

1                                    ***BAPC-assisted* CRISPR/Cas9 System:**  
2                                    **Targeted Delivery into Adult Ovaries for Heritable Germline**  
3                                    **Gene Editing (Arthropoda: Hemiptera)**

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5                                    **Wayne B. Hunter<sup>1</sup>, Maria T. Gonzalez<sup>1</sup>, John Tomich<sup>2</sup>**

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7                                    1. USDA, ARS, 2001 South Rock Road, Fort Pierce, FL, 34945. USA.  
8                                    2. John Tomich, Department of Biochemistry and Molecular Biophysics, Kansas  
9                                    State University, Manhattan, KS, 66506. USA.

10  
11                                    Contacts: [Wayne.hunter@ars.usda.gov](mailto:Wayne.hunter@ars.usda.gov) & [Jtomich@ksu.edu](mailto:Jtomich@ksu.edu)

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13                                    **ABSTRACT**

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

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

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

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## 41 INTRODUCTION

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

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

71 CRISPR/Cas systems have demonstrated important applications in  
72 agriculture, increasing the options for the management of arthropod pests, insect  
73 vectors, and treatments against pathogens of plants, animals and humans (Chen  
74 et al, 2016; Chen et al, 2017; Cui et al, 2017; Taning et al, 2017; Sun et al, 2017;  
75 Gantz & Akbari 2018; Gundersen-Rindal et al, 2017; Sinisterra-Hunter & Hunter  
76 2018). However, one of the hurdles for rapid adoption in arthropods has been  
77 the reliance on embryo injections (Li et al, 2017), which is often unsuccessful in  
78 many arthropod species (Bortesi & Fischer 2015; Boettcher & McManus, 2015;  
79 Chaverra-Rodriguez et al, 2018; Gregory et al, 2016). Thus, an improved delivery  
80 method is needed if gene editing is to be realized for many arthropod species.  
81 This is especially true within the Hemiptera, which have few successful  
82 demonstrations of embryonic transformation through microinjection of eggs.

83 In 2018, Hunter described a direct method that delivered and produced  
84 gene editing using injections into the abdomens of nymphs, pupae, and adults  
85 of hemipterans. Specific examples were the Asian citrus psyllid (*Diaphorina citri*,  
86 Kuwayama, Hemiptera: Liviidae) and glassy-winged sharpshooter leafhopper  
87 (*Homalodisca vitripennis*, (Germar): Hemiptera: Cicadellidae) (Hunter et al,  
88 2018ab). Previous attempts injecting thousands of eggs failed. Switching to  
89 injection of 5<sup>th</sup> instar nymphs and adult females, produced the first successful  
90 trials of gene knockouts in psyllids used the Cas9 protein, co-injected with two  
91 sgRNA, producing about 30% surviving G0 and G1 mutants. To improve the  
92 system, experiments evaluated the incorporation of BAPC-assisted delivery  
93 (Hunter, Gonzalez, Tomich 2018).

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## 96 ***BAPC-assisted* Delivered RNA interference In Arthropods**

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

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

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

115 other arthropods (Hunter & Sinisterra-Hunter 2018; Hunter, Gonzalez, Tomich  
116 2018; Hunter et al, 2019).

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

128 ***BAPC-assisted-CRISPR/Cas9 Delivery System: Adult injection near ovaries for***  
129 ***heritable germline gene editing [Hemiptera: *Diaphorina citri*].***

130 Attempts to deliver CRISPR/Cas9 into psyllid eggs proved to be difficult  
131 and unsuccessful. Injection directly into the 4<sup>th</sup> and 5<sup>th</sup> nymphs, pupae, or adults  
132 proved to be easier, and more effective (Hunter, Gonzalez, Tomich 2018).  
133 Because previous research with BAPC-assisted delivery of dsRNA and plasmids  
134 resulted in efficient delivery into insects and animal cells in cultures (Avila et al,  
135 2018; Sukthankar et al, 2014), an experiment was designed to evaluate the  
136 incorporation of BAPC to improve delivery of CRISPR/Cas9 components into

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

139 **Gene Selection.** *Diaphorina citri*, have at least two Thioredoxins, TRX-1, TRX-2,  
140 with variants in the mitochondria and cytoplasm. Thioredoxin participates in  
141 various redox reactions and catalyzes dithiol-disulfide exchange reactions.  
142 Thioredoxin 2, TRX-2, is preferred over thioredoxin 1, as a reducing substrate of  
143 peroxiredoxin-1. Thioredoxin is required for female meiosis and early embryonic  
144 development. The functions of at least 30 proteins, including enzymes and  
145 transcription factors, the regulation of cellular proliferation and the aging  
146 process are regulated by TRX (Yoshida et al, 2005). The guide RNA's and  
147 protospacer adjacent motif (PAM) were identified and confirmed using software  
148 (Dharmacon, Inc., (Lafayette, CO, 80026). The CRISPR-associated protein 9 (Cas9)  
149 was purchased, and protocols were gleaned from publications on CRISPR (Bassett  
150 et al, 2013; Kistler, et al, 2015; Larson, et al, 2013; Zhang & Reed 2017;  
151 Garczynski et al, 2017). The ACP gene sequence ID: XM\_008487100.1, gene,  
152 thioredoxin-2-like (LOC103521994) was from the DIACI\_2.0 Genome assembly:  
153 [https://citrusgreening.org/organism/Diaphorina\\_citri/genome](https://citrusgreening.org/organism/Diaphorina_citri/genome) (Saha et al,  
154 2017).

### 155 **CRISPR/Cas9 Injections**

156 Injections with CRISPR components, sgRNA's, and a Cas9 protein,  
157 successfully produced knock-out G0 and G1 mutants (Hunter, Gonzalez, Tomich  
158 2018). Subsequent trials incorporated the aid of the *BAPC-assisted*-CRISPR

159 components, successfully produced heritable knockout G2 mutants. Both these  
160 methods successfully demonstrated the first psyllid gene knockouts, KO, with  
161 CRISPR/Cas9. Designs to *Diaphorina citri*, Asian citrus psyllid, ACP, (Hemiptera:  
162 Liviidae) for the knockout used two gRNAs to direct the Cas9 endonuclease to  
163 two sites 556 bp apart (Dharmacon, Inc.). The ACP-TRX-2 KO trials injected 30  
164 nymphs (4<sup>th</sup> and 5<sup>th</sup> instar), and 20 adult females per treatment (co-injection of  
165 two sgRNAs (100 ng/  $\mu$ L of each, with 200 ng/ $\mu$ L of Cas9 protein, plus BAPC (0.1  
166 ng/  $\mu$ L) (Drummond Nanoject III, 3-000-207) (Hunter, Gonzalez, Tomich 2018;  
167 Hunter et al, 2018; Hunter & Sinisterra 2018). Insects were injected ventrally, off  
168 center of midline in the abdomen (FIG. 1). Post injections psyllids were  
169 transferred to a citrus seedling to oviposit (~25 cm tall, sweet orange seedlings).  
170 A cohort of 6 psyllid adult females were individually analyzed 7 d post treatments  
171 using PCR analyses and sequencing. Primers were designed to bracket the gDNA  
172 KO sequence of the TRX ORF at 200 to 250 nt beyond the ends in each direction.  
173 The remaining cohort of ACP nymphs, which were TRX-KO mutants took 6 to 8 d  
174 longer to eclose to adults. The adult psyllids with TRX-KO had significantly  
175 shorter lifespans post eclosion living an average 8.5 d, compared to controls  
176 injected with buffer, or GFP-plasmid (FIG. 2), which lived an average of 16 d post  
177 eclosion (Hunter Gonzalez, Tomich 2018; Hunter & Sinisterra-Hunter 2018;  
178 Hunter et al, 2019). (Plasmid resource, Addgene™: pAc5.1B-EGFP was a gift from  
179 Elisa Izaurralde, Addgene plasmid #21181)(Karlikow et al, 2016; Legaz et al,  
180 2015). Adult female insects producing eggs (FIG. 3), when treated also produced



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

### 183 **Conclusions:**

184 Advances in biotechnologies, like CRISPR and RNAi provides new sustainable  
185 and environmentally friendly strategies to reduce insect vectors, like psyllids  
186 (Andrade & Hunter 2016; 2017; Hunter & Sinisterra-Hunter 2018; Taning et al,  
187 2016; Ghosh et al, 2018). These advances will also aid in the management of  
188 many other insect vectors and pests (Chaverra-Rodriguez et al, 2018;  
189 Darrington et al, 2017; Dong, et al, 2015; 2018; Gantz & Akbari 2018; Ghosh et  
190 al, 2018; Kolliopoulou et al, 2017; Sinisterra-Hunter & Hunter 2018; Taning et  
191 al, 2017; Zotti et al, 2018).

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

201

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228 *status.*

229

230 **Figure\_1. Method of micro-injection of CRISPR/Cas9 components into**  
231 **abdomens of 4<sup>th</sup>, 5<sup>th</sup> instars and adult female, Asian citrus psyllid, *Diaphorina***  
232 ***citri*, (Hemiptera: Liviidae)(Drummond Nanoject III). Nymph on citrus leaf (Left).**  
233 Nymphs and adults were placed onto solidified, chilled, 1% agar for injections.  
234 Shown is artificial dyed solution for easier visualization of method. Abdomen is  
235 most proximal, with the head and two dark antennae more distal.

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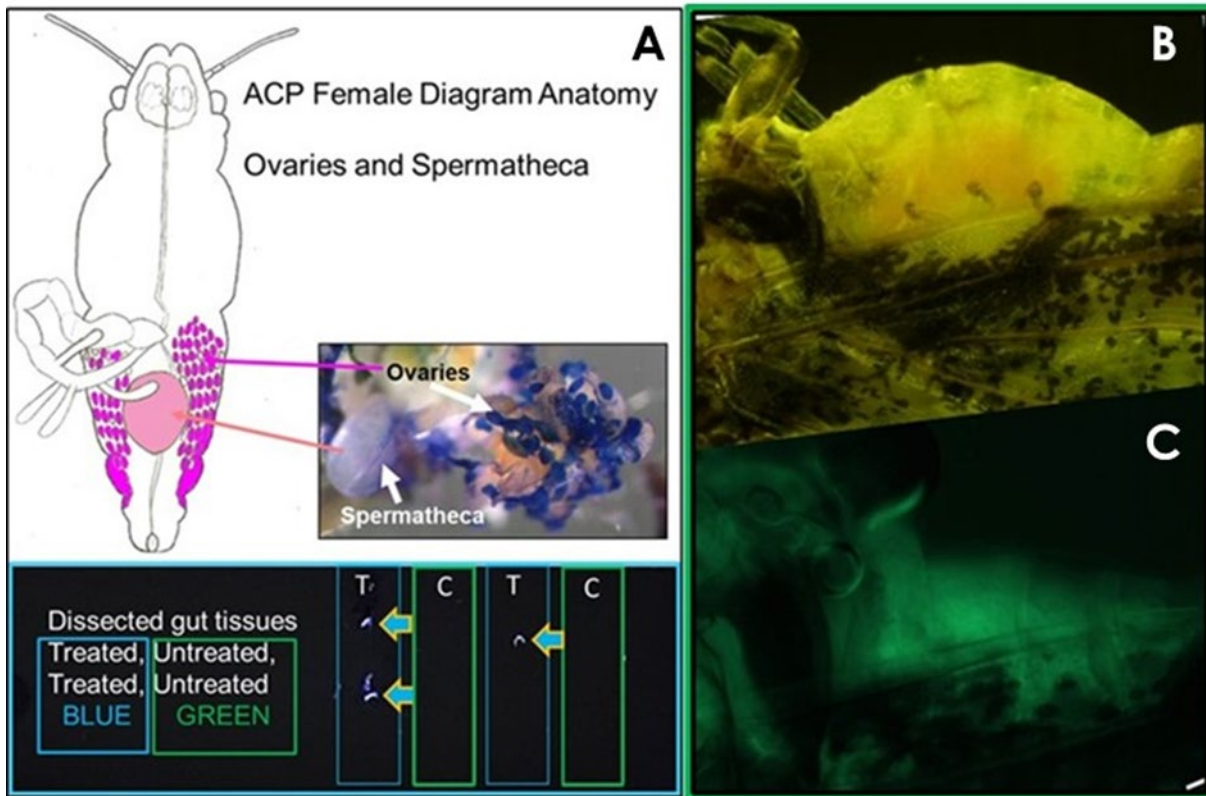
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240 **Figure\_2. Female adult Asian Citrus Psyllid, *Diaphorina citri* Kuwayama. A)**  
241 Female anatomy showing ovaries, and spermatheca in diagram. Fresh dissection  
242 (stained dark blue, Tryptophan). Injection of plasmid-GFP expression, psyllid  
243 dissected 8 d post injection. Illumination of Green fluorescent protein, GFP with  
244 UV light (Blue Arrows). **B)** The GFP-plasmid (MTG-Dc-1, actin) was injected, **C)**  
245 Expression under Dc-Actin promoter identified from *DIACI\_2.0 genome*,  
246 OGS\_0.2v (Saha, et al, 2017). (Plasmid, Addgene, Karlikow et al, 2016).

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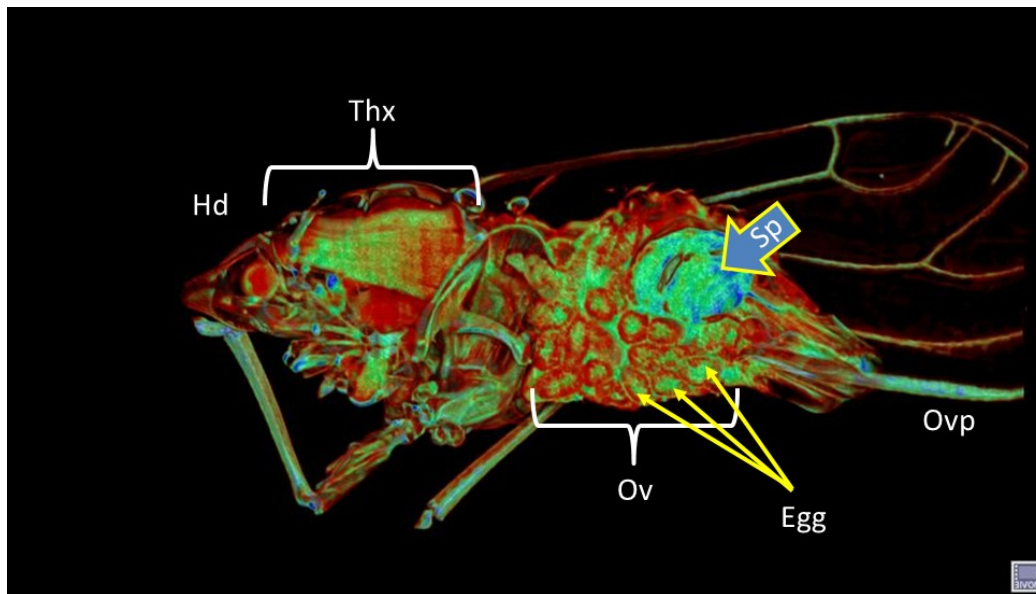
252

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](http://www.citrusgreening.org) [Alba-Tercedor et al, 2018].

256



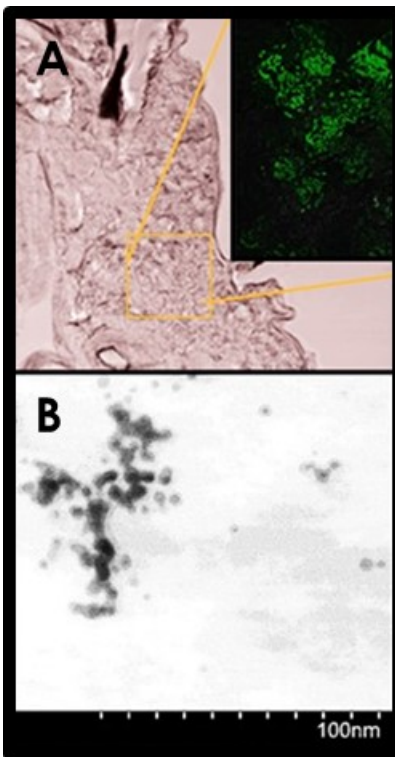
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261 **Figure\_4. BAPC-assisted delivery of GFP-plasmid post ingestion.** Trial using  
262 BAPC-labeled with fluorescent Probe Atto488. Oral delivery to adult psyllid, 5 d  
263 post feeding, from sucrose solution (25%) plus BAPC in feeding satchet. Thick-  
264 section of single psyllid abdomen tissues fixed and parafilm embedded, thick  
265 section, (GFP insert, green). Demonstrated delivery and location post ingestion  
266 of BAPC in psyllid tissues. Psyllid actin promoter designed as previously  
267 mentioned in methods (MTG-Dc-1).



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288 [based-technology-for-highly-specific-pest-control-hispec-](http://www.intechopen.com/books/rna-interference/rna-interference-natural-gene-based-technology-for-highly-specific-pest-control-hispec-)
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