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Short Communication

Efficacy of In Vivo Electroporation on the Delivery of Molecular Agents into Aphid (Hemiptera: Aphididae) Ovarioles

Brendan V. Jamison,[1] Margaret W. Thairu,[2] and Allison K. Hansen[2]

[1]Department of Entomology, University of Illinois, Urbana-Champaign, 505 S. Goodwin Ave, Urbana, IL 61801, [2]Department of Entomology, University of California, Riverside, 417 Entomology Bldg., Riverside, CA 92521, and [3]Corresponding author, e-mail: allison.hansen@ucr.edu

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Abstract

While the wealth of genomic data presently available is increasing rapidly, the advancement of functional genomics technologies for the large majority of these organisms has lagged behind. The Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 system is an emerging gene-editing technology derived from a bacterial adaptive immune system that has proven highly effective in multiple model systems. Here, the CRISPR/Cas9 system was delivered into the ovarioles of the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera, Aphididae), with a new delivery method utilizing in vivo electroporation. To validate gene-editing, a target sequence within the marker *tor* pigment gene was chosen, and gene-editing was predicted to result in white pigmentation in the offspring of treated adult aphids. Adult aphids (10-d old) were injected with the *tor* single guide RNA and Cas9 complex and subsequently subjected to electroporation. Adult aphids were given 4 d to produce viviparous offspring. After offspring developed for 6 d, DNA was extracted and sequenced to validate if CRISPR/Cas9-directed gene editing occurred. A survival rate over 70% was found in treated adult aphids. A distinct white pigmentation was found in 2.5% of aphids; however, gene-editing within the target sequence was not found in any of the individuals screened. Presence of white aphids without gene-editing suggests other mechanisms may have influenced pigmentation. High survival rates in experimental treatments demonstrate the robustness of this new technique, and further refinement of this technique may prove it as an effective functional genomics tool for viviparous insects and/or gene editing at a somatic level.

Key words: *Aphididae*, CRISPR/Cas9, in vivo electroporation

To date, almost 902 animal genomes have been fully sequenced and/or are in draft form (NCBI 2018). Biologists who study any level of biological organization—from cells to molecules, organismal to ecosystems/evolution—are finding strategic ways to extract and analyze this treasure trove of ever-increasing genomic data. Nevertheless, only 0.5% of these eukaryotic genomes are model genetic organisms (Models 2018). In turn, few or no genetic technologies are currently developed for the vast majority of these organisms. Ultimately, the continued development of functional genomic technologies, especially for nonmodel organisms, is crucial for understanding gene functions and their roles in biological processes and in nature.

While the delivery of gene editing systems has been widely developed for many model species, its application for nonmodel systems is still early in development. For example, conditions tested for difficult nonmodel systems are not widely documented partially due to file drawer effects and lack of funding. Currently, no gene editing studies are published in insect systems that display live birth (viviparity), which include pests such as aphids, cockroaches, and flies. In these important systems the delivery of the gene editing technology such as the Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 system or transposable element-mediated transformation using plasmids into the insect's germ line is not straightforward because eggs are not available or efficient to produce in these pest species for gene-editing purposes.

One such difficult system with significant economic and agricultural significance is the pea aphid, *Acyrthosiphon pisum* (*A. pisum*), which resides within the insect order Hemiptera. This common agricultural pest, and model for insect symbiosis and phenotypic plasticity, presents a unique challenge to functional genomics studies. Past gene knockdown exploration in *A. pisum* has been largely unsuccessful. For example, gene knockdown attempts involving RNAi delivery methods have yielded inconsistent results (Jaubert-Possamai

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et al. 2007, Thairu et al. 2017), and this has mostly been attributed to fast-acting RNAses in the insect haemolymph and aphid salivary secretions (Christiaens et al. 2014, however see Thairu et al. 2017). The pea aphid's sexual life stage takes a relatively long time to induce, and a low yield of viable eggs makes gene editing inefficient in this system. In the parthenogenetic life stage, aphids reproduce viviparously, which makes embryonic injection of gene-editing agents especially difficult.

In vivo electroporation following microinjection is a potential approach for CRISPR/Cas9 delivery. In vivo electroporation has been used in a wide range of organisms and involves applying electric shocks to the study subject to promote introduction of molecular agents (Sugimoto and Tsuchida 2015). Recently, Matsumoto et al. (2013) successfully applied in vivo electroporation to perform targeted gene delivery in the cricket brain. This method included five electrical pulses following microinjection and resulted in a high efficiency of plasmid transfer. This technique was also successfully used on the honeybee brain for plasmid transfer (Kunieda et al. 2004). Where injection of gene-editing agents has been inconsistent in insect systems due to robust RNase activity and/or a complex reproductive cycle, subsequent electroporation provides a potential method for delivering gene-editing agents across membranes and directly into the insect's ovarioles.

In this study, the efficacy of developing a new CRISPR/Cas9 delivery method in *A. pisum* by utilizing microinjection and electroporation to introduce the target single guide RNA (sgRNA) and Cas9 into aphid embryonic cells in vivo was evaluated. To test this new technique, the target marker pigment gene (carotene dehydrogenase, *tor*) was targeted for knockout in offspring of adult female aphids. If *tor* is knocked out, white aphid pigmentation is expected to result compared with the wildtype, which displays pink pigmentation (Moran and Jarvik 2010). Sanger sequencing of the target region was also carried out after the treatment to validate the color phenotype results and the efficacy of this new delivery approach in *A. pisum*. The efficacy of green fluorescent protein plasmid delivery in ovarioles was also evaluated using this microinjection and electroporation method.

Methods

The *A. pisum* gene targeted in this study was carotene dehydrogenase, *tor*. Expression of *tor* results in carotenoid pigmentation (pinkish color) through torulene production (Moran and Jarvik 2010). After *tor* is knocked out, the aphid's pigmentation is expected to change to a white/yellowish green phenotype at G1 (Generation 1) because this gene is present as a single copy on one chromosome in the *A. pisum* LSR1 clonal line, which was used in this study (Moran and Jarvik, 2010). See Supplementary methods for the construction of the *tor* sgRNA designed and used in this study.

First, to test the efficacy of the *tor* sgRNA and Cas9 (New England Biolabs, Ipswich, MA) system in nicking the target sequence within the *tor* gene, the New England Biolabs Inc. (NEB) protocol 'In vitro digestion of DNA with Cas9 Nuclease, S. pyogenes' (M0386)' (NEB 2017) was conducted in vitro following the manufacturer's protocol. Briefly, primers (TorScreenF and TorScreenR) that flanked the target protospacer sequence within *tor* were designed (Supplementary Table 1) to amplify a 494-bp fragment from extracted aphid DNA. After the *tor*, sgRNA and Cas9 were incubated with the PCR fragment; the PCR fragment was successfully cut at the expected protospacer region (see Results and Supplementary methods). In turn, in vivo injection and electroporation trials in aphids were then pursued with the *tor* sgRNA.

For trials, even-age cohorts were established where five parthenogenetic female adults were placed on a plant, and after 24 h, all adults were removed leaving 1-d-old aphids. Ten-day-old aphid adults were immobilized before trials by cooling aphids on ice immediately before injections. Thirty adult aphids were injected and subsequently electroporated per treatment per trial. Two trials were carried out and consisted of an experimental treatment (*tor* sgRNA/Cas9) and three control treatments as follows: carrier RNA, carrier RNA + Cas9, and Cas9. For microinjections, concentrations of both sgRNA and Cas9 were 160 ng/ul based on optimization trials in Li et al. (2017), and the control carrier RNA (Qiagen, Hilden, Germany) concentration was also at 160 ng/ul for microinjection trials. The sgRNA and Cas9 (New England Biolabs) solutions were mixed on ice immediately preceding injections. In total, 2 ul of Cas9 was slowly added to 2 ul of sgRNA. This solution was left on ice for 10 min to allow Cas9 to associate with the sgRNA.

Approximately, 0.5 ul of the sgRNA/Cas9 mixture was microinjected into each adult aphid using a glass capillary needle with filament (OD = 1.0 mm, ID = 1.0 mm, length = 4 inches) (World Precision Instruments, Inc., Sarasota, FL). Immediately following microinjection, aphids were moved from the injection platform to an adjacent microscope for electroporation (Fig. 1a). The electroporation device was built by the University of Illinois, Urbana-Champaign's Life Sciences Electric Shop similar to Sugimoto and Tsuchida (2015) (Fig. 1b). Two platinum probes covered with PBS buffer were pressed to either side of the aphid, similar to Matsumoto et al. (2013). Five pulses (approximately 3 s each) at 5 V were delivered to the aphid to promote sgRNA/Cas9 gene editing. Following electroporation, aphids were placed on *Vicia faba* (fava bean) and further monitored.

Following microinjection and electroporation trials, aphids were observed for the next 4 d (Day 1, Day 2, Day 3, and Day 4) for mortality, fecundity, and color phenotype. The treated female cohort was given 24 h to reproduce, and then, all treated aphids were moved to a new *V. faba* plant. This was repeated each day to create even-age cohorts of offspring from the treated females, which were pooled. For each day, three components were measured in both the experimental and control treatments. The three components measured are as follows: mortality of treated adults, total number of nymphs birthed each day for each treatment, and the number of nymphs that were discolored for each treatment.

For each treatment (Day 1, Day 2, Day 3, and Day 4) in both trials, Generation 1 (G1) offspring were screened for *tor* sgRNA/Cas9 editing once they had reached fourth instar. Screening was conducted by selecting five phenotypically normal pink aphids and five discolored aphids per treatment if available. All aphids' DNA was extracted using the Qiagen DNeasy Blood and Tissue kit, and the protocol associated with this kit was followed. After the DNA was extracted, PCR was carried out with the primers TorScreenF and TorScreenR (Supplementary Table 1) to amplify the fragment of the *tor* gene spanning the target region where the sgRNA/Cas9 was expected to nick the dsDNA. Following amplification, the PCR amplicon was cleaned using the QIAquick PCR Purification kit from Qiagen and was sent for Sanger sequencing in both directions at ACGT, Inc. (Wheeling, IL). Sequences were aligned to the reference *A. pisum* LSR1 sequence from Genbank (GU456379) in MEGA7 (Kumar et al. 2016) to detect any possible sgRNA/Cas9 editing.

To determine the efficiency of the electroporation delivery system using control plasmids, we tested the following treatments: 1) Microinjection of plasmid only and 2) Microinjection of plasmid followed by electroporation. Transposable element-mediated transformation has not yet been achieved in aphids. Consequently, for this control experiment, we injected a plasmid into the aphid carrying

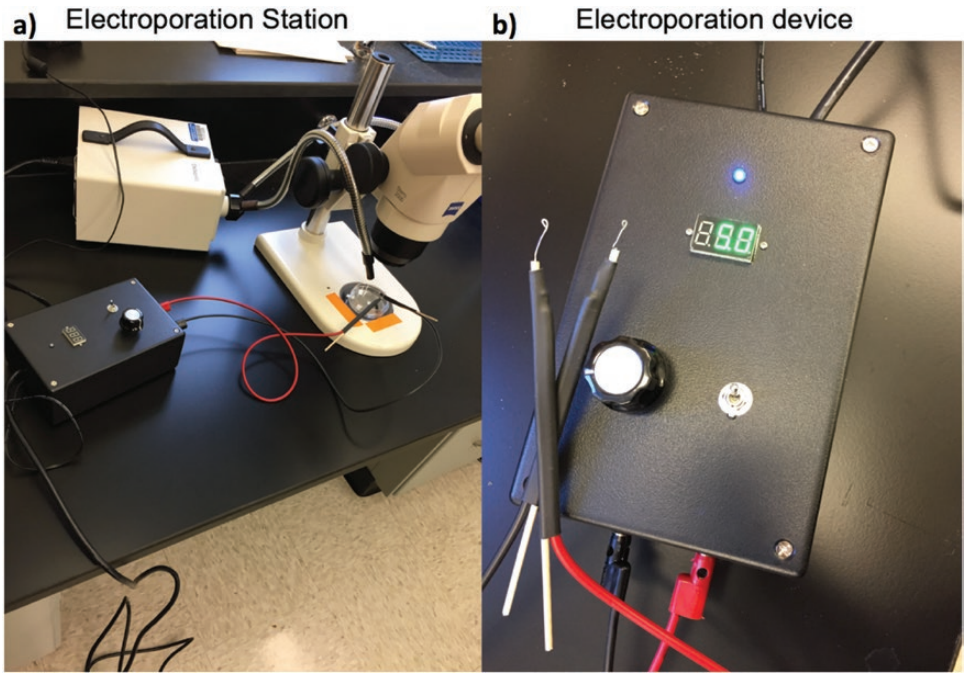

Fig. 1. Image depicting the electroporation station (a) and the electroporation device (b) used to introduce the CRISPR/Cas9 complex into aphid offspring in vivo.

the Green Fluorescent Protein (GFP) gene (see Supplement for plasmid map and sequence (Supplementary Fig. 1)). We then conducted quantitative real-time PCR to compare the relative copy number of the GFP plasmid between both treatments to determine the efficacy of the electroporation delivery method on plasmids into the aphid's ovarioles. For this experiment, as with the sgRNA/Cas9 experiments, 10-d-old aphids were immobilized before trials by cooling aphids on ice immediately before microinjections. In total, 2 ul of the plasmid solution (81ng/uL) was then injected into each aphid. For the microinjection–electroporation treatment, aphids were electroporated using the same conditions in the sgRNA/Cas9 experiments above. Immediately after both treatments, each aphid was dissected in Buffer A (Hansen and Moran 2011), and then, the ovarioles were subsequently triple rinsed in Buffer A. Total DNA was extracted from each aphid's ovarioles using a Qiagen QIAamp DNA micro kit (Qiagen). For each treatment, 22 aphids were used. Control aphid ovarioles (N = 2) (without treatment) were also collected, and DNA was extracted.

Quantitative real-time PCR was conducted on each aphid individual using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX96 Real-Time PCR Detection System using GFP-specific primers (Supplementary Table 2; Hersch et al. 2013). GFP relative abundance was calculated for each sample using the standard curve method for relative quantification (Bookout et al. 2006) and normalized to the aphid housekeeping gene elongation factor 1-alpha (EF-1α) (Supplementary Table 2; Dunbar et al. 2007).

Results

The Kaplan–Meier mortality curves for all CRISPR treatments and controls administered to adult aphids were not significantly different from one another (chi-square = 7.6779, df = 4, P = 0.1041) (Fig. 2). However, one control treatment (carrier RNA + Cas9) was significantly different compared with one of the *tor* CRISPR treatments (trial 1) (chi-square = 5.9863, df = 1, P = 0.0144) where the control treatment had 62.5% higher cumulative mortality compared with the

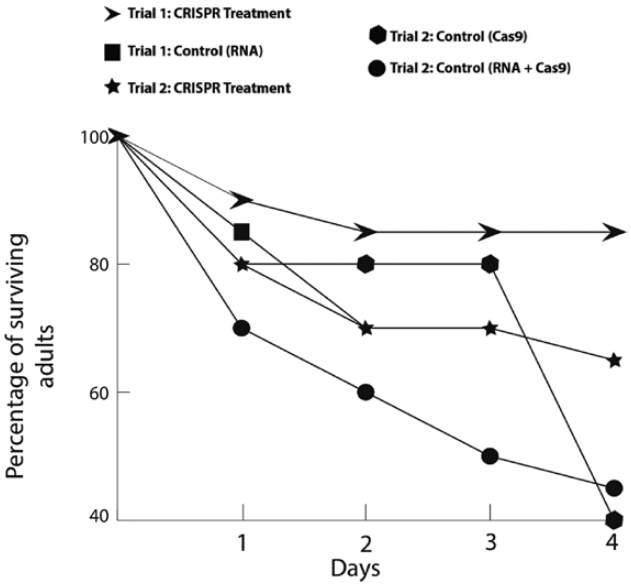

Fig. 2. Adult aphids were microinjected and subsequently electroporated. Survival of each treatment was monitored for 4 d, and mortality data between treatments were statistically analyzed with Kaplan–Meier mortality curves.

tor CRISPR treatment (trial 1). It is unclear why this control treatment had higher levels of mortality. In contrast, the mortality curves of both *tor* CRISPR treatments (trials 1 and 2) were not significantly different (chi-square = 1.7668, df = 1, P = 0.1838) where 85 and 65% survived the *tor* CRISPR treatments (trials 1 and 2), respectively. These results indicate that the *tor* CRISPR treatment conditions for aphid adults are relatively robust in terms of adult survival.

Ten white aphid offspring (2.5%) were observed for trial 1 of the *tor* CRISPR treatment (Fig. 3); however, none were observed for round 2 of the *tor* CRISPR treatment (Fig. 3). The white phenotype appeared distinctly different from pale colored aphids that can be

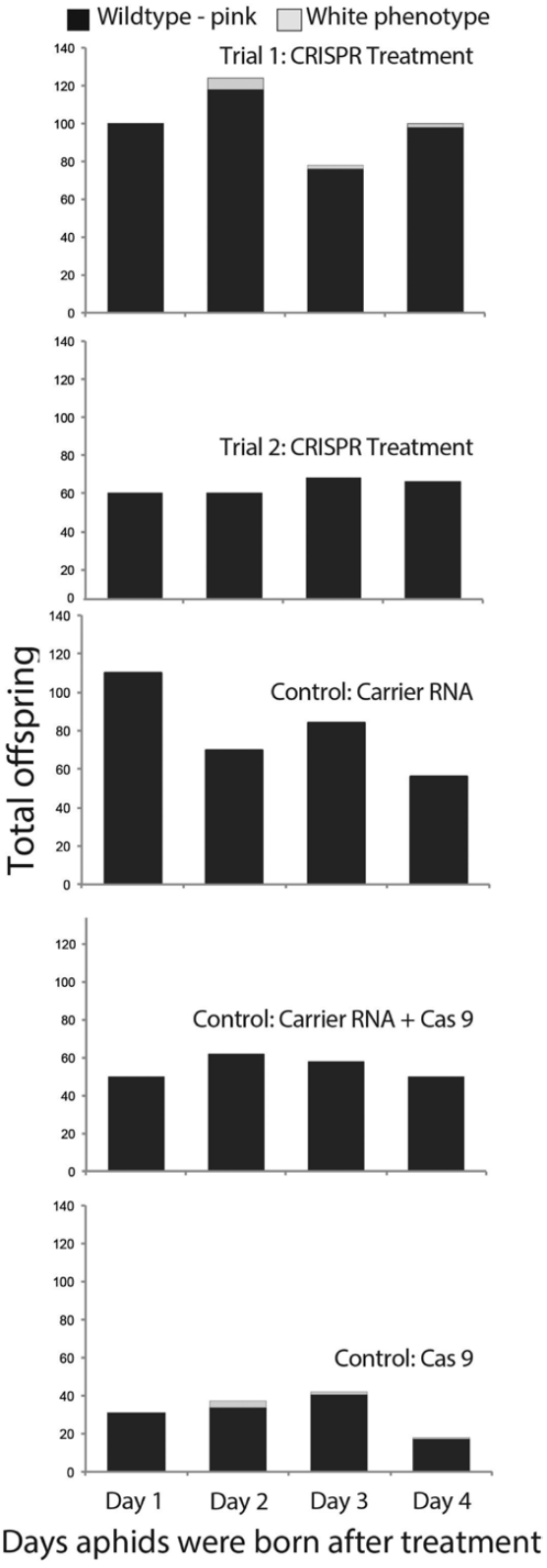

Fig. 3. Bar graphs representing total aphid offspring per day for 4 d following microinjection and electroporation trials. Graphs represent aphid offspring from the Trial 1: CRISPR treatment, the Trial 2: CRISPR treatment, and the three control treatments: Carrier RNA, Carrier RNA + Cas9, and Cas9.

observed in unhealthy/stressed aphid lab cultures (Fig. 4). Five white aphid offspring (4%) were also observed in one control treatment (Cas9). No white aphids were observed in the other two control treatments (carrier RNA, carrier RNA + Cas9) (Fig. 3). These results indicate that the white phenotype may not be the result of editing of the *tor* gene with the CRISPR/Cas9 system but instead is potentially associated with the microinjection of Cas9 into the aphid.

Twenty aphids (N = 5 from each day per treatment (days 1, 2, 3, and 4)) were Sanger sequenced for each *tor* CRISPR treatment (trials 1 and 2). These included four white aphids from round 1, whereas the remaining 36 aphids sequenced from both rounds 1 and 2 displayed a pink phenotype. The four white aphids expressed the white phenotype from birth; however, none lived past 24 h, so all white aphids were sequenced as first instar nymphs rather than fourth instar nymphs. The remaining white aphids in the trial 1 CRISPR treatment (N = 6) could not be sequenced because they did not yield enough high-quality DNA after extractions. None of the Sanger sequences were edited at the expected protospacer region revealing that CRISPR editing did not result from any of the *tor* CRISPR treatments.

For the GFP plasmid experiment, there was no significant difference in the relative number of GFP copies in aphid ovarioles between the inject only treatment and the inject and electroporate treatment (Welches t-value = −0.5526, df = 35; P = 0.5841) (Supplementary Fig. 2). These results suggest that the electroporation treatment did not increase the efficacy of plasmid entry into the ovarioles.

Discussion

In evaluating the efficacy of a new CRISPR/Cas9 and GFP plasmid delivery method in the viviparous insect *A. pisum*, it was found that the approach is fairly robust in terms of aphid survival and in vitro CRISPR/Cas9 editing of *tor*; however, follow-up studies are needed to further establish this promising technique in vivo. Specifically, following intense microinjection and electroporation treatments, high survival rates were observed for both experimental rounds. These mortality results are in agreement with a number of studies where microinjection and electroporation treatments were conducted on insects with survival rates of 60% or higher (Matsumoto et al. 2013, Ando and Fujiwara 2013).

In attempting to knockout the *tor* gene of aphid offspring whose mothers were subjected to the new CRISPR/Cas9 delivery method, a total of 10 offspring expressing the expected white phenotype were observed after the *tor* sgRNA/Cas9 treatment. Discoloration has previously been reported in the pea aphid, *A. pisum*, as a result of poor environmental conditions and/or high-density populations (Valmalette et al. 2012); however, the observed phenotype was considered distinct from unhealthy populations. Results from Sanger sequencing of the target gene, however, revealed no editing in the protospacer region of the *tor* gene. Additionally, the presence of five white aphids in the Cas9 control treatment further indicate that the expected white phenotype was not a result of gene editing but rather potentially a response to the Cas9 protein. Follow-up studies in this system using Cas9 should further control for and evaluate this potential by-product effect of Cas9 on *A. pisum*. In regard to the plasmid transfer experiment, electroporation does not increase the transfer of plasmids into aphid ovarioles. However, the current experiment cannot discern whether or not plasmids entered the embryo for both microinjection only and microinjection plus electroporation treatments. For example, the higher relative copy numbers compared with control ovarioles may reflect plasmid contamination on the outside of dissected embryos. These results highlight that there are major limitations with not being able to measure stable GFP expression in aphids. In turn,

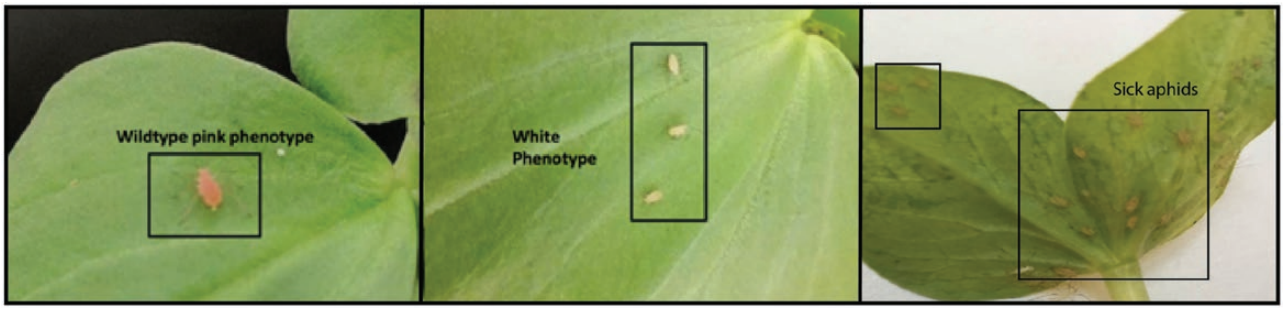

Fig. 4. Image depicting the comparison between the wildtype pink phenotype and white phenotype of offspring from the Trial 1: CRISPR treatment. Sick/stressed aphids are shown in comparison with the white phenotype in trials.

breakthroughs with transposable element-mediated transformation in Hemipterans will greatly accelerate functional genomics in these nonmodel insects.

While the expected gene edit and plasmid transfer were not observed in in vivo experimental treatments, low mortality rates are promising for future development of this technique. Further refinement studies may explore experimentation with aphids in earlier life stages, as preliminary trials at the fourth nymphal instar (results not presented) displayed a similar level of survivorship compared with adults in this study. Moreover, the co-injection of additives such as juvenile hormone (Kelly and Davenport 1976), nanoparticles (Thairu et al. 2017), or Lipofectamine (Dalby et al. 2004) in addition to the CRISPR/Cas9 or plasmid treatment may better facilitate the delivery of molecular agents across the aphid's ovarioles and embryo membranes.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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