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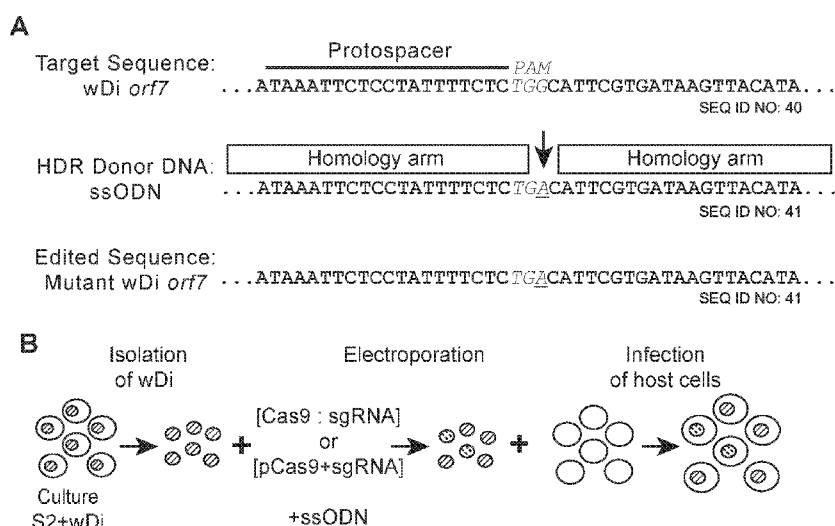
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Figure 8. Strategy of single nucleotide mutation in wDi using homology-directed repair (HDR) with CRISPR/Cas9 system. (A) Nucleotide sequences of wild-type wDi orf7 targeted region, the HDR donor DNA (ssODN) and the resulting mutated wDi orf7. The nucleotide targeted for exchange is highlighted in blue. (B) Schematic diagram outlining the steps used to transform wDi with either the protein or plasmid base systems in combination with ssODN. Grey circles represent S2 cells, yellow circles represent wDi, and red circles represent transformed wDi cells.

(57) **Abstract:** The present disclosure is directed to materials and method for genetically modifying *Wolbachia*, as well as arthropods comprising the modified *Wolbachia*.



**MATERIALS AND METHODS FOR MODIFYING *WOLBACHIA* AND
PARATRANSFORMATION OF ARTHROPODS**

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims the benefit of priority to U.S. Provisional Application No. 62/767,176 filed November 14, 2018, the disclosure of which is incorporated herein by reference in its entirety.

STATEMENT ON U.S. GOVERNMENT INTEREST

[0002] This invention was made with government support under D16AP00031 awarded by The United States Department of Defense. The government has certain rights in the invention

FIELD OF THE INVENTION

[0003] The present disclosure is directed to materials and method for genetically modifying *Wolbachia*, as well as the arthropods comprising the modified *Wolbachia*.

BACKGROUND

[0004] The genus *Wolbachia* are Alpha-proteobacteria that are obligated cytoplasmic endosymbionts of eukaryotic cells (Werren, 1997). These maternally transmitted bacteria, which are widespread among numerous arthropod and nematode species (Stevens et al., 2001; Zug and Hammerstain, 2012), can alter host reproduction by causing parthenogenesis, feminization, embryonic male killing and cytoplasmic incompatibility (Stouthamer et al., 1999; Werren et al., 2008), reproductive manipulations that increase the fitness of infected females and benefits the spread of the bacterium within a host population. Cytoplasmic incompatibility is the most common reproductive phenotype, occurring when sperm from *Wolbachia*-infected males is incompatible with eggs from uninfected females or females that do not harbor the same *Wolbachia* strain, resulting in embryonic death (Yen and Barr, 1971). In addition, *Wolbachia* strains can provide its hosts with protection against different types of infective agents (Teixeira et al., 2008; Moreira et al., 2009; Kambris et al., 2009; Eleftherianos et al., 2013) and potentially interfere the transmission of insect-borne pathogens or parasites (Hancock et al., 2011; Hughes et al., 2011).

[0005] *Wolbachia*'s capacity to alter host reproduction and fitness demonstrates the potential of these bacteria as an instrument for the management of insect vectors.

SUMMARY

[0006] In one aspect, described herein is a method comprising (a) transforming *Wolbachia* bacterium with an exogenous nucleic acid using a CRISPR-Cas9 nuclease to insert the nucleic acid into the genome of the bacterium, thereby producing a genetically transformed bacterium; and (b) introducing the genetically transformed bacterium into an arthropod.

[0007] In some embodiments, the method includes isolating the bacterium from an arthropod host before the transforming step. In this instance, the arthropod host may be the same type of arthropod into which the genetically transformed bacterium is introduced, or the bacterium may be introduced into a different type of arthropod than the host arthropod from which the *Wolbachia* was isolated.

[0008] In some embodiments, the transforming step comprises contacting the bacterium with CRISPR-Cas9 nuclease protein. In some embodiments, the transforming step comprises introducing a nucleic acid encoding a CRISPR-Cas9 nuclease into the bacterium under conditions allowing production of the CRISPR-Cas9 nuclease. In some embodiments, the exogenous nucleic acid is inserted into a capsid gene (e.g., orf7) of the bacterium genome.

[0009] In some embodiments, the arthropod is an insect, an arachnid or a crustacean.

[0010] In some embodiments, the arthropod is an insect is selected from the group consisting of an Asian citrus psyllid (*Diaphorina citri*), Yellow fever mosquito (*Aedes aegypti*), Potato psyllid (*Bactericerca cockerelli*), German cockroach (*Blatella germanica*, Beg bug (*Cimex lectularius*), fruit fly (*Drosophila melanogaster*), house cricket (*Acheta domesticus*), American grasshopper (*Schistocerca Americana*), tobacco hornworm (*Manduca sexta*) and a red flour beetle (*Tribolium castaneum*).

[0011] In some embodiments, the method comprises culturing the bacterium in a host cell before the transforming step. In some embodiments, the host cell is a cell from the same type of arthropod from which the *Wolbachia* was isolated. In some embodiments, the host cell is a cell from a different type of arthropod from which the *Wolbachia* was isolated. In some embodiments, the arthropod is an insect. In some embodiments, the host cell is an insect cell from the same type of insect from which the *Wolbachia* was isolated. In some embodiments, the host cell is an insect cell from a different type of insect from which the *Wolbachia* was isolated. In some embodiments, the host cell is a *Drosophila melanogaster* cell (e.g., an S2 cell).

BRIEF DESCRIPTION OF THE FIGURES

[0012] Figure 1. Verification of wDi in cell culture. The PCR amplification products of the established *in vitro* wDI infected S2 cells (S2+wDi) and an uninfected (S2) cell line using the general-*Wolbachia* (ftsZ) and the specific-wDi (wsp) primer sets. A molecular size standard (Std) is shown (100 base pair (bp) DNA ladder; NEB). The red markers indicate the 100 bp.

[0013] Figure 2. Intracellular location of wDI in S2 cells. Confocal images of fixed S2 and S2-Di cells, acquired using DAPI (blue) and bright field (bf). DAPI stained the S2 cells nuclei in addition to wDi chromosomes.

[0014] Figures 3A-3B. Reduction of wDi infection in S2 cells with tetracycline treatment. Infected and uninfected S2 cells with wDi were untreated (control) and treated with tetracycline for three days in S2 medium (A&B). Treatment with tetracycline had no significant effect on S2 cell survival (A). *Wolbachia* FtsZ mean copy number was determined for control and treated samples. wDi infection in S2 cells was reduced by three day of tetracycline treatment (B).

[0015] Figure 4. Purified wDi cells remain viable. wDi cells were extracted and purified from S2 cells and were visualized with the BacLight live-dead stain. wDi cells were stained with green SYTO-9 dye (top row), and dead wDi cells were stained red with Propidium iodine (bottom row) (fluorescent images). For both fluorescent images, the cells were outlined and counted (second column) using ImageI program (analyzed images). There were 2980 total cells, 2853 live cells and 127 dead cells; percent viability of 95.7% and percent mortality of 4.3%. For all subsequent wDi cell extractions and purifications the percent viabilities were >95% (data not shown).

[0016] Figures 5(A)-5(D). Electroporation of fluorescein-labeled dsRNA on purified wDi. Purified wDi cells were electroporated with fluorescein-labeled dsRNA (dsRNA^{FL}). All cells were mixed in with 30 mM dsRNA^{FL} and were imaged at 48 hours-post-electroporation. The control cells that were not electroporated (A), and cells that were electroporated with the highest number of pulses (25x) (B) had no to little dsRNA^{FL} signal. Samples that were electroporated with 18 (C) and 10 (D) pulses had multiple wDi cells with dsRNA^{FL} signal.

[0017] Figures 6(A)-6(B). Viability of S2+wDi cells after electroporation with pCas9. Cells were counted after two days of reinfection of wDi (A&B). There were two biological replicated for each sample. Control samples were electroporated with no DNA at 18 pulses

(A). Electroporation with 1.5 μ g of pCas9 had significant effect on viability of S2+wDi (A). *Wolbachia* FtsZ mean copy number was determined for each concentration of pCas9 electroporated (B).

[0018] Figures 7(A)-7(B). Detection of DNA and protein from pCas9 in S2+dWi^{pCas9} cells. (A) shows that PCR amplification products of pCas9 (primers were designed to amplify a chloramphenicol resistant gene fragment found in plasmid DNA), (L) 100 bp ladder; DNA of samples are from two day post re-infection; #1-8 are samples that were electroporated with pCas9; sample #9 is the electroporation control with no DNA; and sample #10 is the no DNA and no electroporation control. (B) Confocal images of S2+dWi^{pCas9} cells labeled with Cas9 and DAPI. White arrows indicate cytoplasmic wDi that express Cas9.

[0019] Figures 8(A)-8(B). Strategy of single nucleotide mutation in wDi using homology-directed repair (HDR) with CRISPR/Cas9 system. (A) Nucleotide sequences of wild-type wDi orf7 targeted region, the HDR donor DNA (ssODN) and the resulting mutated wDi orf7. The nucleotide targeted for exchange is highlighted in blue and underlined. (B) Schematic diagram outlining the steps used to transform wDi with either the protein or plasmid base systems in combination with ssODN. Grey circles represent S2 cells, yellow circles represent wDi, and red circles represent transformed wDi cells.

[0020] Figures 9(A)-9(C). Confirmed point mutation in wDi orf7. wDi cells were transfected with CRISPR/Cas9 components followed by a 48 hours incubation period. DNA was isolated, and the targeted region was amplified and subjected to Sanger sequencing. (A) Depicts experiments conducted with the plasmid base system. (B-C) Depicts experiments conducted with the protein base system; and in (C), 1-3 are technical replicates.

[0021] Figures 10(A)-10(B). Approach for gene insertion using homology-directed repair with CRISPR/Cas9 system in wDi. (A) Nucleotide sequences of wild-type wDi orf7 targeted region, the homology-directed repair (HDR) plasmid donor DNA (pDonor) and the resulting incorporation of the transgene in wDi. (B) Schematic diagram outlining the steps used to transform wDi with the protein-based system. Grey circles represent S2 cells, yellow circles represent wDi, and green circles represent transformed wDi cells.

[0022] Figures 11(A)-11(D). Knock-in of GFP in wDi by HDR with CRISPR/Cas9 technology. (A&B) PCR-based genotyping using primer to confirm integration of GFP in wDi cells. Cells electroporated in the absence of pDonor wDi were used as negative controls

(CONT). (C) Using FACS, transformed wDi cells were enriched on the basis of GFP intensity (x-axis) after 2d of electroporation. wDi+dsRNAFlu cells were used as positive controls. Y-axis is the side scatter area (SSC-A). Samples with in the grey area account for GFP+ cells. (D) Wide-field fluorescence microscopy images of live sorted wDi+GFP cells. (E) Confocal miscopy of live S2+wDi+GFP-R cells; samples were stained with Hoechst 33342 (blue).

[0023] Figures 12(A)-12(G). Insertion of toxin genes in wDi by HDR with CRISPR/Cas9 system. (A) Strategy for toxin gene insertion. Electroporation of wDi was conducted with different pDonors; pCry3Aa, pCry3Aa-R, pCyt2Ca1, pCyt2Ca1-R, pGNA3, or pGNA3-R. (B-G) DNA from transformed and non-transformed negative control (NC) cells were used for PCR analysis. Two biological replicates of each sample are shown. Different PCR primers used in the study: Cry3Aa-f, Cry3Aa-r for Cry3Aa (B), Cry3Aa-R-f, Cry3Aa-R-r for Cry3Aa-R (C), Cyt2Ca1-f, Cyt2Ca1-r for Cyt2Ca1 (D), Cyt2Ca1-R-f, Cyt2Ca1-R-r for Cyt2Ca1-R (E), GNA3-f, GNA3-r for GNA3 (F), and GNA3-R-f, GNA3-R-r for GNA3-R (G).

[0024] Figures 13(A)-13(C). Vertical transmission of transformed wDi+ in *D. citri*. (A) *In vivo* visualization of *D. citri* nymphs. Nymph one was injected in the abdominal segment and nymph two was injected twice in the mesothorax with stained Hoechst 33342 (nuclei) wDi+GFP-R. Yellow arrows indicate injection locations. Red arrows indicate exogenous transformed cells that are positive for both nuclei stain and GFP signal. (B&C) Parental *D. citri* were injected with wDi+Cry3Aa-R, their offspring F1 (B) & F3 (C) were individually processed for PCR analysis. (B) Detection of wDi+Cry3Aa-R in *D. citri* adults are labeled with green positive symbol and (C) non-wDi+Cry3Aa-R samples were labeled with red negative symbols. Positive and negative DNA controls were used; (+) pDonor DNA and (-) wild-type *D. citri* DNA.

DETAILED DESCRIPTION

[0025] The present disclosure is based, in part, on the discovery that *Wolbachia* can be genetically modified via CRISPR technology to express an exogenous nucleic acid of interest.

[0026] In one aspect, described herein is a method comprising transforming a *Wolbachia* bacterium with an exogenous nucleic acid using a CRISP-Cas9 nuclease to insert the nucleic acid into the genome of the bacterium, thereby producing a genetically transformed

bacterium; and introducing the genetically transformed bacterium into an arthropod. In some embodiments, the method comprises isolating the *Wolbachia* bacterium from a host arthropod before the transforming step.

[0027] The term “exogenous nucleic acid” is a nucleic acid that is not normally present in a particular host cell. The term “exogenous nucleic acid” as used herein refers to a nucleic acid that is not present in the wild-type *Wolbachia* genome. The *Wolbachia* bacterium has been “genetically modified” or “transformed” or “transfected” by exogenous nucleic acid when such nucleic acid(s) has been introduced inside the cell.

[0028] Nucleic acids include DNA and RNA; can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (i.e., liposomes, including neutral and cationic lipids), electroporation, calcium phosphate precipitation, polyethyleneimine (PEI)-mediated transfection, direct injection, cell fusion, nanoparticle-mediated nucleic acid delivery (including lipid nanoparticles (LNP)), particle bombardment (e.g., particle gun technology), calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector- or bacteriophage-mediated transfer. A general discussion of these methods can be found in Ausubel, et al., *Short Protocols in Molecular Biology*, 3rd ed., Wiley & Sons, 1995. It will be appreciated that the methods described herein are suitable for introducing any exogenous nucleic acid (e.g., encoding a toxin, encoding Cas9 nuclease, or encoding guide RNA) into a host cell.

[0029] Exogenous nucleic acids are typically introduced into host cells using an expression vector. Contemplated expression vectors include, but are not limited to, plasmids, bacteriophage-based vectors, and viral vectors, such as viral vectors based on vaccinia virus, poliovirus, adenovirus, adeno-associated virus, SV40, herpes simplex virus, human immunodeficiency virus, retrovirus (e.g., Murine Leukemia Virus, spleen necrosis virus, Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, a lentivirus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus). Other vectors may be used so long as they are compatible with *Wolbachia*.

[0030] In some embodiments, the exogenous nucleic acid encodes a toxin. Exemplary toxins include, but are not limited to, *Bacillus thuringiensis* crystal toxin (Cry3Aa), *Bacillus thuringiensis* cytolytic toxin (Cyt2Ca1) and *Galanthus nivalis* agglutinin (GNA3) and

ponericins. In some embodiments, the exogenous nucleic acid encodes a peptide. Exemplary peptides include, but are not limited to gut-binding peptides, and insecticidal peptides (e.g., snowdrop lectin (GNA), scorpion venom toxin and indolicidin). Alternatively (or in addition), the bacterium genome is modified to include an exogenous nucleic acid encoding a selectable marker.

[0031] In various aspects, the exogenous nucleic acid is inserted into a region of the *Wolbachia* genome that (1) does not kill the bacterium and (2) allows for the production of a protein encoded by the exogenous nucleic acid. Optionally, the exogenous nucleic acid is inserted into the capsid protein gene, orf7, such as a unique genomic site adjacent to the protospacer adjacent motif (PAM) sequence found in orf7. Expression of the exogenous nucleic acid may be driven by an endogenous (native) promoter in the *Wolbachia* genome, although the use of exogenous promoters also is contemplated. In any event, the exogenous nucleic acid is preferably "operably linked" to regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence. The term "regulatory sequence" is intended to include, for example, promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are well known in the art and are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

[0032] In some embodiments, the *Wolbachia* bacterium is isolated from an arthropod host. Additionally, the genetically transformed *Wolbachia* bacterium optionally serves as a vector for delivering an exogenous protein (by way of the exogenous nucleic acid embedded in the bacterial genome) to an arthropod host. As used herein, the term "arthropod" refers to an invertebrate animal that is characterized by a chitinous exoskeleton and a segmented body with paired, jointed appendages (e.g., legs or feet). Accordingly, an arthropod may be, but is not limited to, an insect (e.g., a mosquito or a fly), a crustacean (e.g., a prawn, a crab or a lobster), or an arachnid (e.g., a tick or a mite). As also used herein, the terms "arthropod vector" or "arthropod vector population" refer to an arthropod, or a population thereof, that is capable of transmitting a pathogen from one host to another.

[0033] Insects include insects of orders such as *Diptera* (e.g., mosquitoes, horseflies, midges, stableflies and tsetse flies), *Phthiraptera* (e.g., lice), *Siphonaptera* (e.g., fleas) and *Hemiptera* (e.g., bedbugs and triatomine bugs). In some embodiments, the insect is selected from the group consisting of an Asian citrus psyllid (*Diaphorina citri*), Yellow fever mosquito (*Aedes aegypti*), Potato psyllid (*Bactericera cockerelli*), German cockroach

(*Blatella germanica*, Beg bug (*Cimex lectularius*), fruit fly (*Drosophila melanogaster*), house cricket (*Acheta domesticus*), American grasshopper (*Schistocerca Americana*), tobacco hornworm (*Manduca sexta*) and a red flour beetle (*Tribolium castaneum*).

[0034] An example of an arachnid is a tick or mite (e.g., of the families *Argasida*, *Trombidiidae* and *Ixodidae*). An example of a crustacean is a prawn or a crab (e.g., of the families *Peneidae* and *Coenobitidae*, such as *Penaeus monodon*, *Marsupenaeus japonicus* and *Litopenaeus vannamei*).

[0035] In some embodiments, the genetically transformed *Wolbachia* bacterium is capable of modifying one or more biological properties of an arthropod host. For example, in some embodiments, the method described herein introduces a reproductive abnormality in an arthropod host such as, but not limited to, parthenogenesis, feminization, male killing, and cytoplasmic incompatibility (CI). Typically, according to this embodiment, the reproductive abnormality reduces fecundity within an arthropod vector population. As used herein, the term "fecundity" refers to the ability of an arthropod, or a population thereof, to reproduce.

[0036] *Isolation of Wolbachia*

[0037] In various aspects, the method described herein comprises the step of isolating *Wolbachia* from a host arthropod. In some embodiments, the *Wolbachia* is isolated from Asian citrus psyllid (*Diaphorina citri*). The term "*Wolbachia*" as used herein is meant to include all strains of bacteria under the genus *Wolbachia*. Exemplary strains of *Wolbachia* include, but are not limited to, strains such as wDi, wMel, wMelPop, wMelPop-CLA, wMeICS, wAu, wRi, wNo, wHa, wMau and wCer2.

[0038] The *Wolbachia* bacterium can be isolated from the host arthropod by any method known in the art. In some embodiments, the *Wolbachia* is isolated as described in Example 1.

[0039] Once isolated, the *Wolbachia* is cultured in a suitable host cell. A "host cell," as used herein, denotes an *in vivo* or *in vitro* prokaryotic cell (e.g., bacterial or archaeal cell), eukaryotic cell, or a cell from a multicellular organism (e.g., a cell line) cultured as a unicellular entity, which eukaryotic or prokaryotic cells can be, or have been, used as recipients for a nucleic acid, and include the progeny of the original cell which has been transformed by the nucleic acid. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. In some

embodiments, the host cell is a cell from the same type of arthropod from which the *Wolbachia* was isolated. In some embodiments, the host cell is a cell from a different type of arthropod from which the *Wolbachia* was isolated.

[0040] In some embodiments, the host cell is an insect cell. In some embodiments, the host cell is an insect cell from the same type of insect from which the *Wolbachia* was isolated. In this regard, for example, the host cell could be a *D. citri* cell and the *Wolbachia* is isolated from *D. citri*. In some embodiments, the host cell is an insect cell from a different type of insect from which the *Wolbachia* was isolated. In this regard, for example, the host cell is a *Drosophila melanogaster* cell and the *Wolbachia* is isolated from *D. citri*.

[0041] In some embodiments, the insect cell is a *Drosophila melanogaster* cell, such as an S2 cell. In some embodiments, the *Wolbachia* bacterium is cultured in an S2 cell line in Schneider medium, optionally under the conditions described in Example 1.

[0042] To aid in identification of insect cells comprising *Wolbachia*, the modified *Wolbachia* may be further manipulated to include a selectable marker gene that is functional in bacteria. Useful selectable markers include, but are not limited to, enzymes which provide for resistance to an antibiotic such as Ampicillin resistance gene (Amp^r), a tetracycline resistance gene (Tc^r), a Cycloheximide-resistance L41 gene, the gene conferring resistance to antibiotic G418 (such as the APT gene derived from a bacterial transposon Tn903), the antibiotic Hygromycin B-resistance gene, a Gentamycin resistance gene, and/or a kanamycin resistance gene, among others. Similarly, enzymes providing for production of a compound identifiable by color change (such as GUS) or luminescence (such as luciferase) are included herein.

[0043] *CRISPR Endonuclease System*

[0044] A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genomic locus can be found in the genomes of many prokaryotes (e.g., bacteria and archaea). In prokaryotes, the CRISPR locus encodes products that function as a type of immune system to help defend the prokaryotes against foreign invaders, such as virus and phage. There are three stages of CRISPR locus function: integration of new sequences into the locus, biogenesis of CRISPR RNA (crRNA), and silencing of foreign invader nucleic acid. Five types of CRISPR systems (e.g., Type I, Type II, Type III, Type U, and Type V) have been identified.

[0045] A CRISPR locus includes a number of short repeating sequences referred to as "repeats." The repeats can form hairpin structures and/or comprise unstructured single-stranded sequences. The repeats usually occur in clusters and frequently diverge between species. The repeats are regularly interspaced with unique intervening sequences referred to as "spacers," resulting in a repeat-spacer-repeat locus architecture. The spacers are identical to or have high homology with known foreign invader sequences. A spacer-repeat unit encodes a crRNA (crRNA), which is processed into a mature form of the spacer-repeat unit. A crRNA comprises a "seed" or spacer sequence that is involved in targeting a target nucleic acid (in the naturally occurring form in prokaryotes, the spacer sequence targets the foreign invader nucleic acid). A spacer sequence is located at the 5' or 3' end of the crRNA.

[0046] A CRISPR locus also comprises polynucleotide sequences encoding CRISPR Associated (Cas) genes. Cas genes encode endonucleases involved in the biogenesis and the interference stages of crRNA function in prokaryotes. Some Cas genes comprise homologous secondary and/or tertiary structures.

[0047] crRNA biogenesis in a Type II CRISPR system in nature requires a trans-activating CRISPR RNA (tracrRNA). The tracrRNA is modified by endogenous RNaseIII, and then hybridizes to a crRNA repeat in the pre-crRNA array. Endogenous RNaseIII is recruited to cleave the pre-crRNA. Cleaved crRNAs are subjected to exoribonuclease trimming to produce the mature crRNA form (e.g., 5' trimming). The tracrRNA remains hybridized to the crRNA, and the tracrRNA and the crRNA associate with a site-directed polypeptide (e.g., Cas9). The crRNA of the crRNA-tracrRNA-Cas9 complex guides the complex to a target nucleic acid to which the crRNA can hybridize. Hybridization of the crRNA to the target nucleic acid activates Cas9 for targeted nucleic acid cleavage. The target nucleic acid in a Type II CRISPR system is referred to as a protospacer adjacent motif (PAM). In nature, the PAM facilitates binding of a site-directed polypeptide (e.g., Cas9) to the target nucleic acid. Type II systems (also referred to as Nmen or CASS4) are further subdivided into Type II-A (CASS4) and II-B (CASS4a). Jinek et al., *Science*, 337(6096):816-821 (2012) showed that the CRISPR/Cas9 system is useful for RNA-programmable genome editing, and International Patent Application Publication Number WO2013/176772 (incorporated herein by reference) provides numerous examples and applications of the CRISPR/Cas endonuclease system for site-specific gene editing.

[0048] Exemplary CRISPR/Cas polypeptides include the Cas9 polypeptides in Fig. 1 of Fonfara et al., *Nucleic Acids Research*, 42: 2577-2590 (2014) (incorporated herein by

reference). The CRISPR/Cas gene naming system has undergone extensive rewriting since the Cas genes were discovered. Fig. 5 of Fonfara, *supra*, provides PAM sequences for the Cas9 polypeptides from various species.

[0049] Cas9 polypeptides can introduce double-strand breaks or single-strand breaks in nucleic acids, e.g., genomic DNA. The double-strand break can stimulate a cell's endogenous DNA-repair pathways (e.g., homology-dependent repair (HDR) or non-homologous end joining (NHEJ) or alternative non-homologous end joining (A-NHEJ) or microhomology-mediated end joining (MMEJ)). NHEJ can repair cleaved target nucleic acid without the need for a homologous template. This can sometimes result in small deletions or insertions (indels) in the target nucleic acid at the site of cleavage, and can lead to disruption or alteration of gene expression. HDR can occur when a homologous repair template, or exogenous nucleic acid, is available.

[0050] Thus, in some cases, homologous recombination is used to insert an exogenous nucleic acid into the genome of the *Wolbachia* bacterium. The modifications of the target DNA due to NHEJ and/or HDR can lead to, for example, mutations, deletions, alterations, integrations, gene correction, gene replacement, gene tagging, transgene insertion, nucleotide deletion, gene disruption, translocations and/or gene mutation. The processes of deleting genomic DNA and integrating non-native nucleic acid into genomic DNA are examples of genome editing.

[0051] In some aspects, the Cas9 nuclease is introduced to the *Wolbachia* as a protein (i.e., a protein-based system). Typically, the *Wolbachia* is treated chemically, electrically, or mechanically to allow Cas9 nuclease entry into the cell. Alternatively, the Cas9 nuclease is introduced to the *Wolbachia* as a nucleic acid (e.g., DNA or mRNA) under conditions which allow production of the nuclease. Guide RNA also is introduced into the *Wolbachia*.

[0052] A genome-targeting RNA is referred to as a “guide RNA” or “gRNA” herein. A guide RNA comprises at least a spacer sequence that hybridizes to a target nucleic acid sequence of interest, and a CRISPR repeat sequence. In Type II systems, the gRNA also comprises a tracrRNA sequence. In the Type II guide RNA, the CRISPR repeat sequence and tracrRNA sequence hybridize to each other to form a duplex. The duplex binds a site-directed polypeptide, such that the guide RNA and site-direct polypeptide form a complex. The guide RNA provides target specificity to the complex by virtue of its association with the

Cas9 nuclease. The guide RNA thus directs the activity of the Cas9 nuclease. In some embodiments, the guide RNA is a single molecule guide RNA (sgRNA).

[0053] A single-molecule guide RNA in a Type II system comprises, in the 5' to 3' direction, an optional spacer extension sequence, a spacer sequence, a minimum CRISPR repeat sequence, a single-molecule guide linker, a minimum tracrRNA sequence, a 3' tracrRNA sequence and an optional tracrRNA extension sequence. The optional tracrRNA extension may comprise elements that contribute additional functionality (*e.g.*, stability) to the guide RNA. The single-molecule guide linker links the minimum CRISPR repeat and the minimum tracrRNA sequence to form a hairpin structure. The optional tracrRNA extension comprises one or more hairpins.

[0054] Exemplary sgRNA for use in the methods described herein include, but are not limited to, the sgRNAs provided in Table 2 in Example 2.

[0055] A nucleic acid encoding the Cas9 nuclease and/or guide RNA is typically delivered in an expression vector. The exogenous nucleic acid can be delivered in the same vector as the Cas9 nucleic acid, or in a second vector. Any of the expression vectors described herein may be used to deliver Cas9 nuclease-encoding nucleic acid into the *Wolbachia*; in many aspects, the expression vector is a plasmid. In some embodiments, an expression vector comprises one or more transcription and/or translation control elements. Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc., may be used

[0056] The Cas9 nuclease-encoding nucleic acid is operably linked to a promoter that drives protein expression. Exemplary prokaryotic promoters include, but are not limited to, wMel WSP Promoter, wDc WSP Promoter and T7. For expressing small RNAs, including guide RNAs used in connection with Cas or Cpf1 endonuclease, promoters such as RNA polymerase III promoters, including for example U6 and H1, can be advantageous. Suitable promoters, as well as parameters for enhancing the use of such promoters, are known in art, and additional information and approaches are regularly being described; see, *e.g.*, Ma, H. *et al.*, *Molecular Therapy - Nucleic Acids* 3, e161 (2014) doi:10.1038/mtna.2014.12.

[0057] In some aspects, the genetically-modified bacterium is introduced into an arthropod. Optionally, an infected arthropod demonstrates a reproductive abnormality, such as an abnormality described herein, resulting in a reduction in pest fecundity. The

genetically-modified bacterium may be introduced into arthropod using any suitable technique, such as a shell vial technique or a microinjection. In some embodiments, the genetically-transformed bacterium is introduced by microinjection into an abdominal segment of the mesothorax (e.g., the ventral first abdominal segment of the mesothorax). In some embodiments, the genetically-transformed bacterium is injected into the dorsal side of the mesothorax. In some embodiments, the genetically-transformed bacterium is electroporated into eggs of an arthropod. In some embodiments, the genetically-transformed bacterium is incorporated into the diet of a target insect and fed to the target insect.

[0058] The *Wolbachia* platform has the potential for ubiquitous use among insects. In some embodiments, the genetically-modified bacterium is modified to express genes that will interfere with reproduction, alter metabolic function, transmission of animal and plant pathogens (e.g. viruses, bacteria, Plasmodium), bactericides, insect toxins, and or dsRNA (for RNAi management). In some embodiments, the *Wolbachia* platform is used to disrupt *Candidatus Liberibacter asiaticus* (Clas) replication and multiplication. Exemplary target mechanisms are set forth below in Table 1.

Table 1. Targets for disruption of Clas replication and multiplication

Potential target mechanism	Gene
Knock-out of wDi gene, facilitates survival of CLAs in <i>D. citri</i>	Holin promoter repressor protein
Knock-in of Profftella gene in wDi, mediates Clas	Polyketide diaphorin
Knock-in genes from wDi, downregulated in ACP infected with CLAs	Iron deficiency- induced protein A Ankyrin Membrane protein Polynucleotide phosphorylase Ribosomal proteins Phosphoribosylamine-glycine ligase Thioredoxin
Knock-in genes from ACP gut downregulated in ACP infected with Clas	Cathepsin B Lipase Facilitated trehalose transporter
Knock-out or -down genes from gut, upregulated in <i>D. citri</i> infected with Clas	Cysteine proteases Mucin

Knock-in genes from <i>D. citri</i> immune system, downregulated in <i>D. citri</i> infected with Clas	Basement membrane- specific heparan sulfate proteoglycan core protein Heat-shock proteins Transferrin-like transcripts
Knock-in genes from the ACP gut mitochondria, downregulated in <i>D. citri</i> infected with Clas	Enzymes regulating metabolic flux during the TCA cycle

[0059] All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification, are incorporated herein by reference, in their entireties.

[0060] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

EXAMPLES

Example 1 – Isolation of *Wolbachia* from *Diaphorina citri*

[0061] *Preparations of Wolbachia from Diaphorina citri (wDi):* The *Diaphorina citri* were collected from Lake Alfred, Florida. The cell line was established following methods described previously with modifications (O'Neill, et al., 1997). Psyllids were placed on a sterile diet rings for two days prior to *Wolbachia* extraction. Individual psyllids were surface sterilized by immersion in 10% bleach (Fisher Scientific, Fair Lawn, NJ) for five minutes and followed by 70% ethanol (Fisher Scientific, Fair Lawn, NJ) wash for one minute. Psyllids were rinsed twice with filtered sterile deionized water. Individual psyllids were transferred in an 1.5mL microcentrifuge tube containing 1mL of Schneider's *Drosophila* (S2) media (Gibco, Carlsbad, CA). Psyllids were homogenized with a pellet pestle. The resulting suspension was centrifuged at 100 x g for five minutes. The supernatant was placed into a new 1.5mL microcentrifuge tube. The supernatant was centrifuged at 400 x g for five minutes. The supernatant was removed and the pellet was resuspended with 1mL of S2 media. The samples were centrifuged at 100 x g for five minutes, then the supernatant was transferred to a new 1.5mL microcentrifuge tube. The supernatant was centrifuged at 4000 x g for five minutes, finally the pellet was resuspended in 1mL of S2 media.

[0062] *Infection of wDi in S2 cells:* S2 cells (Invitrogen, Carlsbad, CA) were placed in a 24-well plate and were seeded at a density of 4.0×10^5 cells per well in a total of 1 mL of S2

media containing 10% heat inactivated fetal bovine serum, 50 units of penicillin (Sigma, St. Louise, Mo) and 50 µg streptomycin sulfate (Gibco, Grand Island, NY) per mL (S2 complete media). S2 cells (2.0×10^5) were collected in 1.5 mL microcentrifuge tubes. Cells were centrifuged at 1000 x g for three minutes. An individual psyllid-*Wolbachia* (wDi) extraction was used to resuspend one tube of pelleted S2 cells. Then samples were centrifuged at 2500 x g for one hour at 15°C. The pelleted cells were resuspended with 1 mL of S2 complete media and added into a sterile 25-cm² flask containing 4mL of S2 complete media. Hereafter, the cells were kept at 28°C. After two days, the cells were transferred to a 75-cm² flask with 4 ml of S2 complete media. The wDi-infected (S2 + wDi) and uninfected-S2 cells were maintained according to standard procedures (Baum and Cherbas, 2008).

[0063] *Diagnostic PCR to screen wDi infection in cell culture:* DNA was extracted from cell culture using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The concentration and purity of DNA was quantified by using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE). The *wsp* (*Wolbachia* outer surface protein) and *ftsZ* (an essential cell division protein in *Wolbachia*) genes were amplified by diagnostic PCR using the GoTaq Colorless Master Mix (Promega, Madison, WI) in a T100 thermal cycler (Bio-Rad, Foster City, CA). The wDi *wsp* (*wsp* Forward: AGG GCT TTA CTC AAA ATT GG (SEQ ID NO: 1) and wDi *wsp* Reverse: CAC CAA CGGT ATG GAG TGA TAG G (SEQ ID NO: 2)), and the *Wolbachia* *ftsZ* (*ftsZ* Forward: ACG AGC CAG AGA AGC AAG AG (SEQ ID NO: 3) and *ftsZ* Reverse: TAC GTC GCA CAC CTT CAA AA (SEQ ID NO: 4)) (Dossi et al., 2014) primers were used for detection of *Wolbachia* under the following conditions: one cycle of 95°C for 3m, followed by 34 cycles of 95°C for 30s, 55°C for 30s, 72°C for 1m, and a final extension cycle of 72°C for 5m. Amplification products were separated on an 1.5% agarose (Fisher Chemical, Fair Lawn, NJ) gel.

[0064] *Localization of wDi in S2 cells:* Coverslips were washed with 80% ethanol and then coated with concavalin A (Vector Labs, Burlingame, CA) to assist cell adhesion to coverslip (Buster et al., 2010). Individual coated coverslips were placed in 35 x 10 mm² tissue culture dishes with 1 mL of S2 media. At a concentration of 1.5×10^6 cells/mL, 150 µL of cell suspension were added to individual coverslips, and were allowed to adhere for one hour at room temperature. The S2 media was removed and coverslips were gently washed with 1x PBS (Fisher Scientific, Fair Lawn, NJ) for five minutes. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for one hour at room temperature. Following fixation, samples were washed three times for five minutes with 1x

PBS at room temperature. Then coverslips were mounted on microscope slides with VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Slides were imaged with a confocal microscope (Leica SP8 Laser-scanning Confocal).

[0065] *Tetracycline treatment on wDi infected cell line:* Tetracycline treatment was used to clear wDi infection from cell culture. Uninfected S2 and infected S2 + wDi cell lines were divided into two separate 75cm² flasks. The control samples were grown in S2 complete media, and the treated samples were grown in S2 complete media supplemented with 0.1 mg/mL tetracycline (Sigma, St. Louis, Mo). Three biological replicates were conducted. Cells were left in treatment for three days. After three days of treatment, survival of S2 and S2+wDi cells were obtained by using trypan blue stain and Countess II FL Automated Cell Counter (Invitrogen, Eugene, OR). In addition, DNA was extracted by the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and quantified by using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE). DNA samples were subjected to quantitative real-time PCR analysis (qRT-PCR) to determine *Wolbachia* (*ftsZ*) copy numbers. qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturer protocol. For each sample, 50 ng/μl of DNA were used in for qRT-PCR analysis. The construction of the standard curve template DNA (*ftsZ* gene), and the subsequent steps for qRT-PCR were conducted as described in Chu et al. (2016).

[0066] *Extraction and purification of wDi from S2 cells:* Extraction and purification of wDi was conducted as previously described by Gamston and Rasgon (2007). Infected S2 cells with wDi were grown in 75-cm² culture flasks in 40 mL S2 complete media to approximately 90% confluence. Cells were dislodged into the media by tapping the flask, scraping the flask surface and by pipetting. The cells were harvested in 50 mL centrifuge tubes. To lyse the cells, approximately 5 mL of sterile 3 mm borosilicate glass beads were added to each sample and were vortexed at the highest speed for five minutes at room temperature (RT). The supernatant (containing S2 and wDi cells) was transferred into a clean and sterile 50 mL centrifuge tube. The supernatant was centrifuged at 2500 x g for ten minutes at 4°C. The supernatant was gently filtered through a sterile 5 μm filter syringe (Whatman, Little Chalfont, Buckinghamshire, UK) and collected in a high-speed centrifuge tube. Samples were centrifuged at 18000 x g at 4°C for ten minutes. The supernatant was removed. The pellet containing wDi was resuspended with 2 mL of S2 complete media. Samples were gently purified with 2.7 μm filter syringes (Whatman, Little Chalfont,

Buckinghamshire, UK) into a long-term storage tube. Purified wDi were held in suspension in S2 complete media at room temperature up to five days.

[0067] *Viability Assays:* S2 cells were counted on an automated cell counter, Countess II FL (Invitrogen, Eugene, OR) with trypan blue staining (Invitrogen, Eugene, OR) following manufactures protocol. The viability of purified wDi was assayed by LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Invitrogen, Eugene, OR) following manufacturer's protocol and using the Countess II FL Automated Cell Counter (Invitrogen, Eugene, OR).

[0068] *Transformation of purified wDi.* The transformation method of wDi was modified from Rachek et al. (1998). For each electroporated sample, 5.0×10^5 of wDi cells were used. Isolated wDi were centrifuged at 1000 x g at 15°C for ten minutes. Supernatant was removed and cells were resuspended with 100 μ L of 250 mM sucrose (Sigma, St. Louis, MO). Samples were centrifuged once more at 1000 x g at 15°C for ten minutes. The supernatant was removed and cells were resuspended with 50 μ L of 250 mM sucrose (Sigma, St. Louis, MO) per electroporated sample. For the initial visualization of electroporated wDi, the 50 μ L samples of wDi suspension were mixed with 10 mM or 30 mM fluorescein-labeled dsRNA (dsRNAFL) oligomer (BLOCK-iT Fluorescent Oligo, Invitrogen, Carlsbad, CA). After establishing the electroporation conditions, samples were electroporated with the plasmid constructed by Jiang et al. (2013) (Addgene plasmid #42876), pCas9. Plasmid was prepared following standard cloning techniques (Sambrook and Russell, 2001). Plasmids were purified with a PureLink HiPure Plasmid Midiprep Kit by Invitrogen (Vilnius, Lithuania). The 50 μ L samples of wDi suspension were mixed with 0.5, 1.0 or 1.5 μ g of circular plasmid DNA (pCas9). Samples were transferred to a 0.1-cm gap electroporation cuvette (BTX Harvard Apparatus, Holliston, MA) and chilled on ice for ten minutes. The cuvette was placed in a BTX Harvard Apparatus Electro Square Porator (ECM 830) (Holliston, MA) and electroporated (voltage at 1.7kV, pulse length at 176 μ s, pulse intervals at 100ms, and number of pulses at 18 times). Cells were placed in a 24-well plate suspended in 500 mL of Hank's Balance Salts (HBS) solution at RT. For samples electroporated with dsRNAFL, plates were covered with foil paper to protect from light. Electroporated cells were allowed to recover for two days.

[0069] After two days of recovery, cells were collected and placed in 1.5 mL microcentrifuge tubes. Cells were centrifuged at 2000 x g for ten minutes. The pellets were resuspended with 200 μ L 1x phosphate buffered saline (PBS). Cells electroporated with dsRNAFL were mounted on a slide and screened under a fluorescent microscope (Olympus

BX61 Epifluorescence microscope). Cells electroporated with pCas9 were centrifuged at 10000 x rpms for ten minutes. Pellets were resuspended with 50 μ L water. Resuspended cells were mixed with RQ1 DNase (Promega) to remove extracellular DNA. The mix samples were incubated at 37°C for one hour. After, samples were centrifuged at 10000 x rpms for ten minutes. The pellets were then resuspended with 500 mL of S2 complete media. Electroporated wDi were re-introduce in S2 cells (6.0×10^5 cell per sample) as described above. To detect pCas9 from cells, DNA was extracted from cell culture to amplify a segment of the chloramphenicol resistant gene found in the pCas9 by PCR as described above. The forward (GCA GTC GGA TAC CTT CCT ATT C – (SEQ ID NO: 5)) and reverse (TCC CTG ATG GTC GTC ATC TA – (SEQ ID NO: 6)) primers were used to detect the plasmid in S2+wDipCas9 DNA samples.

[0070] *Immunocytochemistry*: Immunocytochemistry was performed to visualize the Cas9 protein in S2+wDipCas9 cells. Preparation of cells onto coverslips was conducted as previously describe above. After fixation washes with PBS, an additional wash was conducted with 1x PBS-TritonX-100 (0.5% T) for five minutes at room temperature. Samples were blocked with 5% BSA for one hour at room temperature. Blocking solution was removed, then samples were incubated with anti-CRISPR-Cas9-Alexa Fluor 488 (1:500, Novus Biologicals, Littleton, CO) for one hour at room temperature. Antibody solution was removed and samples were washed with 1x PBS three times for five minutes at room temperature. Samples were mounted with VECTSHIELD antifade mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Samples were imaged with a confocal microscope (Leica SP8 Laser-scanning Confocal).

[0071] *Infection of S2 cells*: Infections were established by centrifugation of infected host material (adult *D. citri*) onto an uninfected *D. melanogaster* Schneider's (S2) cells. This founded a continuous culture of S2 infected wDi cell line (S2+wDi). The presence of wDi infection in the S2 line was confirmed by diagnostic polymerase chain reaction (PCR) assay using primers for the general *Wolbachia* ftsZ and the specific wDi outer surface protein (wsp) genes (Figure 1). Only S2 cells that were infected with wDi had amplification of *Wolbachia* DNA, while S2 only cells had no amplification for *Wolbachia* DNA. To visualize wDi in S2 cells, cells were fixed on concanavalin A slides and mounted with a mounting medium containing DAPI (Buster et al., 2010). Confocal microscopy revealed that wDi localizes intracellularly in S2 cells (Figure 2).

[0072] The treatment of tetracycline has been previously shown to eliminate *Wolbachia* infection (Dobson et al., 2002). To verify the wDi DNA was amplified from living bacteria and not residual exogenous DNA, the S2+wDi cells were divided into two parts; one treated with tetracycline and the other remained untreated (control). Treatment with tetracycline, had no effect on uninfected S2 cells' and S2+wDi cells' survival (Figure 3A), and a drastic reduction of wDi cell density was observed in treated S2+wDi cells (Figure 3B).

[0073] *Extraction and purification of wDi:* Gamston and Rasgon (2007) demonstrated that *Wolbachia* can be isolated and viable for one week outside its host cell, the *Aedes albopictus* embryonic cell line. Thus, large volumes of the established S2+wDi cells were grown for extraction and isolation of wDi cells as previously described by Gamston and Rasgon (2007). After purification of wDi, cells were maintained in S2 complete medium at room temperature and were stained with BacLight alive-dead stain to assess their viability. BacLight alive-dead stain uses two dyes SYTO-9 to stain live and dead cell membranes green, and Propidium iodide to stain cells with compromised cell membranes red (Figure 4). Each fluorescent cell was counted, and all wDi cell samples reported in this study that were isolated and purified remained viable with >95% viability after two days post isolation.

[0074] *Transformation of purified wDi:* For optimization purposes, multiple numbers of pulses (i.e., 25, 18, and 10) were tested. To visualize the exogenous nucleotides, 10 mM and 30 mM of a fluorescein-labeled dsRNA (dsRNAFL) oligomer (BLOCK-iT Fluorescent Oligo) was electroporated. Samples were analyzed at 48 hours-post-electroporation. Samples electroporated with 10 mM had little to no positive wDi cells (data not shown). However, samples electroporated with 30 mM had dsRNAFL signal in wDi cells (Figure 5B-D). Samples with 25 pulses had very little dsRNAFL signal in cells (Figure 5B); while, samples with 18 and 10 pulses had dsRNAFL signal in various wDi cells (Figures 5C & D).

[0075] After transformation of wDi, the purified/transformed wDi was introduced into a host cell to demonstrate that it is viable in host cells after transformation and that it can express an exogenous protein. Thus, we electroporated a CRISPR-Cas plasmid for bacterial expression of Cas9 nuclease, pCas9 (Jiang W et al., 2013). After two days of electroporation with pCas9, wDi cells were harvested and treated with RQ1 DNase (Promega) to remove extracellular plasmid. Next, the transformed wDi cells were mixed with uninfected S2 cells and centrifuged to create S2+wDiCas9 cells. Viability of re-infected S2 cells with control wDi (no DNA; 18 pulses electroporation) was 95% for both biological replicates (Figure 6A: Control); while, viability was drastically reduced for samples electroporated with the highest

pCas9 concentration (1.5µg). The samples electroporated with 0.5 and 1.0 µg of pCas9 had no effect on S2+wDipCas9 cells' viability independent of electroporated pulse number (Figure 6A). In addition, the density of wDi was the highest in these two samples that were electroporated with 18 pulses (Figure 6B).

[0076] The confirmation of transformed wDi in S2 cells was followed by detecting the acquisition of pCas9 DNA and the production of the Cas9 protein in the S2+wDipCas9 samples. The intracellular plasmid was detected by PCR (Figure 7A) and protein expression of Cas9 was detected by immunofluorescence (Figure 7B). Two days post re-infection of S2 with wDipCas9, cells were processed for DNA extraction and immunostaining using anti-Cas9. Amplification of the chloramphenicol resistant gene found in pCas9 was detected in S2+wDipCas9 samples by PCR (Figure 7A). In addition, protein expression of Cas9 was localized in wDi cells found within the host cell (Figure 7B).

[0077] The foregoing Example described the isolation of wDi from the S2 cells and their ability to survive outside the host cell up to one week, which provided a window of opportunity to introduce pCas9 into wDi using an *in vitro* culture system.

Example 2 – Bioengineering of *Wolbachia* using CRISPR/Cas9

[0078] The following Example describes the genetic modification of *Wolbachia* by both plasmid-based and protein-based CRISPR-Cas9 systems.

[0079] *Insects:* An uninfected *Diaphorina citri* Kuwayama population was established in 2005, at the University of Florida Citrus Research and Education Center (UF-CREC, Lake Alfred FL, USA), from a field population collected in Polk Co. (28.0° N, 81.9° W) before detection of Huanglongbind in Florida. *Candidatus Liberibacter asiaticus* (CLas) infected *D. citri* were reared in infected *Citrus sinensis* (L.) Osb. plants. Both *D. citri* colonies were kept in a secure quarantine facility in the UF-CREC (Lake Alfred, FL). All experiments were conducted in a growth chamber at 28°C, 16:8h light:dark photoperiod cycle and 60-80% relative humidity.

[0080] *Insect cell line:* Schneider's *Drosophila* (S2) cells infected with the *Wolbachia* strain from *D. citri* (wDi) (S2+wDi) and uninfected S2 cells were acquired and maintained as described in Example 1. Growth media used was a mixture of S2 media containing 10% heat inactivated fetal bovine serum, 50 unit of penicillin and 50µg streptomycin sulfate per mL (S2 complete media).

[0081] *Target region for genome engineering in wDi:* Selected sgRNAs located in the phage capsid protein gene (orf7) (WDIAC_RS06650 annotated on the Contig75.1; GenBank accession number NZ_AMZJ01000095.1) were analyzed using the draft genome of wDi. The sgRNA selected for the experiments was predicted to target a unique genomic site adjacent to the protospacer adjacent motif (PAM) sequence found in orf7.

[0082] *Plasmid encoded- and recombinant CRISPR-Cas9:* For the plasmid based system, pCas9 was purchased from Addgene (plasmid 42876, donated by Luciano Marraffini). To introduce the single guide RNA (sgRNA) into the plasmid, single-stranded oligonucleotides (Integrated DNA Technologies) containing the spacers were phosphorylated, annealed and ligated as previously described by Jiang et al (2013). The spacer sequences are listed in Table 2. For the protein based system, GeneArt™ Platinum™ Cas9 Nuclease and GeneArt™ Precision gRNA Synthesis Kit were purchased and prepared as described by the manufacturer (Thermo Fisher Scientific). The DNA oligonucleotides used for sgRNA synthesis are listed in Table 2.

[0083] Table 2. sgRNA DNA synthesis oligos.

orf7 spacer for cloning in pCas9	5'-AAACATAAATTCTCCTATTTTCTCG-3' (SEQ ID NO: 7)
orf7 spacer for cloning in pCas9	5'-AAAACGAGAAAATAGGAGAATTTAT-3' (SEQ ID NO: 8)
orf7 forward sgRNA template assembly	5'-TAATACGACTCACTATAGTAAATTCTCCTATTTTCTC-3' (SEQ ID NO: 9)
orf7 reverse sgRNA template assembly	5'-TTCTAGCTCTAAAACGAGAAAATAGGAGAATTTA-3' (SEQ ID NO: 10)

[0084] *Construction of DNA donor template for homology-directed repair:* To generate a single point mutation in orf7, a single-stranded donor template was designed as 150 oligodeoxynucleotides (ssODN) and purchased from Integrated DNA Technologies as PAGE purified long oligos (ultra oligo). This included a single nucleotide change (G to A) flanked by a left 75 nucleotides homology arm and a right 74 nucleotides homology arm; the ssODN sequence is listed in Table 3. The single nucleotide change was located within the PAM sequence preventing re-cutting of the edited region. To insert a complete exogenous gene, the generation of the donor template with the homologues recombination vector system were custom-made by VectorBuilder. The plasmids containing plasmid donor DNA (pDonor) were designed to comprise an insert gene flanked by a left 500 nucleotides homology arm and a right 561 nucleotides homology arm. The DNA sequence of the different insert genes encoding enhanced green fluorescent protein (eGFP), *Bacillus thuringiensis* crystal toxin

(Cry3Aa), *Bacillus thuringiensis* cytolytic toxin (Cyt2Ca1) and *Galanthus nivalis* agglutinin (GNA3); respective reverse sequence of inserts (-R); and the homologous arms are listed in Table 3. Plasmids were prepared by using the QIAGEN Maxi Plasmid Kit (QIAGEN).

[0085] Table 3. Donor DNA. Plasmid donor templates were designed to include the reverse sequence order (-R).

orf7 ssODN	CAAGTAATTCCATTAAAACTTCCCTTGCTTGCTCAATACTTACACCCCT GCTCAATAAATTCTCCTATTTTCTCTGACATTCGTGATAAGTTACATA AACGTATTAATTCAACAACCTTCAGTACGATACTTAGTTAAATTATCA GTTTCTA (SEQ ID NO: 11)
pGFP	ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCT GGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCC GGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT TCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCCACCCTCGTG ACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCA CATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACG TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACC CGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCG AGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCA CAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATGGCCG ACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAA CATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAAC ACCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCT GAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGAT CACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGG CATGGACGAGCTGTACAAGTAAAGCG (SEQ ID NO: 12)
pGFP-R	GCGAAATGAACATGTTCGAGCAGGTACGGCTCTCACTAGGGCCGCCGC CAGTGCTTGAGGTGCTCCTGGTACACTAGCGCGAAGAGCAACCCAG AAACGAGTCCCGCCTGACCCACGAGTCCATACCAACAGCCCGTCGT CGTGCCCCGGCAGCGGCTACCCCCACAAGACGACCATCACCAGCCGC TCGACGTGCGACGGCAGGAGCTACAACACCGCCTAGAACTTCAAGTG GAACTACGGCAAGAAGACGAACAGCCGGTACTATATCTGCAACACC GACAACATCAACATGAGGTGGAACACGGGGTCTTACAACGGCAGGA GGAAGTTCAGCTACGGGAAGTCGAGCTACGCCAAGTGGTCCCACAGC GGGAGCTTGAAGTGGAGCCGCGCCCGAAGATCAACGGCAGCAGGA ACTTCTTCTACCACGCGAGGACCTGCATCGGAAGCCCGTACCGCCTG AACTTCTTCAGCACGACGAAGTACACCAGCCCCATCGCCGACTTCGT GACGTGCGGCATCCAGTCCCACCAAGTGCTCCCACCCGGTCCCGTGCC CGTCGAACGGCCACCACGTCTACTTGAAGTCCCAGTCGAACGGCATC CACCGTAGCGGGAGCGGGAGCGGCCTGTGCGACTTGAACACCGGCA AATGCAGCGGCAGGTTCGAGCTGGTCTTACCCGTGGTGGGGCCACTTG TCGAGGAGCGGGAACGAGTGGTA (SEQ ID NO: 13)
pCry3Aa	TTTGGTGAGCGCTTGTTTCGTTTATACAACTTTTTAAATACTATTT GGCCAAGTGAAGACCCGTGGAAGGCTTTTATGGAACAAGTAGAAGC ATTGATGGATCAGAAAATAGCTGATTATGAAAAAATAAAGCTCTTG CAGAGTTACAGGGCCTTCAAAAATAATGTCGAAGATTATGTGAGTGCA TTGAGTTCATGGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAG CCAGGGGGCGGATAAGAGAGCTGTTTTCTCAAGCAGAAAGTCATTTTC

	GTAATTCAATGCCTTCGTTTGC AATTTCTGGATACGAGGTTCTATTTCTAACAA CATATGCACAAGCTGCCAACATACATTTATTTTACTAAAA GACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATA TTGCTGAATTTTATAAAAAGACAACATAAACTTACGCAAGAATATACT GACCATTGTGTCAAATGGTATAATGTTGGATTAGATAAATTAAGAGG TTCATCTTATGAATCTTGGGTAAACTTTAACC GTTATCGCAGAGAGAT GACATTAACAGTATTAGATTTAATTGCACTATTTCCATTGTATGATGT TCGGCTATACCCAAAAGAAGTTAAAACCGAATTAACAAGAGACGTTT TAACAGATCCAATTGTCTGGAGTCAACAACCTTAGGGGCTATGGAACA ACCTTCTCTAATATAGAAAATTATATTCGAAAACCATCTATTTGAC TATCTGCATAGAATTCAATTTACACGCGGTTCCGACCAGGATACTA TGGAAATGACTCTTTC AATTATTGGTCCGGTAATTATGTTTCAACTAG ACCAAGCATAGGATCAAATGATATAATCACATCTCCATTCTATGGAA ATAAATCCAGTGAACCTGTACAAAATTTAG AATTTAATGGAGAAAAA GTCTATAGAGCCGTAGCAAATACAAATCTTGCGGTCTGGCCGTCCGC TGTATATTCAGGTGTTACAAAAGTGGAATTTAGCCAATATAATGATC AAACAGATGAAGCAAGTACACAAACGTACGACTCAAAAAGAAATGT TGGCGCGGTCAGCTGGGATTCTATCGATCAATTGCCTCCAGAAACAA CAGATGAACCTCCAGAAAAGGGATATAGCCATCAACTCAATTATGTA ATGTGCTTTTTAATGCAGGGTAGTAGAGGAACAATCCAGTGTTAAC TTGGACACATAAAAAGTGTAGACTTTTTTAA CATGATTGATTGAAAA AAATTACACAACCTCCGTTAGTAAAGGCATATAAGTTACAATCTGGT GCTTCCGTTGTCGCAGGTCCTAGGTTTACAGGAGGAGATATCATTCA ATGCACAGAAAATGGAAGTGCGGCAACTATTTACGTTACACCGGATG TGTCGTA CTCTCAAAAACATCGAGCTAGAATTCATTATGCTTCTACAT CTCAGATAACATTTACACTCAGTTTAGACGGGGCACCATTTAATCAA TACTATTTTCGATAAAAACGATAAATAAAGGAGACACATTAACGTATAA TTCATTTAATTTAGCAAGTTTCAGCACACCATT CGAATTATCGGGGAA TAAC TTACAAATAGGCGTCACAGGATTAAGTGCTGGAGATAAAGTTT ATATAGACAAAATTGAATTTATTCCAGTGAATTAA (SEQ ID NO: 14)
pCry3Aa-R	AATTAAGTGACCTTATTTAAGTTAAAACAGATATATTTGAAATAGAG GTCGTGAATTAGGACACTGCGGATAAACATTCAATAAGGGGCTATTA AGCTTACCACACGACTTTGAACGATTTAATTTACTTAATATGCAATTA CACAGAGGAAATAAATAGCAAAATAGCTTTATCATAACTAATTTACC ACGGGGCAGATTTGACTCACATTTACAATAGACTCTACATCTTCGTAT TACTTAAGATCGAGCTACAAAAACTCTCATGCTGTGTAGGCCACATT GCATTTATCAACGGCGTGAAGGTAAAAGACACGTAAC TTA CTATAGA GGAGGACATTTGGATCCTGGACGCTGTTGCCTTCGTGGTCTAACATT GAATATACGGAAATGATTGCCTTCAACACATTA AAAAAAGCTTAGTT AGTACAATTTTTTTCAGATGTGAAAATACACAGGTTCAATTGTGACCC TAACAAGGAGATGATGGGACGTAATTTTTTCGTGTAATGTATTA ACTC AACTACCGATATAGGGAAAAGACCTCCAAGTAGACAACAAAGACCT CCGTTA ACTAGCTATCTTAGGGTCGACTGGCGCGGTTGTAAAGAAAA ACTCAGCATGCAAACACATGAACGAAGTAGACAACTAGTAATATA ACCGATTTAAGGTGAAAACATTGTGGACTTATATGTGCGCTGCCGGT CTGGCGTTCTAAACATAAACGATGCCGAGATATCTGAAAAAGAGGTA ATTTAAGATTTAAAACATGTCCAAGTGACCTAAATAAAGGTATCTTA CCTCTACACTAATATAGTAACTAGGATACGAACCAGATCAACTTTG TATTAATGGCCTGGTTATTA ACTTTCTCAGTAAAGGTATCATAGGACC AGCCTTGGCGCACACTTTAACTTAAGATACGTCTATCAGTTTATCTAC

	ACCAAAAGCTTATATTTAAAAGATATAATCTCTTCCAACAAGGTATCG GGGATTCCAACAACCTGAGGCTGTAAACCTAGACAATTTTGCAGAGAA CAATTAAGCCAAAATTGAAGAAAACCCATATCGGCTTGTAGTATGTT ACCTTTATCACGTTAATTTAGATTATGACAATTACAGTAGAGAGACG CTATTGCCAATTTCAAATGGGTTCTAAGTATTCTACTTGGAGAATTAA ATAGATTAGGTTGTAATATGGTAAACTGTGTTACCAGTCATATAAGA ACGCATTCAAAATCAACAGAAAATATTTTAAGTCGTTATAGAAGAAA AAGCATAGGGGTAAGAAGAGGTTATTTAAACTCGCAGAAAATCATTTT TATTTACATACAACCGTCGAACACGTATACAACAATCTTTATCTTGGA GCATAGGTCTTTAACGTTTGCTTCCGTAACCTAATGCTTTTACTGAAA GACGAACTCTTTTGTCGAGAGAATAGGCGGGGACCGATACACCTAAA GCACTTGAGTGTCTAAAAAAACGGTACTTGAGTTACGTGAGTGTAT TAGAAGCTGTAATAAAACTTCCGGGACATTGAGACGTTCTCGAAATA AAAAACGTATTAGTCGATAAAAGACTAGGTAGTTACGAAGATGAAC AAGGTATTTTCGGAAGGTGCCCAGAAGTGAACCGGTTTATCATAAAT TTTTCAAACATATTTTGCTTTGTTTCGCGAGGTGGTTT (SEQ ID NO: 15)
pCyt2Ca1	ATGTTCTTCAACCGCGTTATCACCTTGACCGTTCCAAGCAGCGATGTC GTTAACCTACAGCGAAATCTACCAGGTCGCTCCACAGTATGTTAACCA GGCTTTGACCTTGGCTAAGTACTTCCAGGGAGCTATCGATGGTAGCA CCTTGAGATTTCGATTTTCGAGAAGGCTTTGCAGATTGCTAACGATATTC CACAGGCAGCCGTGGTCAACACCTTGAATCAGACTGTCCAGCAAGGT ACTGTCCAAGTCAGCGTGATGATCGACAAGATTGTCGACATTATGAA GAATGTGTTGAGCATTGTCATTGATAACAAAAAGTTCTGGGATCAGG TGA CTGCTGCTATTACTAATACCTTCACCAACTTGAACAGCCAAGAA AGCGAAAGATGGATTTTCTACTACAAGGAAGATGCTCACAAGACTAG CTACTATTACAATATCTTGTTTGCTATTCAGGATGAGGAAACTGGTGG CGTTATGGCCACCTTGCCAATCGCCTTTGATATTAGCGTTGATATTGA AAAGGAAAAGGTTTTGTTTGTTACTATCAAGGATACTGAGAATTACG CTGTTACCGTGAAAGCTATCAACGTCGTTTCAGGCTTTGCAAAGCAGC AGAGATAGCAAAGTTGTGGATGCTTTTAAAAGCCCAAGACACTTGCC AAGAAAAAGACACAAAATCTGCAGCAACAGCTAA (SEQ ID NO: 16)
pCyt2Ca1-R	AATCGACAACGACGTCTAAAACACAGAAAAAGAACCGTTCACAGAA CCCGAAAATTTTCGTAGGTGTTGAAACGATAGAGACGACGAAACGTT TCGGACTTGCTGCAACTATCGAAAGTGCCATTGTCGCATTAAGAGTC ATAGGAACTATCATTGTTTGTGTTTGGAAGGAAAAGTTATAGTTGC GATTATAGTTTCCGCTAACCGTTCACCGGTATTGCGGTGGTCAAAG GAGTAGGACTTATCGTTTGTTCTATAACATTATCATCGATCAGAACAC TCGTAGAAGGAACATCATCTTTTAGGTAGAAAGCGAAAGAACCGAC AAGTTCAACCACTTCCATAATCATTATCGTCGTCAGTGGACTAGGGT CTTGAAAAACAATAGTTACTGTTACGAGTTGTGTAAGAAGTATTACA GCTGTTAGAACAGCTAGTAGTGCGACTGAACCTGTCATGGAACGACC TGTCAGACTAAGTTCCACAACCTGGTGCCGACGGACACCTTATAGCAA TCGTTAGACGTTTCGGAAGAGCTTTAGCTTAGAGTTCCACGATGGTA GCTATCGAGGGACCTTCATGAATCGGTTCCAGTTTCGGACCAATTGT ATGACACCTCGCTGGACCATCTAAAGCGACATCAATTGCTGTAGCGA CGAACCTTGCCAGTTCCACTATTGCGCCAACCTTCTTGTA (SEQ ID NO: 17)
pGNA3	ATGGCTAAGGCAAGTCTCCTCATTTTGGCTACCATCTTTCTTGAGTC ATCACACCAGCTTGCCTGAGTGAAAATATTCTGTACTCCGGTGAGAC TCTCCCTACAGGGGGATTTCTCACCTCTGGCAGTTTTGTTTTTATCAT

	GCAAGAGGACTGCAACCTGGTCTTGTACAACGTCGACAAGCCCATCT GGGCAACTAACACAGGCGGCCTCTCCAGTGACTGCTCCCTCAGCATG CAGACCGATGGGAACCTCGTTGTGTACACCCCATCGGACAAACCGAT TTGGGCAAGCGACACTGACGGTCAGAATGGGAATTACGTGTGCATCC ATCAAAAGGATCGGAACGTTGTGATCTACGGAACATAATCGTTGGGCT ACTGGAACCTCACACCGGCGCTGTAGGAATTCCTGCATCACCGCCCTC GGAGAAATATCCTACTGCTGGAATGATAAAGCTTGTGACGGCAAAGT AA (SEQ ID NO: 18)
pGNA3-R	AATGAAACGGCAGTGTTTCGAAATAGTAAGGTCTGCATCCTATAAAGA GGCTCCCGCCACTACGTCCTTAAGGATGTGCGGGCCACACTCAAGGT CATCGGGTTGCTAATCAAGGCATCTAGTGTTGCAAGGCTAGGAAAAC TACCTACGTGTGCATTAAGGGTAAGACTGGCAGTCACAGCGAACGGG TTTAGCCAAACAGGCTACCCACATGTGTTGCTCCAAGGGTAGCCAG ACGTACGACTCCCTCGTCAGTGACCTCTCCGGCGGACACAATCAACG GGTCTACCCGAACAGCTGCAACATGTTCTGGTCCAACGTCAGGAGAA CGTACTATTTTTGTTTTGACGGTCTCCACTCTTTAGGGGGACATCCCT CTCAGAGTGGCCTCATGTCTTATAAAAGTGAGTCCGTTTCGACCACAC TACTGAGGTTCTTTCTACCATCGGTTTACTCCTCTGAACGGAATCGG TA (SEQ ID NO: 19)
Plasmid left homologous arm	ACTCCTTTACTATTTTCCTTTGCGTTTACATCGCTTGCTATTACACCTA TCGCTGTTTGTGTACCATCTGTGGCAGTTGGATTAAAGCTTTAATTA AATTATCTTTAGTATCATAACCAACTACTGTTCTAGCTTCAGATTTT GTCCCTTTGCTACCGTTATTTGGTCTCTTGAATATAAACTTGATGCCT CATACTTTAATAGGTCACCTAGATTATTTTGTTCGGTTATACATGTCA TATTTTCTTTTCTCCTTTTTTCCCTTAGTTTTACCGCCATGTTTGTAGTT ATTGTATGCGGCGGTTATAAAAAAACTTAAATACTTGATTGTGCTCT AGCTTTTGCTACTTGCATCATCAAATCTTCTCCTGAATTCTGTGGTAT TGCACTCAGTATCTCTGTCTTCTTCGTTCTCTCTGCAAGTAATTCCATT AAAACTTCCCTTGCTTGCTCAATACTTACACCCTGCTCAATAAATTCT CCTATTTTCTCTGACAT (SEQ ID NO: 20)
Plasmid right homologous arm	TCGTGATAAGTTACATAAACGTATTAATTCAACAACCTTCAGTACGAT ACTTAGTTAAATTATCAGTTTCTAGGTCACCTTGTAAGTTTGTATTTCAT AGTATTTTCTCCTTTGTTAATAAATTCAAAAAATGTTGTAACCTCCATC TGCAAGACCTATTTCTACTGCTTTCTCACCAAAATATAGACCTGCTTC AGTATTTTTTATTGCTTGTACAGAAAGTCCTCTGTTTCGTGCTATAAG TTCAGTCAGAATTTTCATATAAGCGATTTACTTCCTCCTCTAACTTTT TAAGCTTTCTAACTTATAGGCTCATGTGGATTTAAATCATTCTTTCT CTTGCCCTGCAAAAATAGTTGTGATTTTATTCCTTGCTTTTCATCAAA CCTACTTTGATCTATATGACTTGCTATTACTCCTATACTGCCAACACC AAAAGTTCTGCTCACAAATACCTTTTCAGCACTTGAAGCTATAGCAT ATGCAGCAGAGTACGCATCATTTTGCTATTGCCACAATTCTCTTTT TTGCTCTTGATTTCGTAAATAAAATCAGCCAAGTC (SEQ ID NO: 21)

[0086] *wDi transformation:* Extraction of wDi from S2+wDi cells and electroporation of wDi cells were conducted as described in Example 1. Each electroporation sample contained 500,000 - 850,000 wDi cells. Isolated wDi cells were transformed with (1) 2 μ g of pCas9+sgRNA and 1 μ L of 10 μ M ssODN (plasmid based system) or (2) 1 μ g of GeneArt™

Platinum™ Cas9 nuclease, 200 ng of sgRNAs and either 1 µL of 1 µM ssODN or 500 ng of pDonor DNA (protein based system).

[0087] *PCR analysis:* DNA extractions for both cell culture and *D. citri* samples were conducted using DNeasy Blood and Tissue kit (QIAGEN). DNA concentrations were quantified by spectrophotometry (Nanodrop 2000; Thermo Fisher Scientific). DNA samples were diluted to 10 ng/µL for subsequent PCR analysis. GoTaq Colorless Master Mix (Promega) and T100 thermal cycle (Bio-Rad) were used for the PCR analysis. All samples were screened for wDi presence using the wsp gene (*Wolbachia* outer surface protein). Only samples that were positive for wsp were further analyzed to detect genome editing. The pair of primers for amplifying the targeted region or insert gene were designed using Primer3 v 0.40 software (Untergrasser et al., 2012) and listed in Table 3. The amplicon sequences were verified by Sanger DNA sequencing (Genewiz).

[0088] Table 3. PCR primers used in this study.

Target Gene	Primer sequence (5'-3')	SEQ ID NO:
<i>Wsp</i> - forward	AGG GCT TTA CTC AAA ATT GG	22
<i>Wsp</i> - reverse	CAC CAA CGG TAT GGA GTG ATA GG	23
<i>GFP</i> - forward	GGT GAA CTT CAA GAT CCG CC	24
<i>GFP</i> - reverse	CTT GTA CAG CTC GTC CAT GC	25
<i>GFP</i> -R- forward	CAG GTA CGG CTC TCA CTA GG	26
<i>GFP</i> -R- reverse	TAC CGG CTG TTC GTC TTC TT	27
<i>Cry3Aa</i> - forward	TTG TCG CAG GTC CTA GGT TT	28
<i>Cry3Aa</i> - reverse	AAT GGT GCC CCG TCT AAA CT	29
<i>Cry3Aa</i> -R- forward	GTG TAG GCC ACA TTG CAT TTA TC	30
<i>Cry3Aa</i> -R- reverse	CGT CCC ATC ATC TCC TTG TTA G	31
<i>Cyt2Ca1</i> - forward	GAT CAG GTG ACT GCT GCT ATT A	32
<i>Cyt2Ca1</i> - reverse	CTT TGC TAT CTC TGC TGC TTT G	33
<i>Cyt2Ca1</i> -R- forward	GAA AGC GAA AGA ACC GAC AAG	34
<i>Cyt2Ca1</i> -R- reverse	GCT ACC ATC GTG GAA CTC TAA G	35
<i>GNA3</i> - forward	GGC TAC CAT CTT TCT TGG AGT C	36
<i>GNA3</i> - reverse	CTT TGC CGT CAC AAG CTT TAT C	37
<i>GNA3</i> -R- forward	ACA CTC AAG GTC ATC GGG TT	38
<i>GNA3</i> -R- reverse	CCA GAA CAT GTT GCA GCT GT	39

[0089] *FACS analysis:* wDi cells were collected two days after transformation, then washed and resuspended in phosphate buffer saline (PBS). Fluorescence-activated cell sorting (FACS) analysis of GFP transformed wDi cells were performed using a BD FACSaria II flow cytometer (BD Biosciences) at the Cytometry Core Facility in University of Florida Interdisciplinary Center for Biotechnology Research. Electroporated samples of

wDi without DNA (negative control) and wDi with fluorescein-labeled dsRNA oligo (BLOCK-iTTM Fluorescent Oligo) (positive control) were used to set gating parameters.

[0090] *Nymph microinjections:* Two electroporated wDi samples were combined and resuspended in PBS, followed by microinjection into *D. citri* nymphs (3rd-4th instars) at their ventral side of the first abdominal segment or dorsal side of the mesothorax. The range of injection pressure used was 12 – 15 psi, and approximately 0.2 μ l of wDi cell suspension was injected per nymph. After injection, nymphs were placed on Citrus macrophylla plants to further develop into adults. Psyllids were collected after detecting eggs on the host plants and screened for exogenous gene insert from the transformed wDi.

[0091] *Imaging:* Transformed wDi cells were sorted, and the GFP-positive population (wDi+GFP) was processed for *in vivo* imaging. wDi+GFP cells were suspended in PBS, mounted on a cover glass and placed on a microscope slide. Each slide was imaged in three different locations to screen for GFP-positive cells. wDi-control cells were electroporated with no DNA while two independent electroporated samples with CRISPR/Cas9 components are shown in Figure 11D. Fluorescent images were acquired using an Olympus BX61 fluorescent compound microscope. S2+wDi+GFP-R sorted cells were stained with 17 μ g/ μ l Hoechst 33342 (Thermo Fisher Scientific). Cells were allowed to settle on CELLview™ cell culture dishes (sterile glass bottom) and analyzed with a Leica SP5 inverted confocal microscope (Figure 11E). For the microinjections on *D. citri*, the transformed wDi+GFP-R were stained with Hoechst 33342 before they were microinjected. Due to autofluorescence in the nymph, cells that were positive for both GFP and Hoechst 33342 signal were indicated as transformed wDi+GFP-R (Figure 13A, red arrows). Low-melt agarose was used for mounting the injected nymphs. Nymphs were images 1-2h post-injection. *In vivo* images were taken using a Leica SP8 upright confocal microscope.

[0092] *Results*

[0093] wDi culture undergo gene editing via CRISPR/Cas9-mediated homology-directed repair.

[0094] A single nucleotide change in wDi prophage was produced in order to determine whether wDi cells were responsive to CRISPR/Cas9-mediated homology-directed repair (HDR) gene editing. The targeted location for bioengineering wDi was the minor capsid gene (*orf7*) of the *Wolbachia*-associated phage (WO). CRISPR sgRNA was designed within *orf7* of wDi and the single-stranded DNA oligonucleotide (ssODN) donor, including the two

flanking homology arms and the G to A mutation (sequences are shown in Figure 8A and Table 2). The schematic outline to transform wDi with the CRISPR/Cas9-plasmid or -protein based system strategy is shown in Figure 8B. This strategy made use of pCas9 plasmid, the commercially available Cas9 endonuclease and the synthetic, mutation-inducing oligonucleotide (donor DNA).

[0095] DNA was isolated from cells transfected with the different gene editing strategies (plasmid or protein) in order to analyze the sequence change in wDi after CRISPR/Cas9-mediated HDR (Figure 9). For the plasmid based system, wDi cells were electroporated with either no CRISPR/Cas9 components (control), pCas9 (without the guide sequence), pCas9+sgRNA (without donor DNA), or pCas9+sgRNA + ssODN (Figure 9A). For the protein based system, wDi cells were electroporated with either no CRISPR/Cas9 components (control), Cas9:sgRNA (without donor DNA) or Cas9:sgRNA + ssODN (Figure 9B). In both the plasmid and protein based systems, the detection of single base gene editing occurred; however, as expected, not all cells within an electroporated sample were genetically transformed resulting in a heterogenous population.

[0096] In a separate biological sample, three technical replicates from the sample wDi population were used to analyze the variability of each system. wDi cells that were transfected with pCas9+sgRNA + ssODN resulted in one of three samples with the point mutation (data not shown); whereas, wDi cells transfected with Cas9:sgRNA + ssODN resulted in three of three samples with the point mutation (Figure 9C). The protein based strategy was used for gene insert experiments.

[0097] *Knock-in of eGFP in wDi:* The *Wolbachia* were isolated from host cells and transfected with CRISPR-Cas9 components plus circular pGFP or pGFP-R to integrate an exogenous gene via CRISPR/Cas9-mediated HDR in wDi. S2 cells were infected with two days post-transformed wDi cells (S2+wDi) (Figure 10). Genomic DNA of transfected cells, wDi and S2+wDi, were extracted and analyzed by PCR (Figure 11A & 11B). GFP insertion was confirmed in both wDi and S2+wDi cell cultures by the occurrence of the PCR product in transfected cells and absent in the negative control samples (no pDonor). After two days of transfection, wDi+GFP and wDi+GFP-R cells were subsequently enriched by fluorescence-activated cell sorting (FACS) (Figure 11C). Cells transformed with pGFP or pGFP-R had 4.8% and 6.2% GFP-positive wDi cells, respectively. Both directions of the gene insert were able to produce GFP; however, the GFP intensity signal was higher with the reverse sequence. Successful production of GFP was also demonstrated by *in vivo* imaging,

observing a heterogenous population in terms of signal intensity (Figure 11D, red arrows). In addition, by *in vivo* imaging of S2+wDi+GFP-R cells, the production of GFP by wDi cells in host cells (S2) was visible (Figure 11E). These results demonstrated the ability for CRISPR/Cas9-assisted HDR to knock-in a transgenes, GFP, in wDi; in addition, the transformed wDi cells remain viable in host cells.

[0098] *Generation of wDi+toxin cell lines:* Next, several toxins were introduced into wDi which are promising agents against insect pests. Using the same strategy as before, *Bacillus thuringiensis* crystal toxin Cry3Aa (1686 bp), *Bacillus thuringiensis* cytolytic toxin Cyt2Ca1 (696 bp) or *Galanthus nivalis* agglutinin GNA3 (474 bp) were introduced within the pDonor (Figure 12A). All toxin genes were introduced into the pDonor with the forward and reverse order. PCR assays verified incorporation of toxin genes in S2+wDi+toxin cells (Figure 12B-12G). PCR amplicons were sequenced to confirm their identity to desired transgene. These results demonstrated the successful integration of the donor DNA and that integration described herein is applicable across genes of variant sizes.

[0099] *Paratransgenic D. citri with transformed wDi+:* Paratransgenesis involves genetic manipulation of symbiotic bacteria commonly found in pathogen-transmitting vectors to export anti-pathogen “effector” molecules into the host vector. Paratransgenic strategy to control insect-borne diseases depends on the ability to genetically modify microorganisms from an insect. The ability for the transformed wDi+ to establish and vertically transmit themselves in *D. citri* was tested. To bypass the midgut barrier, *D. citri* nymphs were microinjected with transformed wDi+ to increase the changes of vertical transmission. Early dispersal of wDi+GFP-R in injected nymphs were detected (Figure 6A) by *in vivo* imaging. By 1-2 h post-injection, transformed cells (Figure 13A, red arrows) were visualized near or distal from the injection location (Figure 13A, yellow arrows). Due to concerns with toxic effects of GFP in the insect, further microinjections were conducted with wDi+Cry3Aa or wDi+Cry3Aa-R because there are no known reports of Cry3Aa being toxic to *D. citri*. No live *D. citri* adults were able to collect from plants that had nymphs injected with wDi+Cry3Aa. However, nymphs injected with wDi+Cry3Aa-R did survive to adulthood. No wDi+Cry3Aa-R was detected by PCR in the parental generation (data not shown). By the F1 generation, there were five wDi+Cry3Aa-R positive *D. citri* out of 19 individual that were tested (26.3%) (Figure 13B). The number of positive wDi+Cry3Aa-R *D. citri* increased to 21 out of 24 that were tested of the F3 generation (87.5%) (Figure 13C). Thus, the percent of offspring infected with the transformed wDi+ was higher in F3 compared to F1 generation.

[00100] Genetically engineered *Wolbachia* containing the holin-repressor gene also were generated using the methods described herein. Paratransgenic transformation of *D. citri* with the holin-repressor gene eliminated CLas plant infection, as well as acquisition, suggesting that the paratransgenic *Wolbachia* is useful tool for disrupting of insect-transmitted pathogens. Additional replicates were collected which confirmed successful inhibition of CLas in *D. citri* transformed with *Wolbachia* containing holin-repressor gene (wDi^{-HRP}). CLas was not detected in susceptible citrus plants following exposure to *D. citri* with wDi^{-HRP} following acquisition feeding infected plants. This confirms that CLas transmission was disrupted with paratransgenic *Wolbachia*.

[00101] The foregoing Example describes the successful confirmation of paratransgenesis using the endosymbiont, *Wolbachia*. Transformed *Wolbachia* able to express an exogenous gene were established in the insect host (*D. citri*) and were vertically transmitted from mother to offspring.

Example 3 – Additional paratransgenesis examples

[00102] *Wolbachia* is present in 40% of insect species, rendering it a promiscuous insect symbiont. Given the cosmopolitan nature of the symbiont, it is likely that the *Wolbachia* driver system developed can be incorporated across a wide range of insect species for paratransgenic transformation. The implications of this are particularly important for the potential use of paratransgenic strategies to control insect-borne diseases.

[00103] The *Wolbachia* transformation system and methods described in Example 2 were used to successfully paratransgenic transform insects in three different orders: *Hemiptera*, *Diptera*, and *Lepidoptera* (data not shown).

[00104] This Example demonstrates that paratransgenesis can be used widely across a large range of insect species, without the need to develop a unique endosymbiont cell line for every insect target.

[00105] The foregoing Examples demonstrate that genetically-transformed *Wolbachia* is a flexible tool for, e.g., arthropod control, and facilitates the exogenous expression of a number of foreign genes, including insecticidal toxins, Gfp proteins, and CLas-associated genes. Significantly, the use of the genetically-transformed *Wolbachia* to paratransgenically engineer *D. citri* that do not transmit CLas was demonstrated. This finding is a significant demonstration that paratransgenesis can be used to reduce the spread of vector-borne

pathogens, which may be extended to other pathosystems, such as mosquito-borne arboviruses, *Plasmodium*, etc.

Example 4 – Insecticidal Activity

[00106] *Honeydew assays.* Honeydew will be collected from paratransgenic *D. citri* produced according to the methods described in Example 2 to evaluate production of Cry toxins by *D. citri*. Honeydew produced during insect feeding will be collected on the underside of an inverted Petri dish placed under a leaf disc. A glass pipette will be used to collect honeydew for subsequent detection of Cry toxins via Western blotting. In the event no toxins are detected, whole insects will be ground, centrifuged, and the supernatant collected for Cry toxin detection. Extracted honeydew will be used in subsequent insecticidal bioassays.

[00107] *Insecticidal activity bioassays.* Honeydew (or whole-body extracts) produced by paratransgenic *D. citri* will be used to assess Cry activity against Coleopteran larvae. *Diaprepes abbreviatus*, also pests of citrus, are maintained in colony in an insect rearing facility at the University of Florida Citrus Research and Education Center. Honeydew extracts from paratransgenic *D. citri* will be incorporated into artificial diet media in a 0.5 ul PCR tube. Neonate *D. abbreviatus* will be placed in tubes. Tubes will be inverted and stored at 27°C for 2 weeks. Mortality will be recorded by inspecting larvae under a dissecting microscope. Mortality of larvae in response to paratransgenically-derived Cry toxin will be compared to mortality of beetles in response to optimum doses of exogenous Cry toxins incorporated in the artificial diet.

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What is claimed is:

1. A method comprising
 - (a) transforming *Wolbachia* bacterium with an exogenous nucleic acid using a CRISPR-Cas9 nuclease to insert the nucleic acid into the genome of the bacterium, thereby producing a genetically transformed bacterium; and
 - (b) introducing the genetically transformed bacterium into an arthropod.
2. The method of claim 1, wherein the method includes isolating the bacterium from an arthropod host before step (a).
3. The method of claim 1, wherein step (a) comprises contacting the bacterium with CRISPR-Cas9 nuclease protein.
4. The method of claim 1, wherein step (a) comprises introducing a nucleic acid encoding a CRISPR-Cas9 nuclease into the bacterium under conditions allowing production of the CRISPR-Cas9 nuclease.
5. The method of claim 1, wherein the exogenous nucleic acid is inserted into a capsid gene of the bacterium genome.
6. The method of claim 4, wherein the capsid gene is orf7.
7. The method of claim 1, wherein the arthropod is selected from the group consisting of an insect, an arachnid and a crustacean.
8. The method of claim 1, wherein the insect is selected from the group consisting of an Asian citrus psyllid (*Diaphorina citri*), Yellow fever mosquito (*Aedes aegypti*), Potato psyllid (*Bactericerca cockerelli*), German cockroach (*Blatella germanica*), Beg bug (*Cimex lectularius*), fruit fly (*Drosophila melanogaster*), house cricket (*Acheta domesticus*), American grasshopper (*Schistocerca Americana*), tobacco hornworm (*Manduca sexta*) and a red flour beetle (*Tribolium castaneum*).
9. The method of claim 1, wherein the exogenous nucleic acid encodes a selectable marker.
10. The method of claim 1, wherein the exogenous nucleic acid encodes a toxin.
11. The method of claim 1, wherein the toxin is selected from the group consisting of *Bacillus thuringiensis* crystal toxin (Cry3Aa), *Bacillus thuringiensis* cytolytic toxin (Cyt2Ca1) and *Galanthus nivalis* agglutinin (GNA3).

12. The method of claim 1, further comprising the step of culturing the bacterium in a *Drosophila melanogaster* cell before step (a).
13. The method of claim 12, wherein the *Drosophila melanogaster* cell is an S2 cell.
14. The method of claim 13, wherein the S2 cell is cultured in Schneider medium.

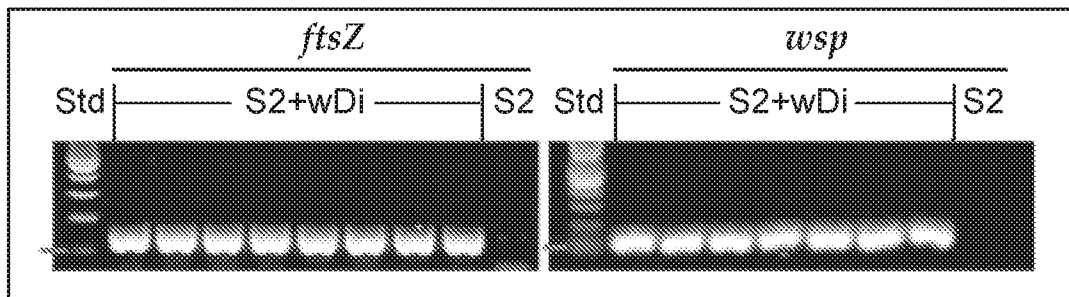


Figure 1. Verification of wDi in cell culture. The PCR amplification products of the established *in vitro* wDi infected S2 cells (S2+wDi) and an uninfected (S2) cell lines using the general-*Wolbachia* (*ftsZ*) and the specific-wDi (*wsp*) primer sets. A molecular size standard (Std) is shown (100 base pair (bp) DNA Ladder; NEB). The red markers indicate the 100 bp.

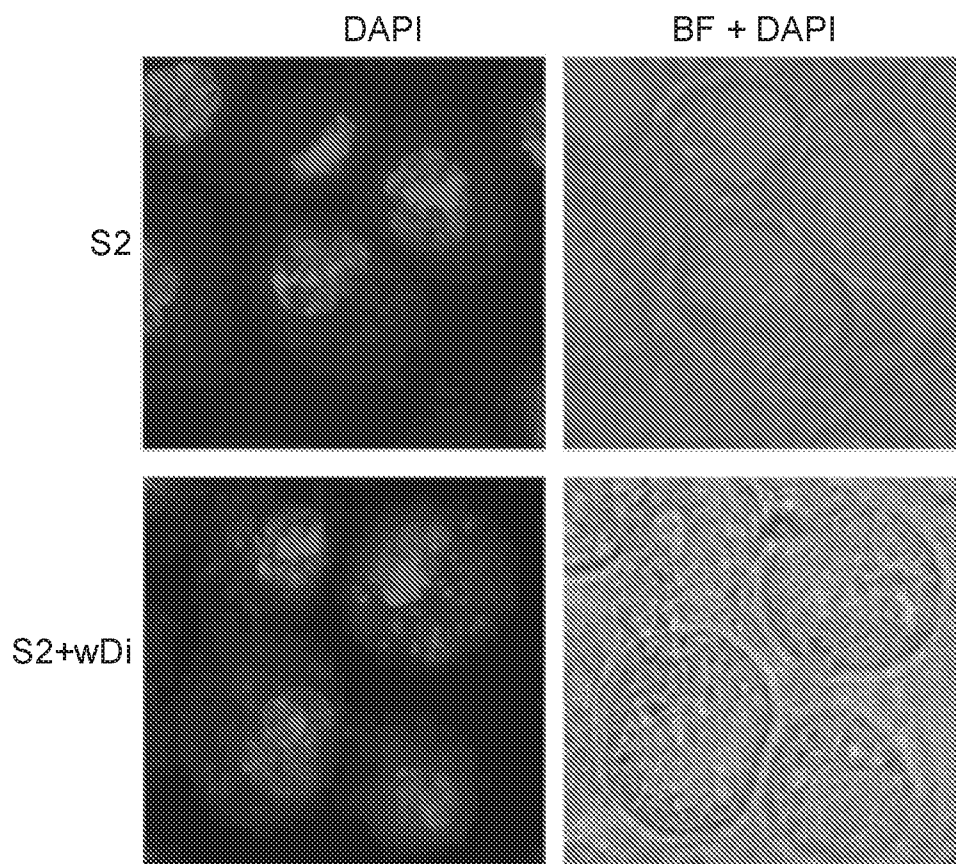


Figure 2. Intracellular location of wDi in S2 cells. Confocal images of fixed S2 and S2+wDi cells, acquired using DAPI (blue) filter and bright field (bf). DAPI stained the S2 cells nuclei in addition to wDi chromosomes.

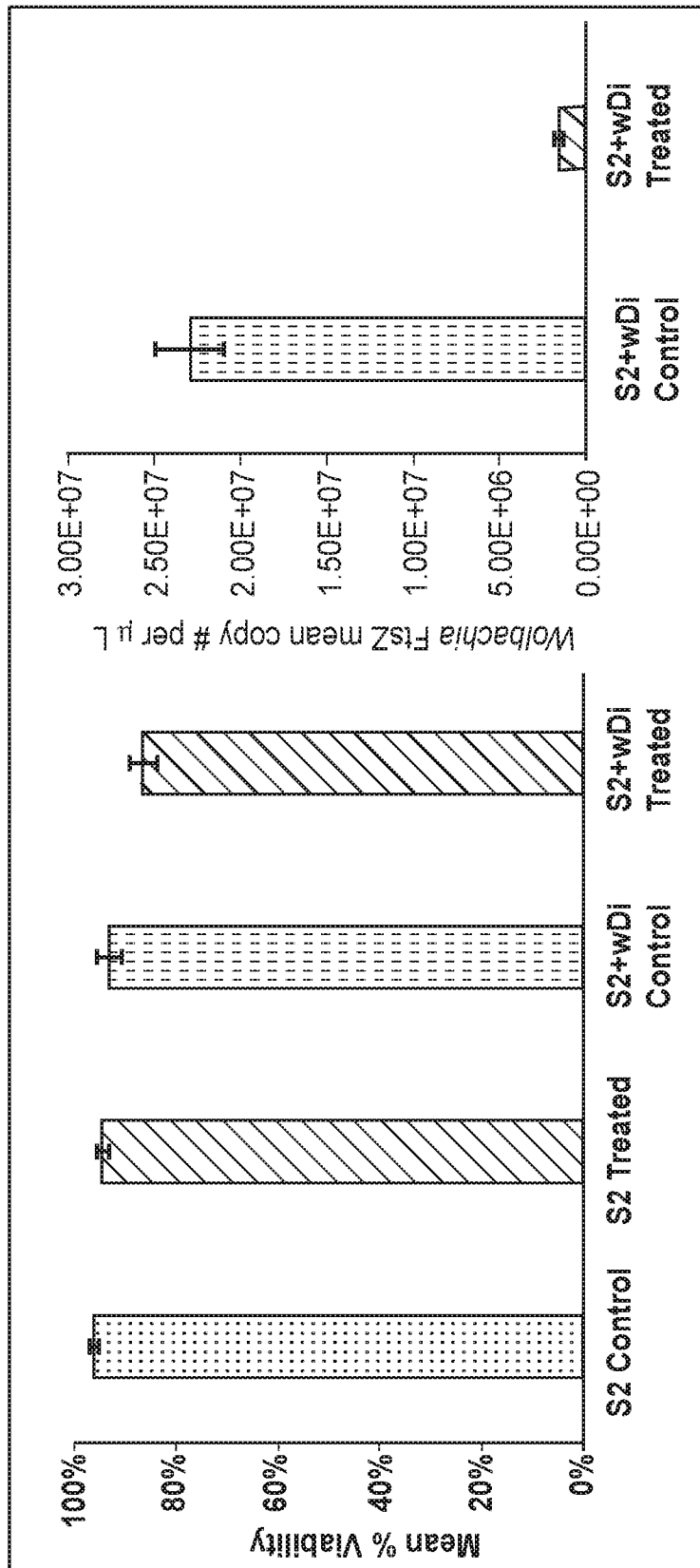


Figure 3. Reduction of wDi infection in S2 cells with tetracycline treatment. Infected and uninfected S2 cells with wDi were untreated (control) and treated with tetracycline for three days in S2 medium (A & B). Treatment with tetracycline had no significant effect on S2 cells survival (A). *Wolbachia* FtsZ mean copy number was determined for control and treated samples. (B) shows that wDi infection in S2 cells was reduced by three day of tetracycline treatment.

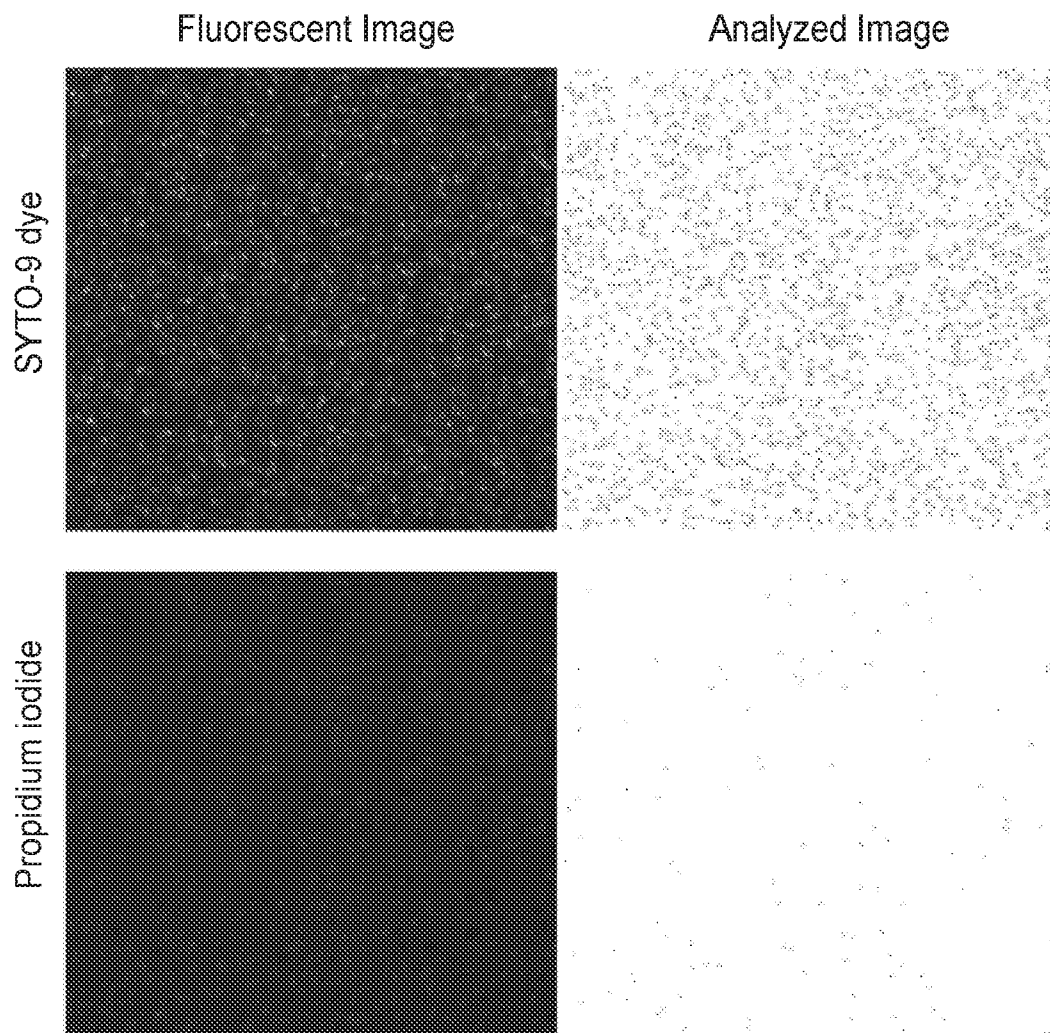


Figure 4. Purified wDi cells remain viable. wDi cells were extracted and purified from S2 cells, and were visualized with the BacLight live-dead stain. wDi cells were stained with green SYTO-9 dye (top row), and dead wDi cells are stained red with Propidium iodine (bottom row) (fluorescent images). For both fluorescent images, the cells were outlined and counted (second column) using ImageJ program (analyzed images). There were 2980 total cells, 2853 live cells and 127 dead cells; percent viability of 95.7% and percent mortality of 4.3%. For all subsequent wDi cell extractions and purifications the percent viabilities were >95% (data not shown).

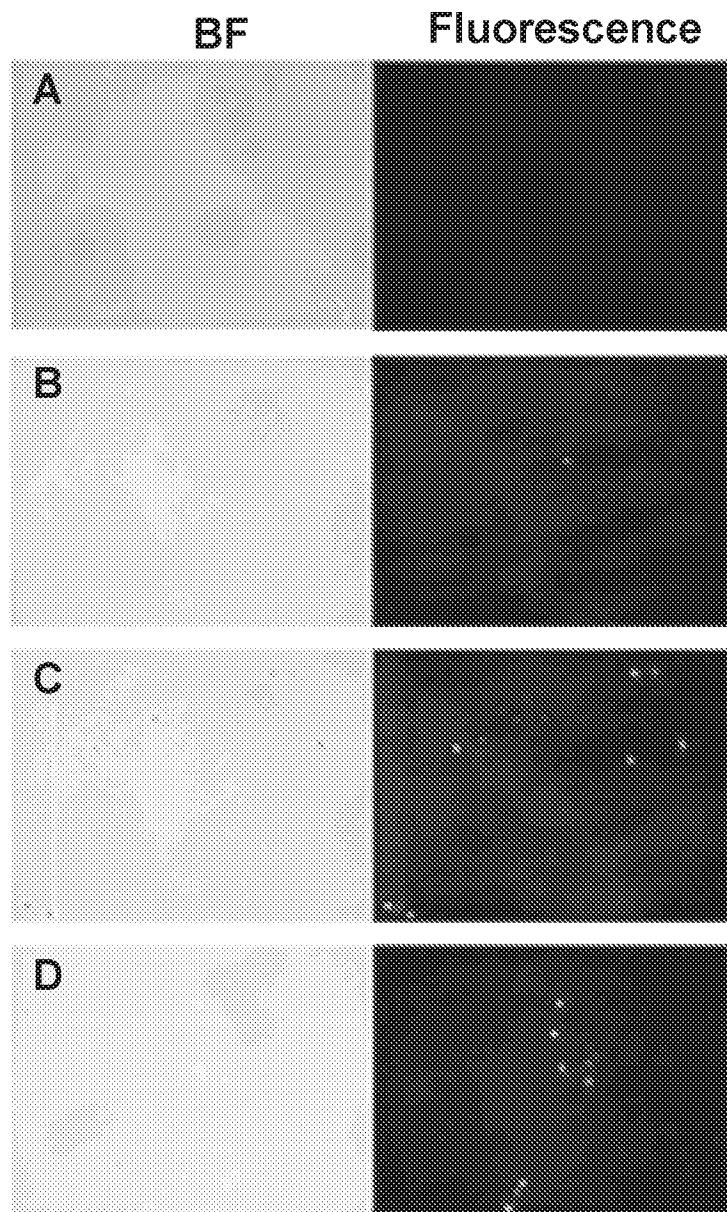


Figure 5. Electroporation of fluorescein-labeled dsRNA on purified wDi. Purified wDi cells were electroporated with fluorescein-labeled dsRNA (dsRNA^{FL}). All cells were mixed in with 30mM dsRNA^{FL} and were imaged at 48 hours-post-electroporation. The control cells that were not electroporated (A), and cells that were electroporated with the highest number of pulses (25x) (B) had no to little dsRNA^{FL} signal. Samples that were electroporated with 18 (C) and 10 (D) pulses had multiple wDi cells with dsRNA^{FL} signal.

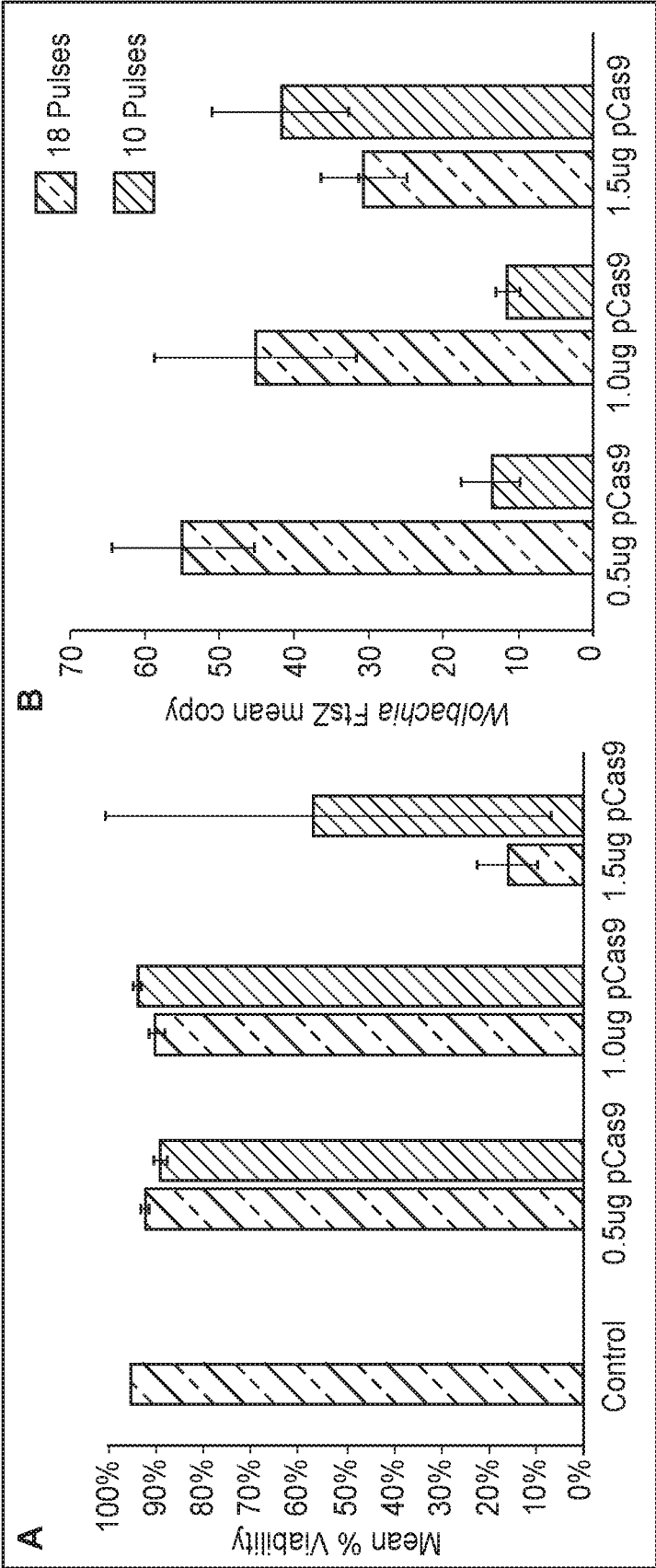


Figure 6. Viability of S2+wDi cells after electroporation with pCas9. Cells were counted after two days of reinfection of wDi (A & B). There were two biological replicated for each sample. Control samples were electroporated with no DNA at 18 pulses (A). Electroporation with 1.5ug of pCas9 had significant effect on viability of S2+wDi (A). *Wolbachia* FtsZ mean copy number was determined for each concentration of pCas9 electroporated (B).

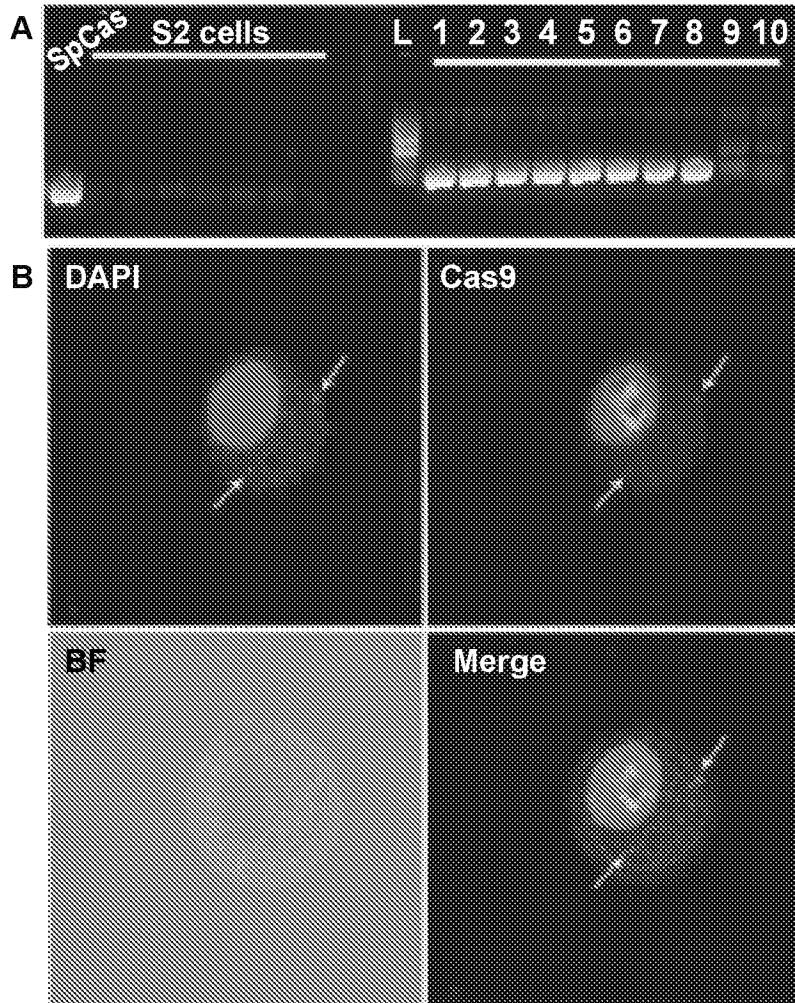


Figure 7. Detection of DNA and protein from pCas9 in S2+wDi^{pCas9} cells. (A) are the PCR amplification produces of pCas9 (primers design to amplify a *chloramphenicol resistant* gene fragment found in the plasmid DNA), (L) 100 bp ladder; DNA of samples are from two day post re-infection; #1-8 are samples that were electroporated with pCas9; sample #9 is the electroporation control with no DNA; and sample #10 is the no DNA and no electroporation control. Confocal images of S2+wDi^{pCas9} cells labeled with Cas9 and DAPI (B). White arrows indicate cytoplasmic wDi that express Cas9.

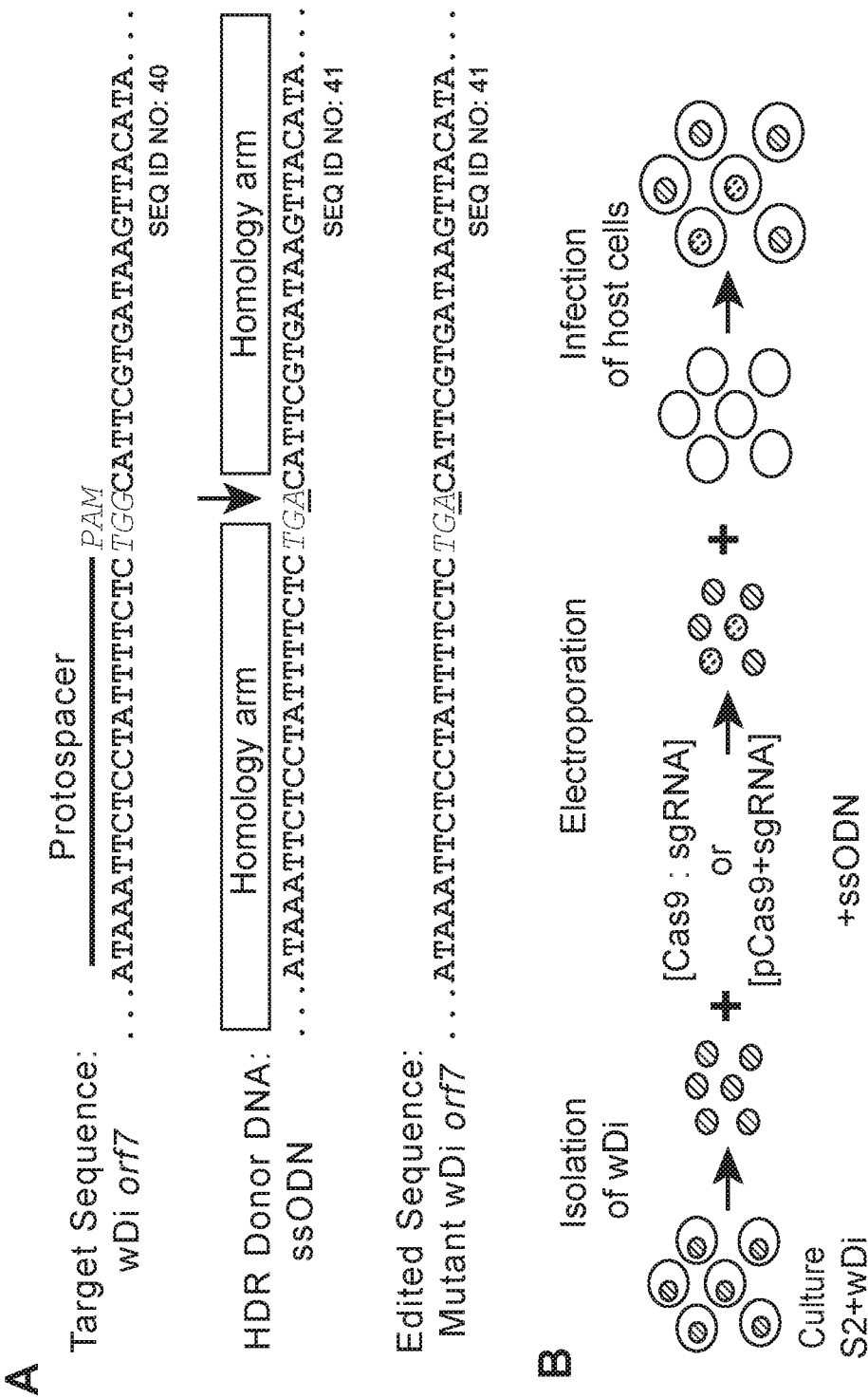
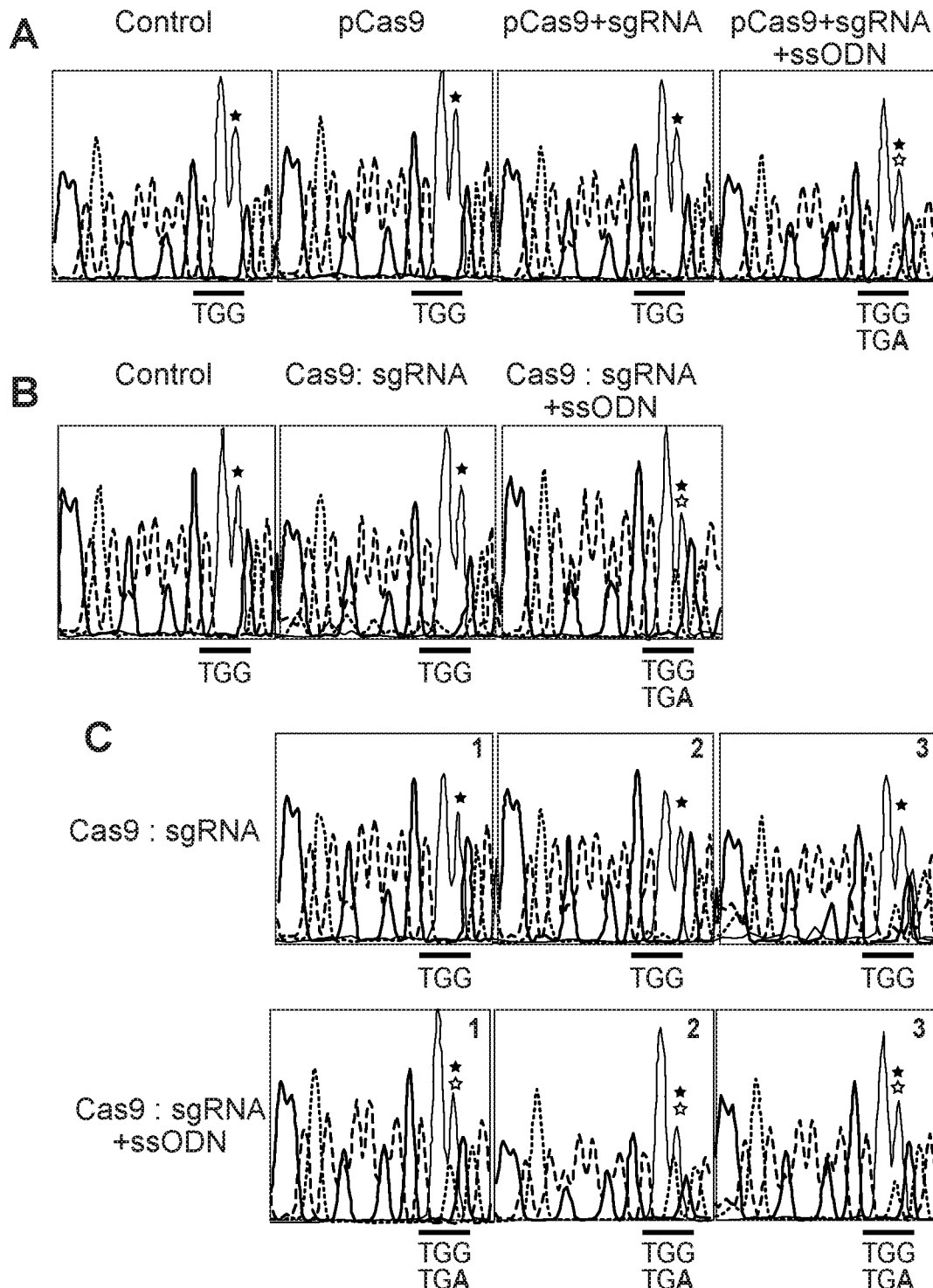


Figure 8. Strategy of single nucleotide mutation in wDi using homology-directed repair (HDR) with CRISPR/Cas9 system. (A) Nucleotide sequences of wild-type wDi *orf7* targeted region, the HDR donor DNA (ssODN) and the resulting mutated wDi *orf7*. The nucleotide targeted for exchange is highlighted in blue. **(B)** Schematic diagram outlining the steps used to transform wDi with either the protein or plasmid base systems in combination with ssODN. Grey circles represent S2 cells, yellow circles represent wDi, and red circles represent transformed wDi cells.

Figure 9. Confirmed point mutation in wDi *orf7*. wDi cells were transfected with CRISPR/Cas9 components followed by a 48 hours incubation period. DNA was isolated, the targeted region was amplified and subjected to Sanger sequencing. (A) Depicts experiments conducted with the plasmid base system. (B-C) Depicts experiments conducted with the protein base system; and in (C), 1-3 are technical replicates.



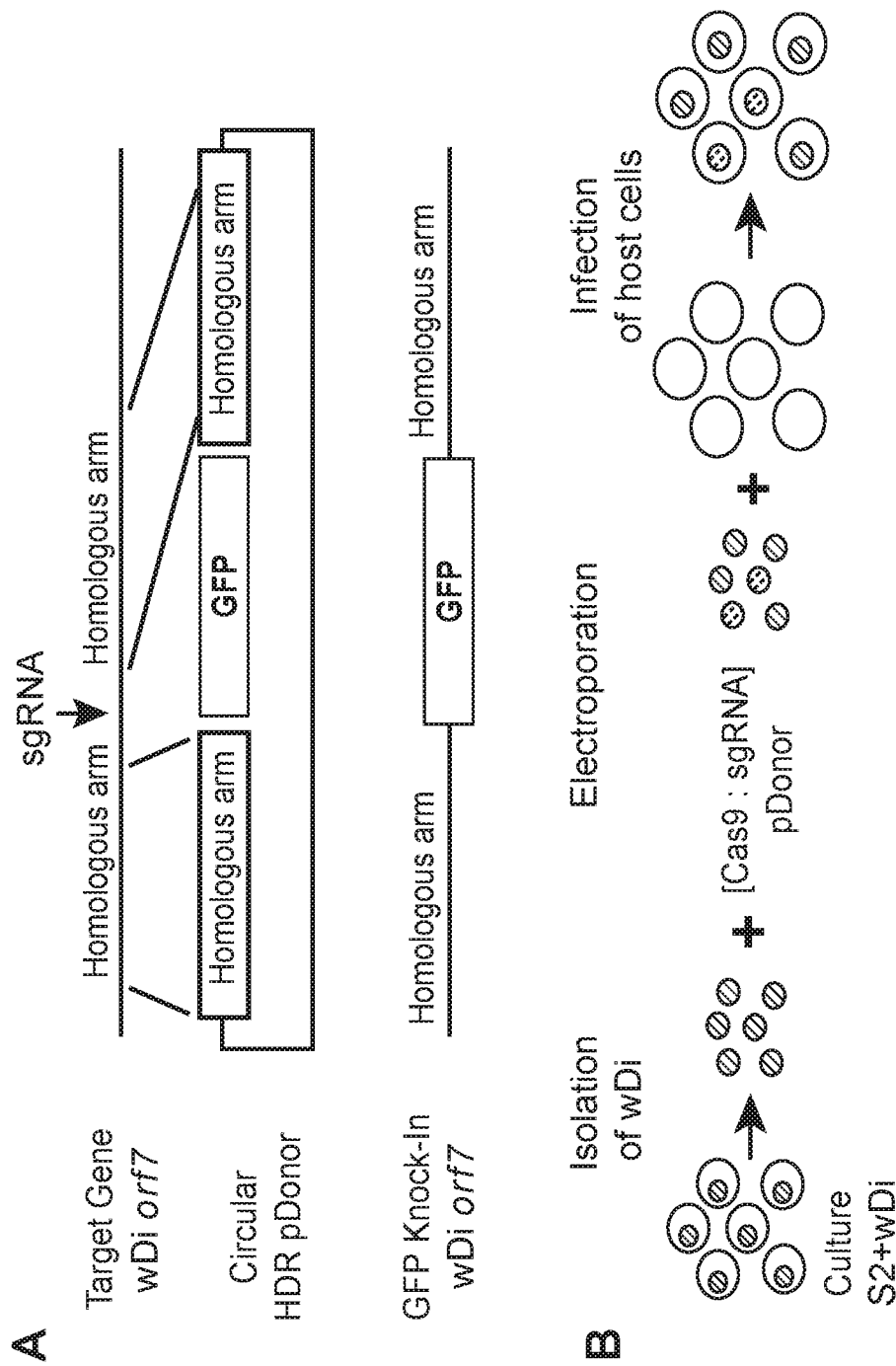
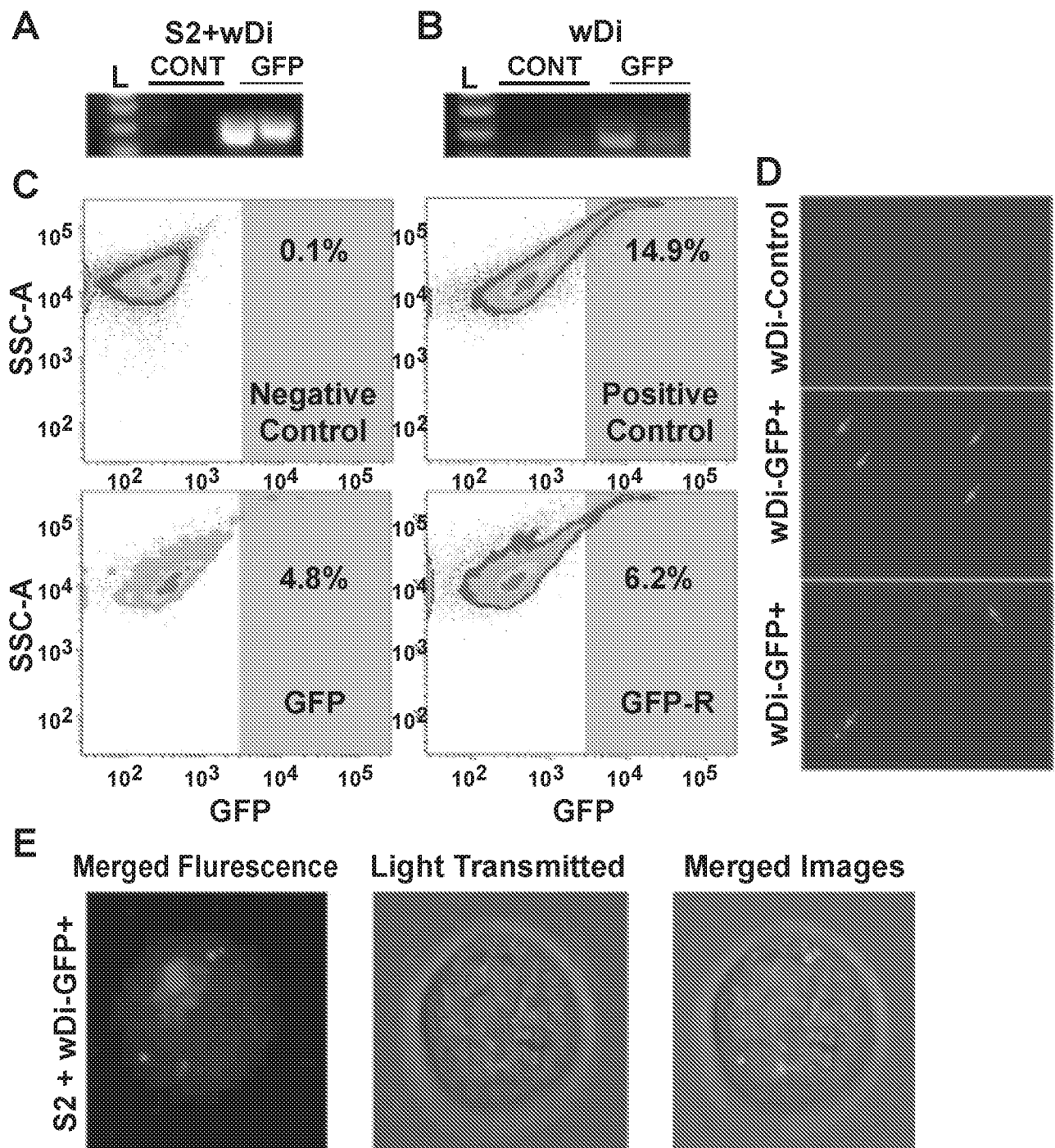


Figure 10. Approach for gene insertion using homology-directed repair with CRISPR/Cas9 system in wDi. (A) Nucleotide sequences of wild-type wDi *orf7* targeted region, the homology-directed repair (HDR) plasmid donor DNA (pDonor) and the resulting incorporation of the transgene in wDi. (B) Schematic diagram outlining the steps used to transform wDi with the protein base systems. Grey circles represent S2 cells, yellow circles represent wDi, and green circles represent transformed wDi cells.

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Figure 11. Knock-in of GFP in wDi by HDR with CRISPR/Cas9 technology. (A & B) PCR-based genotyping using primer to confirmed integration of GFP in wDi cells. Electroporated with no pDonor wDi cells were used as negative controls (CONT). (C) Using FACS, transformed wDi cells were enriched on the basis of GFP intensity (x-axis) after 2d of electroporation. wDi^{+dsRNAFlu} cells were used as positive controls. Y-axis is the side scatter area (SSC-A). Samples with in the grey area account for GFP+ cells. (D) Wide-field fluorescence microscopy images of live sorted wDi^{+GFP} cells. (E) Confocal miscopy of live S2+wDi^{+GFP-R} cells; samples were stained with Hoechst 33342 (blue).



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Figure 12. Insertion of *toxin* genes in wDi by HDR with CRISPR/Cas9 system. (A) Strategy for *toxin* gene insertion. Electroporation of wDi were conducted with different pDonors; pCry3Aa, pCry3Aa-R, pCyt2Ca1, pCyt2Ca1-R, pGNA3, pGNA3-R. (B-G) DNA from transformed and non-transformed negative control (NC) cells were used for PCR analysis. Two biological replicates of each sample are shown. Different PCR primers used in the study: Cry3Aa-f, Cry3Aa-r for *Cry3Aa* (B), Cry3Aa-R-f, Cry3Aa-R-r for *Cry3Aa-R* (C), Cyt2Ca1-f, Cyt2Ca1-r for *Cyt2Ca1* (D), Cyt2Ca1-R-f, Cyt2Ca1-R-r for *Cyt2Ca1-R* (E), GNA3-f, GNA3-r for *GNA3* (F), and GNA3-R-f, GNA3-R-r for *GNA3-R* (G).

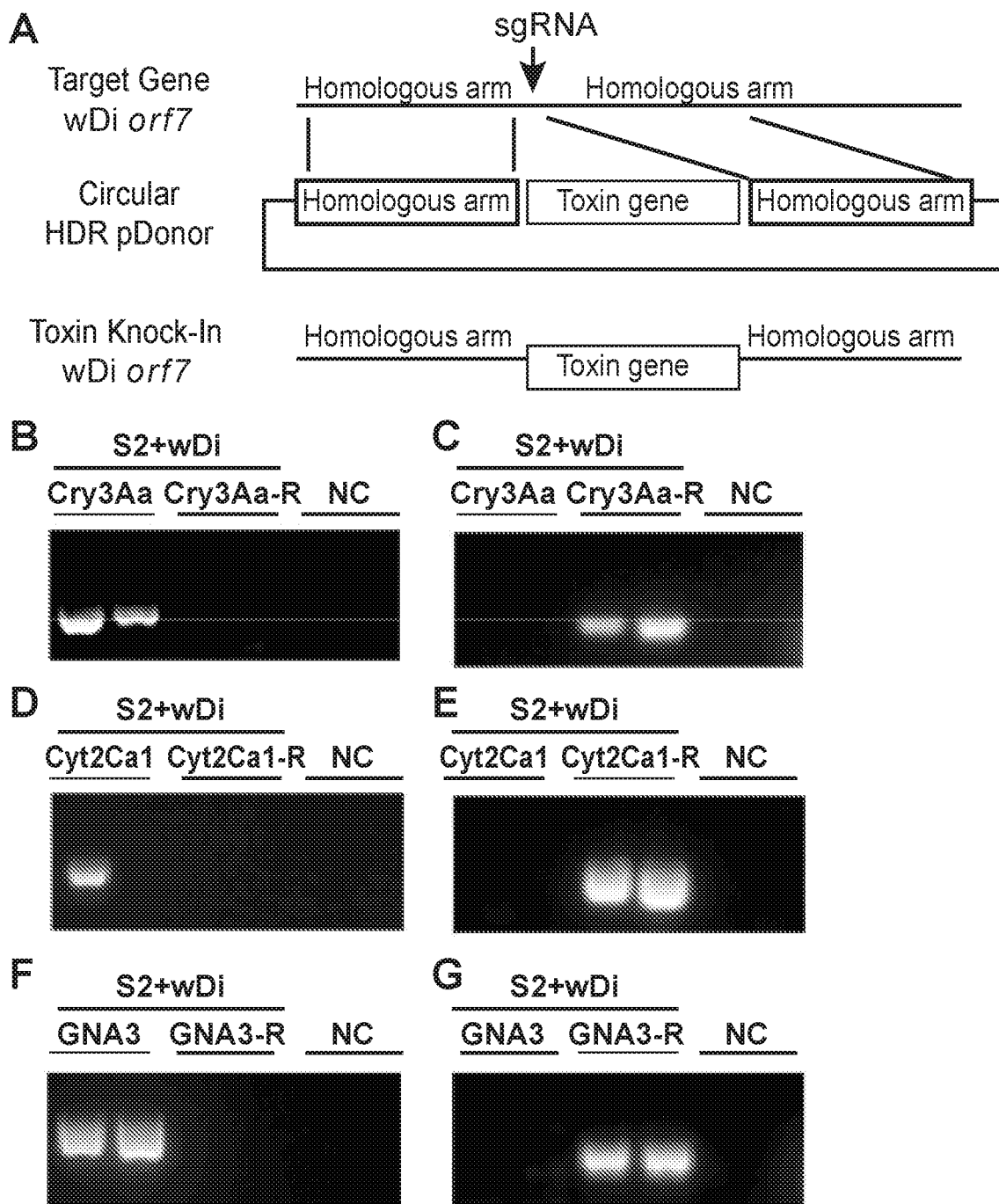
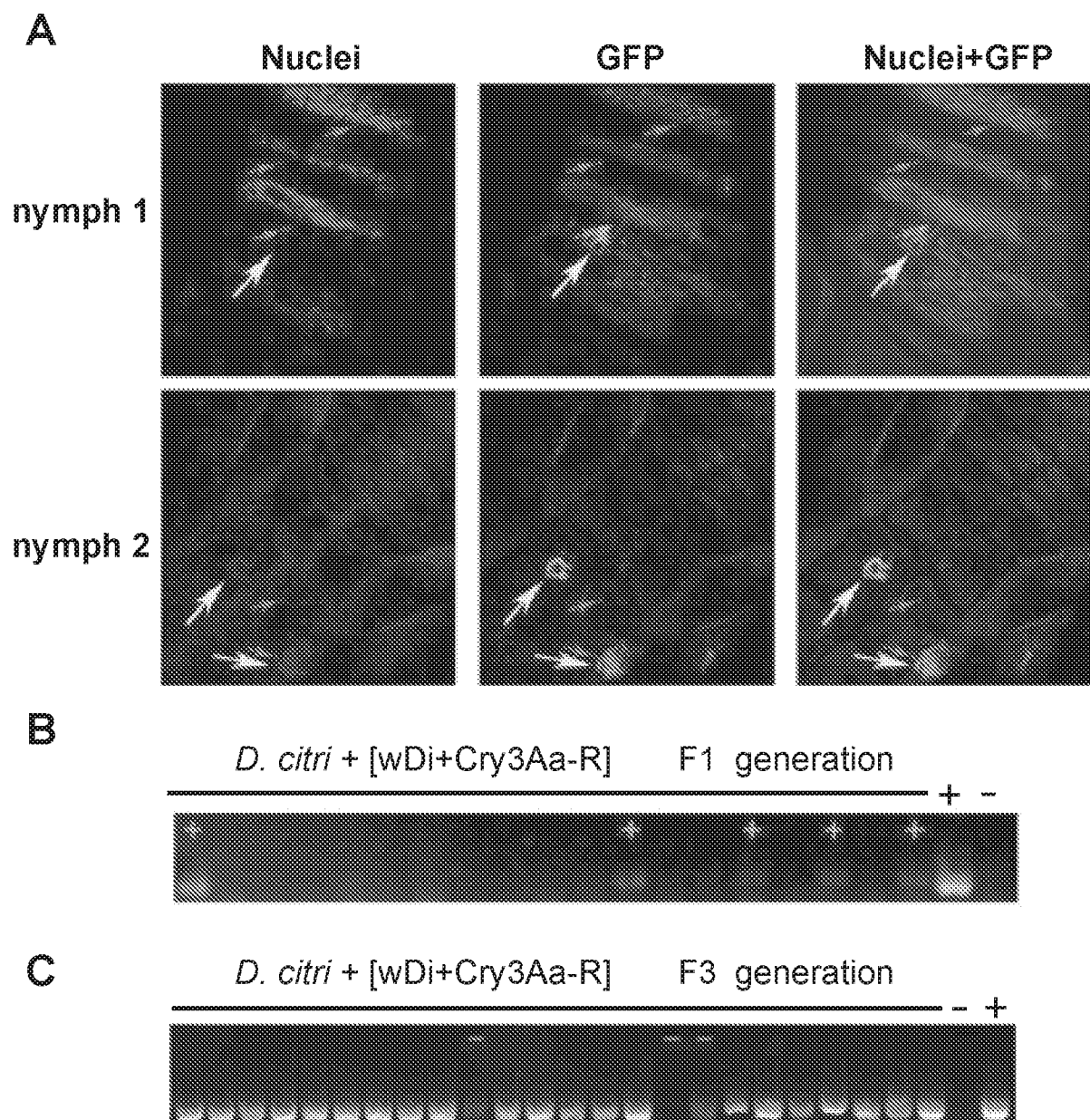


Figure 13. Vertical transmission of transformed wDi⁺ in *D. citri*. (A) *In vivo* visualization of *D. citri* nymphs. Nymph one was injected in the abdominal segment and nymph two was injected twice in the mesothorax with stained Hoechst 33342 (nuclei) wDi⁺GFP-R. Yellow arrows indicate injection locations. Red arrows indicate exogenous transformed cells that are positive for both nuclei stain and GFP signal. (B & C) Parental *D. citri* were injected with wDi⁺Cry3Aa-R, their offspring F1 (B) & F3 (C) were individually processed for PCR analysis. (B) Detection of wDi⁺Cry3Aa-R in *D. citri* adults are labeled with green positive symbol and (C) non-wDi⁺Cry3Aa-R samples were labeled with red negative symbols. Positive and negative DNA controls were used; (+) pDonor DNA and (-) wild-type *D. citri* DNA.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/61094

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12R 1/01; C12N 15/74, 15/86; A01K 67/033 (2020.01)

CPC - C12N 15/74, 15/86, 15/8509; A01K 67/0339

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	(THIEM, S) A Genetic Manipulation System For Wolbachia In Mosquitoes. Grant proposal [online]. USDA Research, Education, & Economics Information System. 30 September 2017 [retrieved on 9 January 2020]. Retrieved from the Internet: <URL: https://rees.usda.gov/web/crisprojectpages/1003653-a-genetic-manipulation-system-for-wolbachia-in-mosquitoes.html >; page 1, "Non Technical Summary" paragraph; page 2, "Goals/Objectives" and "Project Methods" paragraphs; page 3, "Impacts" paragraph; page 4, "Impacts" paragraph; page 5, "Outputs" paragraph;	1-4, 7, 9 ----- 5-6, 8, 10-14
Y	(KENT, BN et al.) Phage WO of Wolbachia: lambda of the endosymbiont world. Trends in Microbiology. April 2010, Epub 18 January 2010, Vol. 18, No. 4; pages 173-181; page 2, 2nd paragraph; page 7, 3rd paragraph; DOI: 10.1016/j.tim.2009.12.011	5-6
Y	WO 2017/181043 A1 (VANDERBILT UNIVERSITY) 19 October 2017; page 19, lines 4-6; page 21, line 27; page 24, lines 7-11; page 50, lines 12-24	8, 12-14
Y	(SALLES, JF et al.) Use Of Endophytic Diazotrophic Bacteria As A Vector To Express The cry3A Gene From Bacillus Thuringiensis. Brazilian Journal of Microbiology. September 2000, Vol. 31, No. 3; pages 154-160; abstract; page 160, 1st column, 3rd paragraph; DOI: 10.1590/S1517-83822000000300001	10-11

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

22 January 2020 (22.01.2020)

Date of mailing of the international search report

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