Arthropoda: Hemiptera)

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ABSTRACT

Innovative gene targeting strategies are often limited in application across arthropod species due to problems with successful delivery. In hemipterans, embryonic injections often used to deliver CRISPR components fail due to nearly complete embryo mortality. The Asian citrus psyllid, *Diaphorina citri*, Kuwayama, (Hemiptera: Liviidae), is the vector for a pathogenic bacterium, *Candidatus* Liberibacter asiaticus, CLas, which is devastating the U.S. citrus industries. The disease called, Huanglongbing, HLB, (aka. Citrus greening disease), is transmitted during psyllid feeding. Infection causes severe tree decline, loss of fruits, and eventually tree death. The citrus tree pathogen, CLas, is a fastidious alpha-proteobacterium, which has spread into all citrus growing

regions worldwide. The economic losses are estimated in the billions of dollars, in U.S.A., Brazil, and China. Innovative technologies aimed at reducing psyllid populations using targeting RNA suppression, like RNAi, or gene-editing tools, like CRISPR/Cas9 have potential to reduce psyllid vectors and the pathogen in a highly specific manner. Breakthroughs that improve gene editing in psyllids, such as the *BAPC-assisted*-CRISPR/Cas9 System, enabled delivery by injection of CRISPR/Cas9 components directly into nymphs and adult females. Injection near ovaries produced heritable germline gene editing in subsequent generations. This method opens the world of gene editing across arthropods and bypasses the need for microinjection of eggs. Effective development of therapeutic treatments to reduce insect vectors, and stop pathogen transmission would provide sustainable citrus and grapevine industries.

Keywords: Asian citrus psyllid, Citrus, Gene edit, RNAi Leafhoppers, Huanglongbing, management, pest control

INTRODUCTION

Biotechnologies provide techniques that greatly improve the level of safety and target specificity in the management of pests and pathogens (Bhaya et al, 2011; Garneau et al, 2010; Fire et al, 1998; Doudna & Charpentier 2014). These techniques include targeted RNA suppression, gene regulation, and gene editing in all organisms: bacteria, plants, animals, and humans. As traditional chemical insecticides fail to provide adequate pest management, due to development of chemical resistance, dependence upon biotech strategies for management have

become the best options for development of therapeutic treatments to reduce arthropod vectors, the pathogens, or to cause disruption of vector pathogen acquisition and transmission (Andrade & Hunter 2016; Baum & Roberts 2014; Gantz & Akbari 2018; Hunter & Sinisterra-Hunter 2018; Kolliopoulou et al, 2017; Roberts et al, 2015; Petrick et al, 2013;2016; Scott et al, 2013; Sinisterra-Hunter & Hunter 2018; Taning et al, 2017; Zotti et al, 2018). The rapid emergence of gene editing techniques, like CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein, Cas9, provide precise editing of genes across all species (Doudna & Charpentier 2014; Peng et al., 2014; Wang et al. 2016). As a natural mechanism in bacteria, numerous studies have described the mode of function of the CRISPR defense system that is an adaptive mechanism, which enables bacteria to suppress invading viruses (Garneau et al, 2010; Doudna & Charpentier 2014). The technology has now been co-adapted to target genes within insect pests (Taning et al, 2017). The relative ease of use, normally by injection into eggs/embryos, combined with improved methods for increase efficacy in CRISPR/Cas9 systems has led CRISPR/Cas to become the primary gene editing tool in the life sciences. For reviews on CRISPR/Cas9 systems see: (Boettcher & McManus 2015; Dominguez et al. 2016; Gupta & Shukla 2016; La Russa & Qi 2015; Liang et al. 2015; Taning et al, 2017; Wang et al. 2016; Wilson & Doudna 2013). Reviews on other gene editing systems with Zinc finger nucleases and TAL effector nucleases, TALENS, see: (Gaj et al, 2013; Bortesi & Fischer 2015; Markert et al, 2016).

CRISPR/Cas systems have demonstrated important applications in agriculture, increasing the options for the management of arthropod pests, insect vectors, and treatments against pathogens of plants, animals and humans (Chen et al, 2016; Chen et al, 2017; Cui et al, 2017; Taning et al, 2017; Sun et al, 2017; Gantz & Akbari 2018; Gundersen-Rindal et al, 2017; Sinisterra-Hunter & Hunter 2018). However, one of the hurdles for rapid adoption in arthropods has been the reliance on embryo injections (Li et al, 2017), which is often unsuccessful in many arthropod species (Bortesi & Fischer 2015; Boettcher & McManus, 2015; Chaverra-Rodriguez et al, 2018; Gregory et al, 2016). Thus, an improved delivery method is needed if gene editing is to be realized for many arthropod species. This is especially true within the Hemiptera, which have few successful demonstrations of embryonic transformation through microinjection of eggs.

In 2018, Hunter described a direct method that delivered and produced gene editing using injections into the abdomens of nymphs, pupae, and adults of hemipterans. Specific examples were the Asian citrus psyllid (*Diaphorina citri*, Kuwayama, Hemiptera: Liviidae) and glassy-winged sharpshooter leafhopper (*Homalodisca vitripennis*, (Germar): Hemiptera: Cicadellidae) (Hunter et al, 2018ab). Previous attempts injecting thousands of eggs failed. Switching to injection of 5th instar nymphs and adult females, produced the first successful trials of gene knockouts in psyllids used the Cas9 protein, co-injected with two sgRNA, producing about 30% surviving G0 and G1 mutants. To improve the system, experiments evaluated the incorporation of BAPC-assisted delivery (Hunter, Gonzalez, Tomich 2018).

BAPC-assisted Delivered RNA interference In Arthropods

The **Branched Amphiphilic Peptide Capsules (BAPC),** are a new class of inert, self-assembling peptide nano-capsular spheres (Phoreus Biotechnology, Inc., Olathe, KS, USA). The peptide-based nano-assemblies show promise as nano-delivery vehicles for the safe, targeted transport of drugs, plasmids, dsRNA, and siRNA, to specific tissues and organs with minimal off target accumulation (Gudlur et al, 2012; Sukthankar et al, 2013; 2014; Avila et al, 2015).

Studies on RNAi report that incorporation of BAPC with dsRNA to specific beetle genes, caused significant increase in mortality compared to controls upon ingestion (Avila et al, 2018). In those studies, BAPC-dsRNA was fed to *Tribolium castaneum* (Coleoptera), and the pea aphid, *Acyrthosiphon pisum* (Hemiptera). The authors report an improved delivery of dsRNA into cells most likely due to presence of the BAPC, which prevents degradation by nucleases, producing slower controlled release of the dsRNA upon entering cells, resulting in increased RNAi efficacy and subsequent increased mortality of the insects.

Based on the physical properties of BAPC with nucleic acids we hypothesized that BAPC mixed with guide RNA's and CRISPR/Cas9 components would result in improved delivery and produce a new method for heritable germline gene editing suitable for injection into adult ovaries of psyllids and

other arthropods (Hunter & Sinisterra-Hunter 2018; Hunter, Gonzalez, Tomich 2018; Hunter et al, 2019).

Similar proofs-of-concepts in flies are reported by Chaverra-Rodriguez et al, (2018) in mosquitoes using the P2C peptide mediated transduction of the Cas9 plasmid from the female hemolymph into the developing mosquito oocytes. Their technology, termed "Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control), was shown to work well within the Order: Diptera (Mosquitoes). Another delivery system reported by Wang et al, (2018), in mammals, used microvesicles. Extracellular vesicles, known as *arrestin domain containing protein 1* [ARRDC1]-mediated microvesicles (ARMMs). These ARMMs, could package and deliver intracellularly of a myriad of macromolecules, including the tumor suppressor p53 protein, RNAs, and the genome-editing CRISPR-Cas9/guide RNA complex into mammalian cells.

BAPC-assisted-CRISPR/Cas9 Delivery System: Adult injection near ovaries for heritable germline gene editing [Hemiptera: _Diaphorina citri_).

Attempts to deliver CRISPR/Cas9 into psyllid eggs proved to be difficult and unsuccessful. Injection directly into the 4th and 5th nymphs, pupae, or adults proved to be easier, and more effective (Hunter, Gonzalez, Tomich 2018). Because previous research with BAPC-assisted delivery of dsRNA and plasmids resulted in efficient delivery into insects and animal cells in cultures (Avila et al, 2018; Sukthankar et al, 2014), an experiment was designed to evaluate the incorporation of BAPC to improve delivery of CRISPR/Cas9 components into

arthropod ovaries of adult hemipterans (Psyllids and Leafhoppers) for heritable embryonic gene editing.

Gene Selection. *Diaphorina citri*, have at least two Thioredoxins, TRX-1, TRX-2, with variants in the mitochondria and cytoplasm. Thioredoxin participates in various redox reactions and catalyzes dithiol-disulfide exchange reactions. Thioredoxin 2, TRX-2, is preferred over thioredoxin 1, as a reducing substrate of peroxiredoxin-1. Thioredoxin is required for female meiosis and early embryonic development. The functions of at least 30 proteins, including enzymes and transcription factors, the regulation of cellular proliferation and the aging process are regulated by TRX (Yoshida et al, 2005). The guide RNA's and protospacer adjacent motif (PAM) were identified and confirmed using software (Dharmacon, Inc., (Lafayette, CO, 80026). The CRISPR-associated protein 9 (Cas9) was purchased, and protocols were gleaned from publications on CRISPR (Bassett et al, 2013; Kistler, et al, 2015; Larson, et al, 2013; Zhang & Reed 2017; Garczynski et al, 2017). The ACP gene sequence ID: XM_008487100.1, gene, thioredoxin-2-like (LOC103521994) was from the DIACI_2.0 Genome assembly: https://citrusgreening.org/organism/Diaphorina_citri/genome (Saha et al, 2017).

CRISPR/Cas9 Injections

Injections with CRISPR components, sgRNA's, and a Cas9 protein, successfully produced knock-out G0 and G1 mutants (Hunter, Gonzalez, Tomich 2018). Subsequent trials incorporated the aid of the *BAPC-assisted*-CRISPR

components, successfully produced heritable knockout G2 mutants. Both these methods successfully demonstrated the first psyllid gene knockouts, KO, with CRISPR/Cas9. Designs to *Diaphorina citri*, Asian citrus psyllid, ACP, (Hemiptera: Liviidae) for the knockout used two gRNAs to direct the Cas9 endonuclease to two sites 556 bp apart (Dharmacon, Inc.). The ACP-TRX-2 KO trials injected 30 nymphs (4^{th} and 5^{th} instar), and 20 adult females per treatment (co-injection of two sgRNAs (100 ng/ µL of each, with 200 ng/µL of Cas9 protein, plus BAPC (0.1 ng/ µL) (Drummond Nanoject III, 3-000-207) (Hunter, Gonzalez, Tomich 2018; Hunter et al, 2018; Hunter & Sinisterra 2018). Insects were injected ventrally, off center of midline in the abdomen (FIG. 1). Post injections psyllids were transferred to a citrus seedling to oviposit (~25 cm tall, sweet orange seedlings). A cohort of 6 psyllid adult females were individually analyzed 7 d post treatments using PCR analyses and sequencing. Primers were designed to bracket the gDNA KO sequence of the TRX ORF at 200 to 250 nt beyond the ends in each direction. The remaining cohort of ACP nymphs, which were TRX-KO mutants took 6 to 8 d longer to eclose to adults. The adult psyllids with TRX-KO had significantly shorter lifespans post eclosion living an average 8.5 d, compared to controls injected with buffer, or GFP-plasmid (FIG. 2), which lived an average of 16 d post eclosion (Hunter Gonzalez, Tomich 2018; Hunter & Sinisterra-Hunter 2018; Hunter et al, 2019). (Plasmid resource, Addgene™: pAc5.1B-EGFP was a gift from Elisa Izaurralde, Addgene plasmid #21181)(Karlikow et al, 2016; Legaz et al, 2015). Adult female insects producing eggs (FIG. 3), when treated also produced

G2 mutants. Delivery of a GFP-plasmid, when mixed with BAPC, was successfully ingested and expressed in adult psyllids (FIG. 4).

Conclusions:

Advances in biotechnologies, like CRISPR and RNAi provides new sustainable and environmentally friendly strategies to reduce insect vectors, like psyllids (Andrade & Hunter 2016; 2017; Hunter & Sinisterra-Hunter 2018; Taning et al, 2016; Ghosh et al, 2018). These advances will also aid in the management of many other insect vectors and pests (Chaverra-Rodriguez et al, 2018; Darrington et al, 2017; Dong, et al, 2015; 2018; Gantz & Akbari 2018; Ghosh et al, 2018; Kolliopoulou et al, 2017; Sinisterra-Hunter & Hunter 2018; Taning et al, 2017; Zotti et al, 2018).

CRISPR/Cas9 gene editing in hemipterans was shown to be feasible using nymphs and adult psyllids, as the recipient for gene editing CRISPR components. The method was a significant improvement over efforts using injection of eggs. Furthermore, incorporation of *BAPC-assisted* delivery of CRISPR/Cas9 components into nymph and adults provides an innovative breakthrough in hemipteran gene editing. Production of G2 mutants, from *BAPC-assisted*-CRISPR/Cas9 injected, adult female psyllids further supports the viability of this method. Improvements in efficacy, by adjusting component concentration ratios still need to be evaluated across several hemipteran species.

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226 *programs and activities on the basis of race, color, national origin, gender,*
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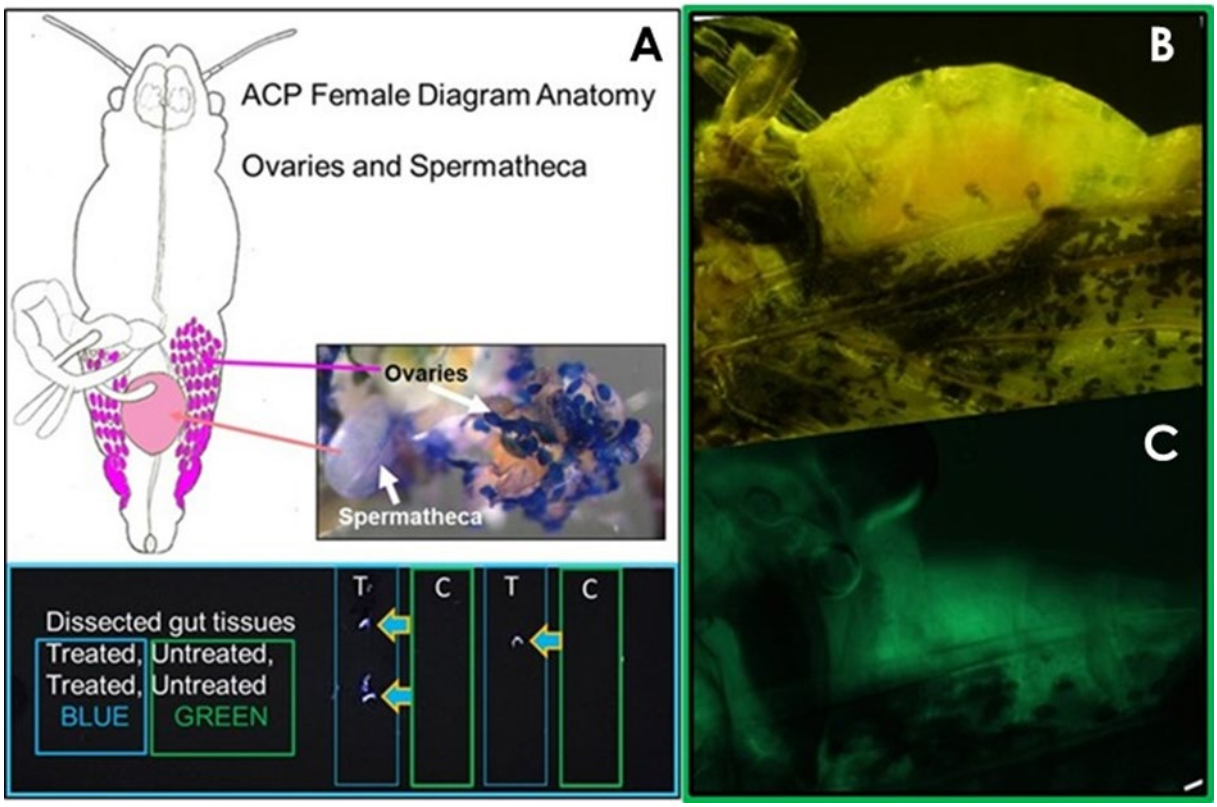
Figure_1. Method of micro-injection of CRISPR/Cas9 components into abdomens of 4th, 5th instars and adult female, Asian citrus psyllid, *Diaphorina citri*, (Hemiptera: Liviidae)(Drummond Nanoject III). Nymph on citrus leaf (Left). Nymphs and adults were placed onto solidified, chilled, 1% agar for injections. Shown is artificial dyed solution for easier visualization of method. Abdomen is most proximal, with the head and two dark antennae more distal.

Figure_2. Female adult Asian Citrus Psyllid, *Diaphorina citri* Kuwayama. A) Female anatomy showing ovaries, and spermatheca in diagram. Fresh dissection (stained dark blue, Tryptophan). Injection of plasmid-GFP expression, psyllid dissected 8 d post injection. Illumination of Green fluorescent protein, GFP with UV light (Blue Arrows). **B)** The GFP-plasmid (MTG-Dc-1, actin) was injected, **C)** Expression under Dc-Actin promoter identified from *DIACI_2.0 genome,* OGS_0.2v (Saha, et al, 2017). (Plasmid, Addgene, Karlikow et al, 2016).



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253 **Figure_3. Micro-CT imaging of adult female Asian citrus psyllid**, *Diaphorina*

254 *citri*, Kuwayama (Hemiptera: Liviidae). (Alba-Tercedor, J. and Hunter, W.B. 2016).

255 www.citrusgreening.org [Alba-Tercedor et al, 2018].

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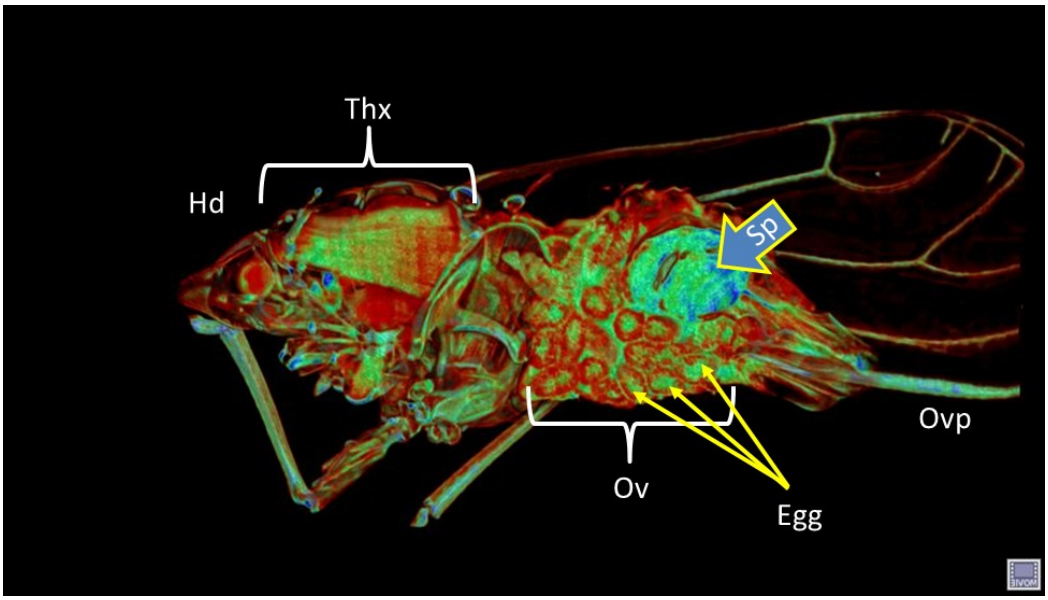

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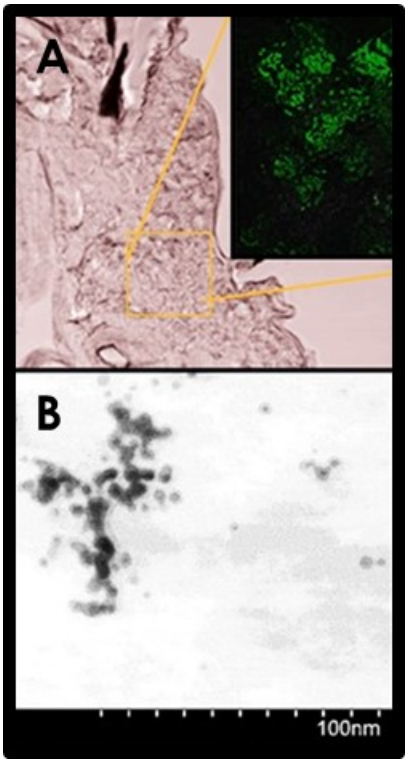
Figure_4. BAPC-assisted delivery of GFP-plasmid post ingestion. Trial using BAPC-labeled with fluorescent Probe Atto488. Oral delivery to adult psyllid, 5 d post feeding, from sucrose solution (25%) plus BAPC in feeding satchet. Thick-section of single psyllid abdomen tissues fixed and parafilm embedded, thick section, (GFP insert, green). Demonstrated delivery and location post ingestion of BAPC in psyllid tissues. Psyllid actin promoter designed as previously mentioned in methods (MTG-Dc-1).



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