(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 June 2024 (27.06.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/137926 A1

(51) International Patent Classification:
C12N 15/11 (2006.01) *C12N 15/63* (2006.01)
C12N 15/09 (2006.01) *C12N 15/87* (2006.01)

(21) International Application Number:
PCT/US2023/085330

(22) International Filing Date:
21 December 2023 (21.12.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/477,043 23 December 2022 (23.12.2022) US

(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US).

(72) Inventors: **ELZINGA, Dezi A.**; c/o The Regents of the University of California, 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US). **WALLING, Linda L.**; c/o The Regents of the University of California, 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US). **ATKINSON, Peter W.**; c/o The Regents of the University of California, 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US). **VALENZUELA, Alex H.**; c/o The Regents of the University of California, 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US).

(74) Agent: **YAO, Zhengzheng** et al.; Kilpatrick Townsend & Stockton LLP, Mailstop: IP Docketing - 22, 1100 Peachtree Street, Suite 2800, Atlanta, Georgia 30309 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) Title: PLANT-MEDIATED INSECT GENE EDITING AND USE OF THE METHODS FOR INSECT GENETIC CONTROL

(57) Abstract: The present disclosure provides methods and compositions for introducing macromolecules such as CRISPR system components into the eggs of insects, wherein the eggs are present on a leaf and contact the leaf through a pedicel or analogous structure.

WO 2024/137926 A1

PLANT-MEDIATED INSECT GENE EDITING AND USE OF THE METHODS FOR INSECT GENETIC CONTROL

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 63/477,043, filed December 23, 2022, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under DARPA contract D19AC00019 awarded by the U.S. Department of Defense. The Government has certain rights in the invention.

BACKGROUND

[0003] Although sap-feeding insects such as whiteflies are major pests of US and international agriculture, few or no genetic techniques have been applied to them as mechanisms of control. However, chemical insecticides present numerous problems, including that they quickly select for insects that have developed resistance to them, that they are not target-specific and can harm beneficial insect species, and that they are toxic to vertebrates. Biological control approaches are also problematic, as they can take many years to develop, they often involve the introduction of non-native species, and they are usually not target population-specific and so can threaten native populations of the pest species in regions where the insect is not a pest.

[0004] For these reasons, genetic-based approaches, such as CRISPR/Cas9 editing or RNA interference, for the control of sap-feeding insects are urgently needed. However, the delivery of the macromolecular components into organisms of such species is a limiting factor in the extension and effectiveness of this genetic technology. Currently, the standard approach is to directly inject the insect egg after it has been laid and attached to the plant; alternatively, the

egg can be removed from the plant, secured to a glass microscope slide, and injected. Typically, both approaches result in significant mortality to the egg, especially when the egg is small. Recently, an alternative technique has been developed for a range of insect species and applied to one sap-feeding insect, the whitefly *Bemisia tabaci*. This method injects the editing macromolecules into the ovaries of the mother and edited mutants are recovered at low frequency (Heu et al. 2020). This technology requires the use of a carrier molecule derived from the target species (the vitellogenin protein) to deliver the Cas9 machinery. However, this technology has yet to generate gain-of-function mutations in any species. Also, similar to egg (embryo) injections, there is significant mortality to the injected adult. Other insect control methods have been deployed in transgenic plants, but these rely on expression of compounds that are consumed by insects via phloem-sap or by maceration and consumption of leaf tissue. While these methods can control feeding adults and their immature stages, they do not impact the developing embryo.

[0005] There is therefore a need for widely applicable, efficient, economical, and lower technology methods to deliver macromolecules such as those of the CRISPR/Cas machinery into the eggs of sap-feeding insects. The present disclosure satisfies this need and provides other advantages as well.

BRIEF SUMMARY

[0006] In one aspect, the present disclosure provides a method of introducing a macromolecule into an egg of a sap-feeding insect, the method comprising providing a leaf of a host plant upon which the egg is present, and introducing the macromolecule into the leaf in the proximity of the egg, wherein the egg comprises and/or is anchored by a pedicel and wherein the macromolecule is transferred from the leaf into the egg through the egg's pedicel.

[0007] In some embodiments, the insect is a whitefly. In some embodiments, the species of the whitefly is *Bemisia tabaci*. In some embodiments, the leaf upon which the egg is present is a rooted leaf disc. In some embodiments, the plant is a Brassica plant. In some embodiments, the macromolecule is introduced into the leaf by *in planta* injection. In some embodiments, the macromolecule is introduced into the leaf by vacuum infiltration. In some embodiments, the macromolecule is a protein. In some embodiments, the protein is Cas9. In some embodiments, protein is subsequently detectable in the egg. In some embodiments, the

introduction of the macromolecule into the leaf results in a phenotypic change in a nymph or adult developing from the egg.

[0008] In some embodiments, the macromolecule is a nucleic acid. In some embodiments, the nucleic acid is RNA. In some embodiments, the RNA is selected from the group consisting of a guide RNA, double-stranded RNA (dsRNA), miRNA, siRNA, and shRNA. In some embodiments, the introduction of the RNA into the leaf results in an alteration in expression of a target gene and/or a phenotypic change in a nymph or adult developing from the egg. In some embodiments, the target gene is a gene involved in an essential whitefly embryonic function. In certain embodiments, the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function. Examples of genes involved in segmentation include, but are not limited to, *fushi tarazu* and *even-skipped*. Examples of genes involved in neural development and/or nervous system function include, but are not limited to, *scute* and *Notch*. Examples of genes involved in organogenesis include, but are not limited to, *Tinman* and *Pannier*. Examples of genes involved in endocrine system function include, but are not limited to, *Notch* and *Hey*.

[0009] In some embodiments, the nucleic acid is DNA. In some embodiments, the DNA is a plasmid. In some embodiments, the plasmid comprises a coding sequence for Cas9 protein and/or a guide RNA, operably linked to a promoter. In some embodiments, the plasmid comprises a coding sequence for a dsRNA, miRNA, siRNA, or shRNA, operably linked to a promoter. It will be appreciated that, as used herein, "coding" or "encoding" with respect to a DNA molecule (e.g., a DNA plasmid) can refer either to the transcription of the DNA giving rise to an mRNA that is then translated into a polypeptide product (e.g., Cas9 protein), or the transcription of the DNA to give rise, directly or indirectly, to an RNA product such as dsRNA, miRNA, siRNA, shRNA, etc. In some embodiments, the introduction of the DNA into the leaf results in an alteration in expression of a target gene and/or a phenotypic change in a nymph or adult developing from the egg, and/or the detectable expression of a protein or RNA encoded by the plasmid. In some embodiments, the target gene is a gene involved in an essential whitefly embryonic function. In certain embodiments, the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function. Examples of genes involved in segmentation include, but are not limited to, *fushi tarazu* and *even-skipped*. Examples of genes involved in neural development and/or nervous system function include, but are not limited to, *scute* and *Notch*. Examples of genes involved in organogenesis include, but are not limited to, *Tinman* and *Pannier*. Examples of genes

involved in endocrine system function include, but are not limited to, *Notch* and *Hey*. In some embodiments, the macromolecule is introduced into the insect by genetically modifying the host plant, such that the macromolecule is expressed in the host plant leaf and transferred to the egg through the pedicel. In some embodiments, the macromolecule is a Cas9 protein and/or a guide RNA. In some embodiments, the macromolecule is an inhibitory RNA molecule, e.g., a molecule selected from the group consisting of dsRNA, miRNA, siRNA, and shRNA.

[0010] In another aspect, the present disclosure provides a method of manipulating the genome of a sap-feeding insect, comprising providing a leaf of a host plant upon which an egg of the insect is present and introducing a macromolecule into the leaf in the proximity of the egg, wherein the egg comprises and/or is anchored by a pedicel, wherein the macromolecule is transferred from the leaf into the egg through the egg's pedicel, wherein the macromolecule is or encodes a component of a CRISPR-Cas system targeting a sequence within the insect genome, and wherein the CRISPR-Cas system introduces a modification of the insect genome at or in proximity to the targeted sequence.

[0011] In some embodiments, the macromolecule is a Cas9 protein and/or a guide RNA, or a plasmid encoding a Cas9 protein and/or a guide RNA, wherein the guide RNA targets the sequence within the insect genome. In some embodiments, the modification is a deletion, insertion, or nucleotide substitution at the targeted sequence. In some embodiments, the modification alters the expression of a gene in the sap-feeding insect. In some embodiments, the gene is a gene involved in an essential whitefly embryonic function. In certain embodiments, the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function. Examples of genes involved in segmentation include, but are not limited to, *fushi tarazu* and *even-skipped*. Examples of genes involved in neural development and/or nervous system function include, but are not limited to, *scute* and *Notch*. Examples of genes involved in organogenesis include, but are not limited to, *Tinman* and *Pannier*. Examples of genes involved in endocrine system function include, but are not limited to, *Notch* and *Hey*.

[0012] In some embodiments, the modification results in a phenotypic change in a nymph or adult developing from the egg. In some embodiments, the sap-feeding insect is a whitefly. In some embodiments, the whitefly is *Bemisia tabaci*. In some embodiments, the leaf is a rooted leaf disc. In some embodiments, the plant is a Brassica plant. In some embodiments,

the macromolecule is introduced into the leaf by *in planta* injection. In some embodiments, the macromolecule is introduced into the leaf by vacuum infiltration. In some embodiments, the macromolecule is introduced into the insect by genetically modifying the host plant, such that the macromolecule is expressed in the host plant leaf and transferred to the egg through the pedicel.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIGS. 1A-1B. The plant-pedicel connection- a macromolecule superhighway. We have developed two technologically simple methods for indirectly introducing macromolecules (DNA, RNA and protein) into hemipteran eggs. We have extensively tested with Cas9 protein, *white2* gRNAs and plasmids that produce a Cas9-mCherry fusion protein or *white2* gRNAs. FIG. 1A. Vacuum infiltration of macromolecules. FIG. 1B. *In planta* injection of macromolecules. Whitefly egg (embryo is within the eggshell; tan) and pedicel are inserted into the leaf. Rooted leaf disc with the adaxial (upper) side in contact with the phytoagar plate. Macromolecules (navy blue) are delivered by infiltration or *in planta* injection of the abaxial (lower) side of the leaf disc.

[0014] FIGS. 2A-2J. Summary of data supporting the plant-pedicel connection. FIG. 2A: Vacuum-infiltration of sterile red dye and eGFP plasmid (*3xP3:eGFP*) into leaf discs. Red dye and plasmid are taken up by the egg pedicel, enter the embryo, and the *3xP3:eGFP* plasmid was expressed as evidenced by egg fluorescence. FIG. 2B: *In planta* injection of phenol red indicates it is taken up by the embryo. FIG. 2C: *In planta* injection of *PUbA:mCherry* plasmids. DNAs were extracted from a pool of eggs and transformed into *E. coli*. PCR confirmed the presence of the plasmid in the 8 colonies chosen for analysis. FIG. 2D: *In planta* injection of Cas9 protein and immunoblot analysis of protein extracts from eggs indicates the intact Cas9 protein entered the embryo. FIG. 2E: *In planta* injection of *exu:Cas9-T2A-mCherry*. Confocal microscopy of eggs after 72 hr indicates the plasmid is transcribed and RNA translated as mCherry protein is visualized in embryos. FIG. 2F: Vacuum infiltration of *exu:Cas9-T2A-mCherry*. Confocal microscopy of eggs after 48 hr indicates the plasmid is transcribed and RNA translated as mCherry protein is visualized in embryos. This image was enhanced using the Red hot table in Image J. FIG. 2G. Leaf disc injection scheme for Cas9 protein and gRNAs. FIG. 2H: Left eye and right eye close ups and wing defects in mutant are shown. Areas of mosaicism in eyes are indicated by orange arrows. FIG. 2I: T7 endonuclease assay of mosaic (w2) and wild-type whiteflies (lanes 1-7)

showing the presence of a heteroduplex in the w2 mosaic. FIG. 2J: *white* gene sequences from the w2 mosaic mutant showing CRISPR/Cas9-mediated deletions at the target site. Mutant #18 (not shown) has a 2-bp substitution and a 4-bp deletion.

[0015] FIG. 3. Macromolecule delivery into plant cells revealed the plant cell-egg pedicel-embryo communication channel.

[0016] FIG. 4. Transgenic plant delivery of dsRNAs or Cas endonuclease and sgRNAs to target whitefly genes. The target whitefly genes are essential for embryo development. By silencing these genes with dsRNAs or inactivating these genes using CRISPR/Cas editing, embryos cannot develop and eggs will not hatch and/or insects cannot develop into adults.

[0017] FIG. 5. The principle of hairpin RNA (hpRNA) for whitefly control (Strategy 1). Using a strong constitutive promoter (35S) or an epidermal cell-specific promoter (not shown), a hpRNA gene is transcribed within the plant nucleus. This RNA has sense and antisense target sequences (dark blue arrows) that pair to form a hairpin-like structure. The *pdk* intron forms the loop of the hairpin (light blue). The hpRNA can be taken up directly or via vesicles into the whitefly pedicel; within the embryo, the hpRNA can be processed to form sRNAs for whitefly gene silencing. Alternatively, the processing of hpRNAs can occur within the plant cell and delivered directly or via vesicles to the whitefly embryo. Within the embryo the sRNAs will interfere with target gene RNA stability or translation. In this figure as single hpRNA is illustrated. We will express four or more hpRNAs in each transgenic plant for Strategy 1.

[0018] FIG. 6. The hpRNA cassettes for whitefly control. We will make eight hpRNA chimeric genes that target essential whitefly embryonic functions such as segmentation (gap genes), neural development, organogenesis, and endocrine system. Four hpRNA constructs will be expressed using strong constitutive or epidermal cell specific promoters. Cassette 1 and Cassette 2 will use different essential whitefly genes. Transgenic plant 1 and transgenic plant 2 can be crossed. Homozygous transgenic plants will express eight different hpRNA genes for maximum control. Different selectable markers will be used for cassette 1 and 2. Different plant promoters will be used to avoid transgene silencing.

[0019] FIG. 7. Transgenic plants expressing Cas9 and target gene sgRNAs to abolish embryo viability.

[0020] FIG. 8. Engineering resistance to whiteflies with CRISPR/Cas9. Transgenic plants expressing multiple U6:gRNA genes (see Strategy 1 in Example 5 for gene identities) and 35S:Cas9-cytosol (not shown) or 35S:Cas9-Golgi will be made. These Cas9 proteins lack the nuclear localization sequence (NLS). We postulate that Cas9 and gRNAs will be packaged in vesicles within the epidermal cell and fused to the pedicel in the epidermal cell or within the apoplast. Upon uptake into the whitefly embryo, the Cas9 and gRNAs will edit the four or more target genes, rendering the embryo inviable.

DETAILED DESCRIPTION

[0021] The present disclosure is based on the surprising discovery that the pedicel of the insect egg, which anchors the egg to the surface of the leaf and serves as a conduit for the transport of water to the egg, can also transport macromolecules from the plant to the developing insect embryo within the eggshell. These macromolecules can be, e.g., DNA, RNA or protein. The herein-described methods based on this discovery can be used, for example, to indirectly introduce the protein and RNA components of CRISPR genetic technology to the insect egg via the plant cell. Macromolecules can be introduced, e.g., by injection or by vacuum infiltration into the leaf in close proximity to a developing egg. Once transported into the insect egg through the pedicel, these components reach the embryo and result, e.g., in targeted CRISPR/Cas9-mediated mutagenesis of the insect genome and resulting changes in phenotype.

[0022] The present methods can also be used to introduce plasmid DNA into the insect egg. Once within the embryo, the genes located on the plasmids are transcribed and translated to produce, e.g., the proteins and RNAs required for CRISPR genome editing. These technologies provide a very simple and efficient platform for the genetic analysis of these agriculturally important insect pest species. The present methods can be used, e.g., to create loss-of-function mutants in whiteflies using *in planta* injection or vacuum infiltration delivery methods for, e.g., (1) Cas9 protein and gRNAs, (2) plasmids expressing Cas9 protein in the embryo plus gRNAs, or (3) plasmids that express both Cas9 protein and gRNAs in the embryo. The ability to deliver plasmids to the embryo via the plant:pedicel continuum also provides a simple and effective way of making gain-of-function mutants via homology-directed repair following CRISPR-mediated mutagenesis.

[0023] The present methods also provide new approaches for the control of insect pests of plants that have embryos that imbibe water and other materials from leaves of their host

plants. For example, the discovery of the power of the plant:pedicel continuum opens the opportunity for engineering host plants to produce macromolecules *in planta* for delivery to the embryo; macromolecules can be taken up by the embryo that, e.g., interfere with essential insect functions. For example, a plant that produces Cas9 protein and gRNAs specific for the insect pest could deliver these macromolecules to the insect egg via exosomes. Their activity within the embryo could result in mutations in essential genes (e.g., genes involved in essential whitefly embryonic functions, such as segmentation (gap genes), neural development, organogenesis, and endocrine system function) and thereby kill the developing embryo before it can hatch as a sap-feeding nymph. This strategy is a powerful method of insect control, as it can prevent insect predation, any damage to the plant, and pest insect population expansion.

[0024] An alternative and complementary strategy described herein is to engineer the plant to produce double-stranded small-interfering RNAs that are taken up by the insect egg with the same lethal or debilitating outcome for the insect pest. Preventing the rapid population expansions that occur when pests infest agricultural or horticultural crops will mitigate the need for the use of chemical insecticides and promote "green control methods" such as biocontrol.

Definitions

[0025] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0026] The terms "a," "an," or "the" as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the agent" includes reference to one or more agents known to those skilled in the art, and so forth.

[0027] The terms "about" and "approximately" as used herein shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Any reference to "about X" specifically indicates at least the values X, 0.8X, 0.81X, 0.82X, 0.83X, 0.84X, 0.85X, 0.86X, 0.87X, 0.88X, 0.89X, 0.9X, 0.91X, 0.92X, 0.93X, 0.94X,

0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, 1.05X, 1.06X, 1.07X, 1.08X, 1.09X, 1.1X, 1.11X, 1.12X, 1.13X, 1.14X, 1.15X, 1.16X, 1.17X, 1.18X, 1.19X, and 1.2X. Thus, "about X" is intended to teach and provide written description support for a claim limitation of, *e.g.*, "0.98X."

[0028] The "CRISPR-Cas" system refers to a class of bacterial systems for defense against foreign nucleic acids. CRISPR-Cas systems are found in a wide range of eubacterial and archaeal organisms. CRISPR-Cas systems fall into two classes with six types, I, II, III, IV, V, and VI as well as many sub-types, with Class 1 including types I and III CRISPR systems, and Class 2 including types II, IV, V and VI; Class 1 subtypes include subtypes I-A to I-F, for example. See, *e.g.*, Fonfara et al., *Nature* 532, 7600 (2016); Zetsche et al., *Cell* 163, 759-771 (2015); Adli et al. (2018). Endogenous CRISPR-Cas systems include a CRISPR locus containing repeat clusters separated by non-repeating spacer sequences that correspond to sequences from viruses and other mobile genetic elements, and Cas proteins that carry out multiple functions including spacer acquisition, RNA processing from the CRISPR locus, target identification, and cleavage. In class 1 systems these activities are mediated by multiple Cas proteins, with Cas3 providing the endonuclease activity, whereas in class 2 systems they are all carried out by a single Cas, Cas9.

[0029] Endogenous systems function with two RNAs transcribed from the CRISPR locus: crRNA, which includes the spacer sequences and which determines the target specificity of the system, and the transactivating tracrRNA. Exogenous systems, however, can function which a single chimeric guide RNA that incorporates both the crRNA and tracrRNA components. In addition, modified systems have been developed with entirely or partially catalytically inactive Cas proteins that are still capable of, e.g., specifically binding to nucleic acid targets as directed by the guide RNA, but which lack endonuclease activity entirely, or which only cleave a single strand, and which are thus useful for, e.g., nucleic acid labeling purposes or for enhanced targeting specificity. Any of these endogenous or exogenous CRISPR-Cas system, of any class, type, or subtype, or with any type of modification, can be utilized in the present methods. In particular, "Cas" proteins can be any member of the Cas protein family, including, *inter alia*, Cas3, Cas5, Cas6, Cas7, Cas8, Cas9, Cas10, Cas12 (including Cas12a, or Cpf1), Cas13, Cse1, Cse2, Csy1, Csy2, Csy3, GSU0054, Csm2, Cmr5, Csx11, Csx10, Csf1, Csn2, Cas4, C2c1, C2c3, C2c2, and others. In particular embodiments, Cas proteins with endonuclease activity are used, e.g., Cas3, Cas9, or Cas12a (Cpf1).

[0030] The term "nucleic acid sequence encoding a polypeptide" refers to a segment of DNA, which in some embodiments may be a gene or a portion thereof, that is involved in producing a polypeptide chain (e.g., an RNA-guided nuclease such as Cas9). A gene will generally include regions preceding and following the coding region (5' and 3' flanking regions and/or 5' and 3'untranslated regions) involved in the transcription/translation of the gene product and the regulation of the transcription/translation. A gene can also include intervening sequences (introns) between individual coding segments (exons, which can also include 5' and 3' untranslated regions, or UTRs). Leaders (5' flanking regions and/or UTRs), trailers (3' flanking regions and/or UTRs), and introns can include regulatory elements that are necessary during the transcription and the translation of a gene (e.g., promoters, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions, etc.). A "gene product" can refer to either mRNA or other RNA (e.g. sgRNA) or protein expressed from a particular gene.

[0031] The terms "expression" and "expressed" refer to the production of a transcriptional and/or translational product, e.g., of a nucleic acid sequence encoding a protein (e.g., a guide RNA or RNA-guided nuclease). In some embodiments, the term refers to the production of a transcriptional and/or translational product encoded by a gene (e.g., a gene encoding a protein) or a portion thereof. The level of expression of a DNA molecule in a cell may be assessed on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of protein encoded by that DNA produced by the cell.

[0032] The term "recombinant" when used with reference, e.g., to a polynucleotide, protein, vector, or cell, indicates that the polynucleotide, protein, vector, or cell has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. For example, recombinant polynucleotides contain nucleic acid sequences that are not found within the native (non-recombinant) form of the polynucleotide.

[0033] As used herein, the terms "polynucleotide," "nucleic acid," and "nucleotide," refer to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof. The term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, and DNA-RNA hybrids, as well as other polymers comprising purine and/or pyrimidine bases or other natural, chemically modified, biochemically modified, non-natural,

synthetic, or derivatized nucleotide bases. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), homologs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)).

[0034] The terms "vector" and "expression vector" refer to a nucleic acid construct, e.g., plasmid or viral vector, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid sequence (*e.g.*, a guide RNA and/or RNA-guided nuclease) in a cell, e.g., an embryonic cell. In some embodiments, a vector includes a polynucleotide to be transcribed, operably linked to a promoter, e.g., a constitutive or inducible promoter. Other elements that may be present in a vector include those that enhance transcription (e.g., enhancers), those that terminate transcription (e.g., terminators), those that confer certain binding affinity or antigenicity to a protein (e.g., recombinant protein) produced from the vector, and those that enable replication of the vector and its packaging (e.g., into a viral particle). In some embodiments, the vector is a viral vector (i.e., a viral genome or a portion thereof).

[0035] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid polymers in which one or more amino acid residues are an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

Insects and host plants

[0036] The present methods can be used with any insect species that lays eggs on host plant leaves and that extracts water from the leaf through a pedicel or analogous structure such as a hydropyle. In some embodiments, the insect is a hemipteran. In some embodiments, the

hemipteran is of the suborder Sternorrhyncha. In particular embodiments, the insect is a whitefly, or member of the Aleyrodoidea superfamily. Any species within any of these taxa can be used, including any whitefly species or species complex, e.g., *Aleurocanthus woglumi*, *Aleyrodes proletella*, *Bemisia tabaci*, and *Trialeurodes vaporariorum*. The present disclosure is based on the surprising discovery that, when such eggs are present on a leaf of a host plant, with the pedicel of the egg contacting the leaf to obtain moisture for the embryo, macromolecules present in the leaf in the proximity of the egg can be taken up through the pedicel (or analogous structure) and reach the embryo. The present methods can be used with eggs that have pedicels that contact the leaves through the stoma, as well as pedicels that contact the leaves outside of the stoma.

[0037] Other members of the Aleyrodidae family that can be used include, but are not limited to: members of the following genera: *Acanthobemisia* (*e.g., Acanthobemisia distylii* and *Acanthobemisia indicus*), *Aleurotrachelus socialis*, *Apobemisia* (*e.g., Apobemisia celti* and *Apobemisia kuwanai*), Asterobem*isia* (*e.g., Asterobemisia atraphaxius*, *Asterobemisia carpini*, *Asterobemisia curvata*, *Asterobemisia dentate*, *Asterobemisia lata*, *Asterobemisia obenbergeri*, *Asterobemisia paveli*, *Asterobemisia salicaria*, *Asterobemisia silvatica*, *Asterobemisia takahashii*, *Asterobemisia trifolii*, and *Asterobemisia yanagicola*), *Bemisaleyrodes* (*e.g.,* Bemisaleyrodes balachowskyi, *Bemisaleyrodes brideliae*, *Bemisaleyrodes grjebinei*, and *Bemisaleyrodes pauliani*), *Bemisia* (*e.g., Bemisia tabaci* species complex (comprising 35 species), *Bemisia afer*, *Bemisia alni*, *Bemisia antennata*, *Bemisia bambusae*, *Bemisia berbericola*, *Bemisia capitate*, *Bemisia caudasculptura*, *Bemisia centroamericana*, *Bemisia combreticula*, *Bemisia confuse*, and *Bemisia cordylinidis*), *Bemisiella* (*e.g., Bemisiella artemisiae* and *Bemisiella lespedezae*), *Heterobemisia* (*e.g., Heterobemisia alba*), *Metabemisia* (*e.g., Metabemisia distylii*, *Metabemisia filicis*, and *Metabemisia palawana*), and *Parabemisia* (*e.g., Parabemisia aceris*, *Parabemisia indica*, *Parabemisia jawani*, *Parabemisia lushanensis*, *Parabemisia maculate*, *Parabemisia myricae*, *Parabemisia myrmecophila*), and *Trialeurodes* (*e.g., Trialeurodes vaporarium, Trialeurodes viribalis*, etc.). Other sap-feeding insects including members of other genera within hemiptera, for example, aphids psyllids, sharpshooters, and leafhoppers. These species include the Glassy Winged Sharp Shooter (GWSS, *Homalodisca coagulata*), Asian Citrus Psyllid (ACP, *Diaporhorina citri* Kuwayama), the potato/tomato psyllid (*Bactericerca cockerelli* (Sulc)), Bagrada bug, the beet leafhopper (*Circulifer tenellus*), and any insect

species in which the egg is accessible and attached to the leaf surface through a pedicel or analogous structure.

Introduction of macromolecules into leaves

[0038] The present methods can be used to indirectly introduce any macromolecule or combination of macromolecules into a pedicel-containing egg present on a leaf of a host plant. In some embodiments, a "leaf" as used herein refers to a rooted leaf disc, i.e., a portion of a leaf that has been transferred to a phytoagar medium and allowed to grow roots. In some embodiments, the macromolecule is a protein, e.g., a Cas9 protein. In some embodiments, the macromolecule is a nucleic acid, e.g., a DNA vector encoding a protein and/or RNA molecule, or an RNA molecule such as a guide RNA (e.g., sgRNA), or double-stranded RNA (dsRNA). In some embodiments, a combination of macromolecules is introduced, e.g., a ribonucleoprotein (RNP) comprising a protein (e.g,. a Cas9 protein) complexed with a CRISPR guide RNA. In some embodiments, a combination of macromolecules in introduced comprising a protein (e.g., Cas9 protein), RNA (e.g., sgRNA), and DNA (e.g., homologous donor template for introducing gain of function mutations in a target gene). In some embodiments, the macromolecule is a DNA plasmid encoding one or more CRISPR element, e.g., Cas9 or a guide RNA.

[0039] In some embodiments, a protein and gRNA mix is used, e.g., in the form of an RNP. For example, a Cas9 protein and gRNA mix can be prepared in water to a final concentration of, e.g., 200 ng/μl of Cas9 protein and 300 ng/μl gRNA, and the gRNA is loaded ono the Cas9 nuclease by incubating the injection mix at, e.g., 37 °C for 5 min. In some embodiments, a plasmid and gRNA mix is used. For example, plasmids encoding Cas9 and a gRNA are used, e.g., by adding 1 volume of IDTE buffer (e.g., 10 mM Tris, 0.1 mM EDTA) to 1 volume of sterile water, with the Cas9 plasmid present, e.g., at a concentration of 25 ng/μl and the gRNA present, e.g., at a concentration of 300 ng/μl. In some embodiments, a plasmid mix is used. For example, to 1 volume of sterile water, 1 volume of IDTE buffer is added with plasmid DNA at a final concentration of 50 ng/μl; when multiple plasmids are used, each plasmid DNA can have a final concentration of 25 ng/μl.

[0040] In some embodiments, an RNA sequence is used to suppress or silence gene expression in an insect. For example, the ability to suppress gene function in a variety of organisms using double-stranded (ds) RNA is well known. RNAi (*e.g.*, siRNA, miRNA) appears to function by base-pairing to complementary RNA or DNA target sequences. When

bound to RNA, the inhibitory RNA molecules trigger either RNA cleavage or translational inhibition of the target sequence.

[0041] A short hairpin RNA or small hairpin RNA (shRNA) is an artificial RNA molecule with a hairpin turn that can be used to silence target gene expression *via* the small interfering RNA (siRNA) it produced in cells. Expression of shRNA in cells can be accomplished, e.g., by delivery of plasmids. Once the vector has integrated into the host genome, the shRNA is then transcribed in the nucleus typically by RNA polymerase II or RNA polymerase III. The resulting pre-shRNA is exported from the nucleus and then processed by the Dicer enzyme and loaded into the RNA-induced silencing complex (RISC). The sense strand is degraded by RISC and the antisense strand directs RISC to an mRNA that has a complementary sequence. An AGO protein in the RISC then cleaves the mRNA, or in some cases, represses translation of the mRNA, thus, leading to its destruction and an eventual reduction in the protein encoded by the mRNA. Thus, the shRNA leads to targeted gene silencing. shRNA is an advantageous mediator of siRNA in that it has relatively low rate of degradation and turnover.

[0042] MicroRNAs (miRNAs) are noncoding RNAs of about 19 to about 24 nucleotides in length that are processed from longer precursor transcripts that form stable hairpin structures. miRNAs base pair with complementary sequences within the mRNA transcript. As a result, the mRNA transcript may be silenced by one or more of the mechanisms such as cleavage of the mRNA strand, destabilization of the mRNA through shortening of its poly(A) tail, and decrease translation efficiency of the mRNA transcript into proteins by ribosomes. In some embodiments, miRNAs resemble the siRNAs of the shRNA pathway, except that miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins, which are also called pri-miRNA. Once transcribed as pri-miRNA, the hairpins are cleaved out of the primary transcript in the nucleus by an enzyme called Drosha in association with the RNA binding protein GRC8. The hairpins, or pre-miRNA, are then exported from the nucleus into the cytosol. In the cytosol, the loop of the hairpin is cleaved off by the Dicer enzyme. The resulting product is now a double-stranded RNA with overhangs at the 3' end, which is then incorporated into RISC. Once in the RISC, the second strand is discarded and the miRNA that is now in the RISC is a mature miRNA, which binds to mRNAs that have complementary sequences.

[0043] The macromolecules can be introduced into the leaves in any of multiple ways. In one aspect, the macromolecules are introduced into the egg by *in planta* injection or by vacuum infusion. For example, in particular embodiments, a rooted leaf disc is used, with the adaxial side in contact with the phytoagar plate, and the macromolecules delivered by infiltration or *in planta* injection of an abaxial side of the disc. Such methods are useful, e.g., for generating genetically modified insects, e.g., for the genetic analysis of pest species, e.g., by introducing genetic modifications, including gain-of-function (e.g., using a homologous template) or loss-of-function (e.g., using a homologous template or in the absence of a template) mutations, and analyzing the resulting phenotypes.

[0044] In some embodiments, Brassica leaf discs are used. Such discs can be used for either in planta injection or vacuum infiltration. They provide a small footprint rearing system that allows macromolecule delivery and the development of whiteflies from egg to adult. The preparation of use of such discs is described, e.g., in WO 2019/140010, the entire disclosure of which is herein incorporated by reference.

[0045] In some embodiments, leaf discs are prepared by excision from, e.g., 4-week old Brassica plants, and are placed with their abaxial side up on, e.g., phytoagar media (e.g., Gamborg's media with vitamins) with, e.g., 1 mg/L napthalene acetic acid (NAA). The leaf-disc plate may also be constructed using a sorghum leaf, a citrus leaf, a tomato leaf, or an eggplant leaf. The discs are then incubated for, e.g., 5-9 days, and allowed to form roots. Once roots have formed, the rooted discs are transferred to phytohormone-free media and used for subsequent steps of the herein-described methods.

[0046] In some applications, it may be useful to adjust the pH of the leaf-disc plate system depending on the type of plant used and/or insect to be reared. In some embodiments, the pH of the leaf-disc system is at least about 5.0-5.2, 5.3-5.5, 5.6-5.8, 6.0-6.2, or 6.3-6.5 but no greater than about pH 7.0. In some embodiments, the pH of the leaf-disc system is at least about pH 5.8 but no greater than about pH 7.0.

[0047] In some embodiments, the method can further comprise adding a surface sterilization solution to wash the disc leaf. In some cases, the surface sterilization is applied as a pretreatment. In some cases, the surface sterilization is applied during the growing phase of the leaf-disc. In some embodiments, the surface sterilization may comprise an ethanol-based solution. In some embodiments, the surface sterilization may comprise sodium hypochlorite. In some embodiments, the surface sterilization may comprise ethanol and

sodium hypochlorite. In some embodiments, the surface sterilization may comprise 70% ethanol and 0.24% sodium hypochlorite.

[0048] In some embodiments, to prevent potential fungal growth, an antifungal agent that does not interfere with the growth and health of the whiteflies or leaf may be applied to the leaf-disc plate. Examples of antifungal agents include, but are not limited to: amphotericin B, micanazole, benzimidazole, nipagin anidulafungin, caspofungin, micafungin, candicidin, filipin, natamycin, nystatin miconazole, bifonazole, clotrimazole, econazole, ketoconazole, and oxiconazole. The antifungal agent may be dissolved in an appropriate solvent (*e.g.*, dimethyl sulfoxide (DMSO) or water) that is not toxic to either the insects or the leaf-disc.

[0049] Once the rooted leaf discs are obtained, oviposition is allowed in order to obtain eggs on the discs for use in the present methods. In some embodiments, adults, e.g., 200-300 *Bemisia tabaci* adults are collected and then released onto a rooted leaf disc. Females are allowed to oviposit, e.g., for 1 hour at 28 °C. Plates can be checked, e.g., at 30 minutes and 1 hour after insect release, and oviposition can continue until, e.g., about 50-80 eggs are obtained per disc. The adults are then removed from the discs and the eggs are allowed to develop for, e.g., 3-6 hours. The eggs are then suitable for *in planta* injection or vacuum infiltration of macromolecule mixes as described herein.

In planta injection

[0050] In some embodiments, the macromolecules are introduced into the leaf disc by *in planta* injection. Any of the herein-described macromolecular mixes or any combinations thereof can be injected including, but not limited to, protein (e.g., Cas9), protein + RNA (e.g., Cas9 + gRNA), RNA (e.g., gRNA, dsRNA), DNA (e.g., plasmid), DNA + RNA (e.g., plasmid encoding Cas9 + gRNA), protein + DNA (e.g., Cas9 + plasmid expressing gRNA(s) and/or a gene of interest), and others. For such methods, the injection mix is prepared, e.g,. as described elsewhere herein, and are injected using siliconized, beveled, quartz needles. In some embodiments, the needle is backloaded with, e.g., 2 µl of injection mix, and then brought into close proximity to the leaf and eggs. The leaf can then be injected under or otherwise close to the pedicel, e.g., at an angle of 30°-50°. Each egg can be targeted, with the leaf being injected near each egg or, for multiple closely located eggs, a larger area of the leaf can be injected that targeted all of the clustered eggs. Successful injection can be determined by observing the soaking of the leaf immediately around the site of injection.

[0051] Once the leaves have been injected, the rooted discs are transferred together with their agar to, e.g., 6-cm phytoagar plates. The injected discs can then be covered, e.g., with a plastic lid with a mesh top, and then sealed with parafilm and place upside down in a 28 °C incubator at, e.g., 60% RH and a 16:8 D:N cycle.

[0052] Subsequently, eggs can be harvested, e.g., at 24, 48, or 72 hours post-injection, and assessed for protein levels, e.g., by blot or fluorescence. Eggs can also be allowed to develop to maturity on the discs, with the discs being removed and inspected regularly (e.g., daily), to control fungal growth on the agar substrate until eclosion. Nymphs and adults can be scored, e.g., for phenotypic changes, and molecular and sequencing analysis (e.g., of the targeted genomic region) using standard techniques.

Vacuum infiltration

[0053] In some embodiments, macromolecules are introduced into the leaf disc by vacuum infiltration. Any of the herein-described macromolecules or macromolecular mixes can be used (including those disclosed in paragraph [0041]. In particular embodiments, plasmids (e.g., plasmids encoding Cas9 and/or gRNAs), plasmids and RNA (e.g., Cas9-encoding plasmid with gRNA), or protein and RNA (e.g., Cas9 with gRNA) are used. In some embodiments, an infiltration mix is prepared as described herein, and drops of the mix are deposited onto the abaxial side of the disc comprising eggs (e.g., *B. tabaci* eggs). For example, 20 x 10 µl drops are deposited, with the drops spaced such that each of the eggs is in proximity to at least one of the drops.

[0054] The leaf disc plate can then be placed on the shelf of a vacuum chamber, and once the lid has been closed and the vacuum host connected, the vacuum can be applied for, e.g., 3 minutes. Once the vacuum has been stopped and the chamber opened, e.g., as described in more detail in the Examples, the remaining drops of the macromolecule mix can be removed, e.g., with a Gilson pipette. Rescued mix can be reused once, e.g., for introduction of macromolecules into new rooted leaf discs with deposited eggs.

[0055] Following vacuum infiltration, the rooted discs are transferred together with their agar to, e.g., 6-cm phytoagar plates. The injected discs can then be covered, e.g., with a plastic lid with a mesh top, and then sealed with parafilm and place upside down in a 28 °C incubator at, e.g., 60% RH and a 16:8 D:N cycle.

[0056] Subsequently, eggs can be harvested, e.g., 24, 48, or 72 hours post-injection, and assessed for, e.g., protein levels (e.g., by blot or fluorescence). Eggs can also be assessed for the presence of plasmid DNA, e.g., by extracting the DNA from the eggs using standard methods, transforming and culturing competent cells (e.g., JM109 cells), and analyzing the DNA, e.g., by PCR. Eggs can also be allowed to develop to maturity on the discs, with the discs being removed and inspected regularly (e.g., daily), to control fungal growth on the agar substrate until eclosion. Nymphs and adults can be scored, e.g., for phenotypic changes, and molecular and sequencing analysis (e.g., of the targeted genomic region) using standard techniques.

Transgenic plants

[0057] In another aspect, the present disclosure provides methods of delivering macromolecules to insect eggs by generating transgenic plants that produce macromolecules that are then transferred to the egg through the pedicel, e.g., via exosomes. Such transgenic plants can be generated, e.g., using conventional methods. For example, in some embodiments, a plant that can be targeted by any of the herein-described insects can be modified such that it produces Cas9 protein and gRNA specific for the insect pest, to allow CRISPR-mediated genomic editing as described in more detail elsewhere herein. The modified plant could therefore deliver the macromolecules to the insect egg, e.g., via exosomes. Once present in the embryo, the activity of the molecules could generate mutations in essential genes (e.g., genes involved in essential whitefly embryonic functions, such as segmentation (gap genes), neural development, organogenesis, and endocrine system function), thereby killing the developing embryo before it can hatch as a sap-feeding nymph.

[0058] In some embodiments, a plant is engineered to produce inhibitory RNA molecules, e.g., double-stranded small-interfering RNAs, siRNA, miRNA, that can similarly be taken up by the insect egg and lead to lethal or debilitating outcomes for the insect pest by altering expression of targeted genes (e.g., genes involved in essential whitefly embryonic functions, such as segmentation (gap genes), neural development, organogenesis, and endocrine system function) in the developing organism.

Genomic Modifications

[0059] In particular embodiments of the present methods, macromolecules comprising elements of a CRISPR-Cas system are introduced into the embryo. Such systems can be used, e.g., to modify one or more target genes in the genome of the insect (e.g., genes involved in

essential whitefly embryonic functions, such as segmentation (gap genes), neural development, organogenesis, and endocrine system function). Examples of genes involved in segmentation include, but are not limited to, *fushi tarazu* and *even-skipped*. Examples of genes involved in neural development and/or nervous system function include, but are not limited to, *scute* and *Notch*. Examples of genes involved in organogenesis include, but are not limited to, *Tinman* and *Pannier*. Examples of genes involved in endocrine system function include, but are not limited to, *Notch* and *Hey*. Other methods can also be used, e.g., transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and others. Any type of genetic modification can be performed, including insertions of one or more sequences into the insect genome, deletions of one or more sequences in the insect genome, replacement of one or more sequences in the insect genome, and alteration of one or more nucleotides in the insect genome.

[0060] In particular embodiments of the disclosure, a CRISPR-Cas system is used, e.g., Type II CRISPR-Cas system. The CRISPR-Cas system includes a guide RNA, e.g., sgRNA, that targets the genomic sequence to be altered, and a nuclease that interacts with the guide RNA and cleaves or binds to the targeted genomic sequence. The guide RNA can take any form, including as a single guide RNA, or sgRNA (e.g. a single RNA comprising both crRNA and tracrRNA elements) or as separate crRNA and tracrRNA elements. Standard methods can be used for the design of suitable guide RNAs, e.g., sgRNAs, e.g. as described in Cui et al. (2018) Interdisc. Sci.: Comp. Life Sci. 10(2):455-465; Bauer et al. (2018) Front. Pharmacol: 12 July 2018, doi.org/10.3389/fphar.2018.00749; Mohr et al. (2016) FEBS J., doi.org/10.1111/febs.13777, the entire disclosures of which are herein incorporated by reference.

[0061] Any CRISPR nuclease can be used in the present methods, including, but not limited to, Cas9, Cas12a/Cpf1, or Cas3, and the nuclease can be from any source, e.g. *Streptococcus pyogenes* (e.g. SpCas9), *Staphylococcus aureus* (SaCas9), *Streptococcus thermophiles* (StCas9), *Neisseria meningitides* (NmCas9), *Francisella novicida* (FnCas9), *and Campylobacter jejuni* (CjCas9). The guide RNA and nuclease can be used in various ways to effect genomic modifications in the insect embryo. For example, two guide RNAs can be used that flank an undesired gene or genomic sequence, and cleavage of the two target sites leads to the deletion of the gene or genomic sequence. In some embodiments, a guide RNA targeting a gene or genomic sequence of interest is used, and the cleavage of the gene or genomic sequence of interest and subsequent repair by the cell leads to an insertion,

deletion, or mutation of nucleotides at the site of cleavage. In some such embodiments, one or more additional polynucleotides are introduced into the embryo together with the guide RNA and nuclease, e.g., a polynucleotide comprising a sequence sharing homology to the targeted genomic sequence, and the one or more additional polynucleotides can produce a deletion, insertion, or alteration of the cleaved genomic sequence via homologous recombination (homology-directed repair).

[0062] In particular embodiments, one or more polynucleotides are introduced into the embryo encoding a guide RNA and encoding the RNA-guided nuclease, e.g., Cas9. For example, a vector, e.g. a plasmid vector, encoding one or more guide RNAs and encoding an RNA-guided nuclease is introduced into the egg, wherein the one or more guide RNAs and the RNA-guided nuclease are expressed in the embryo. In some embodiments, one or more guide RNAs are preassembled with RNA-guided nucleases as ribonucleoproteins (RNPs), and the assembled ribonucleoproteins are introduced into the egg.

Kits

[0063] In another aspect, kits are provided herein. In some embodiments, the kit comprises one or more element for producing genetically modified insects according to the present invention. The kit can comprise, e.g., one or more elements described herein for practicing the present methods, including a leaf disc, a macromolecule mixture, a needle for injection, phytoagar plate or media, guide RNA, RNA-guided nuclease, a CRISPR-Cas RNP, culture medium, transfection reagents, etc.

[0064] Kits of the present invention can be packaged in a way that allows for safe or convenient storage or use (e.g., in a box or other container having a lid). Typically, kits of the present invention include one or more containers, each container storing a particular kit component such as a reagent, a leaf disc, and so on. The choice of container will depend on the particular form of its contents, e.g., a kit component that is in liquid form, powder form, etc. Furthermore, containers can be made of materials that are designed to maximize the shelf-life of the kit components. As a non-limiting example, kit components that are light-sensitive can be stored in containers that are opaque.

[0065] In some embodiments, the kit contains one or more containers or devices, e.g. vacuum apparatus, injection needle, jars (e.g., 60 ml Nalgene jars), flask, for practicing the present methods. In yet other embodiments, the kit further comprises instructions for use, e.g., containing directions (*i.e.*, protocols) for the practice of the methods of this invention

(e.g., instructions for using the kit for preparing leaf discs, introducing macromolecules into leaves, effecting genomic modifications on insect embryos, etc.). While the instructional materials typically comprise written or printed materials, they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

Example 1. Methods

A. Preparation of rooted Brassica leaf discs

[0066] Leaf disc technology is important for the success of these *in planta* injection and vacuum-infiltration technologies. It provides a small footprint rearing system that allows macromolecule delivery and the development of whiteflies from egg to adult.

[0067] The steps involved in preparing the discs include:

1. Leaf discs are excised from 4-week-old Brassica plants and placed with their abaxial side up on phytoagar media (Gamborg's media with vitamins) with 1 mg/L NAA.
2. Leaf discs are incubated for 5-9 days and allowed to form roots.
3. Rooted discs are transferred to phytohormone-free media and used once roots are established.

B. Oviposition on rooted leaf discs

[0068] Leaf discs containing eggs are prepared as follows:

1. *Bemisia tabaci* adults (200-300) are collected prior to experiment initiation.
2. Whiteflies are released onto a rooted leaf disc.
3. Females are allowed to oviposit for 1 hr at 28°C. Plates are checked at 30 min and 1 hr after insect release. Approximately 50-80 eggs per disc should be present on a disc. If needed, the oviposition period can be extended to meet this egg density.
4. The adults are removed and the eggs allowed to develop for 3-6 hr.

5. *In planta* injection or vacuum infiltration is used to deliver macromolecule mixes to the leaf disc. (see below)

C. Macromolecule mixes

[0069] All plasmids used for this discovery are listed in **Table 1**. Plasmids using the *Bemisia tabaci exuparentia* (*exu*) and *polyubiquitin* (*PUbA*) promoters and 3'-untranslated regions (3'-UTRs) were constructed. Certain mixes were prepared as follows:

1. **Protein and gRNA mixes.** A Cas9 protein (PNBio) and gRNA (Synthego) mix is prepared in water to a final concentration of 200 ng/µl for Cas9 protein and 300 ng/µl gRNA (Synthego). The gRNA is loaded ono the Cas9 nuclease by incubating the injection mix at 37°C for 5 min. The injection mix is stored at -80°C in 2.5-µl aliquots. Initial experiments used a *white* gRNA (gRNA-w2).

Table 1. Plasmids used in the present experiments

Plasmid name	Purpose	Funding Source
exu:Cas9-T2A-mCherry:exu	Expression of the Cas9 and mCherry proteins in whitefly germline cells.	DARPA, BARD
U6-4:gRNA-w2	Production of the w2 gRNA in whitefly cells.	DARPA
U6-4:gRNA-w2	Production of the w2 gRNA in whitefly cells.	DARPA
PUbA:mCherry	Expression of Cas9 protein constitutively and used in plasmid rescue experiments	DARPA, BARD
PUbA:Cas9-T2A-mCherry:PUbA	Expression of the Cas9 and mCherry proteins in whitefly cells (constitutive).	DARPA
CRISPaint-3xP3:GFP	General promoter *(3xP3)* for expression of GFP in insect cells	AddGene
pU6-(BbsI)_CBh-Cas9-T2A-	Source of Cas9-T2A-mCherry for whitefly constructs above	AddGene

mCherry		
pCDF1-dU6:1gRNA	Source of vector backbone and the gRNA scaffold for the five *BtU6:gRNA* constructs above	AddGene

2. **Plasmid and gRNA mixes.** To 1 volume of sterile water (Synthego or IDT), add 1 volume of IDTE buffer with a Cas9 plasmid at a concentration of 25 ng/µl and 300 ng/µl gRNA.

3. **Plasmid mixes.** To 1 volume of sterile water (Synthego or IDT), add and 1 volume of IDTE buffer with plasmid DNA at a final concentration of 50 ng/µl. If multiple plasmids are used, each plasmid DNA has a final concentration of 25 ng/µl.

D. *In planta* injections

[0070] Cas9 protein/gRNA, Cas9 plasmid/gRNA or Cas9 and U6:gRNA plasmids are used in the *in planta* injections. Injections were performed as follows:

1. Prepare injection mix as above.

2. Siliconized, beveled, quartz needles are used for *in planta* injections of leaves and are prepared using standard techniques.

3. The injection needle is carefully backloaded with 2-4 µl of injection mix.

4. The needle is brought to be in close proximity to the leaf and *B. tabaci* eggs. The leaf is injected under the pedicel or as close to the pedicel as possible at an angle of attack between 30°-50°.

5. Proof of injection is easily obtained by observing the soaking of the leaf immediately around the site of injection. Several closely located eggs and their pedicels can be above a soaked section of leaf following a single microinjection.

6. *In planta* inject near every egg if possible, to allow an accurate assessment of egg hatch and mutation frequency.

7. After delivery of the macromolecules, rooted discs with their agar are transferred to 6-cm phytoagar plates.

8. The injected disc is covered with a plastic led with a mesh top, sealed with parafilm and placed upside down in a 28°C incubator at approximately 60% RH and a 16:8 D:N cycle.
9. If protein is to be analyzed by protein blot or fluorescence, 50-60 eggs are harvested at 24, 48 or 72 h post injection.
10. Alternatively, insects develop to maturity on discs.
11. Discs are removed for inspection and curated daily (if needed) to control fungi growth on the agar substrate until adults eclose.
12. Nymphs and adults are scored for phenotypic changes.
13. Molecular and sequencing analysis of the *w2* target region follows standard techniques.

E. Vacuum infiltration of leaf discs

[0071] Cas9 plasmid with gRNAs or Cas9 plasmids and *U6:gRNA* plasmids are used in the vacuum infiltration experiments. Infiltration of leaves with Cas9 protein, while feasible, may be too expensive to be practical.

1. Prepare infiltration mix as described above.
2. Deposit 20 x 10 µl drops using a P20 pipette onto the abaxial of the rooted leaf disc containing *B. tabaci* eggs. Space these drops across the leaf surface so that all eggs are in proximity to at least one drop of the macromolecule mix.
3. Place the leaf disc plate into the shelf of a plastic vacuum chamber, place lid, and connect the hose to the house vacuum. Turn on the vacuum and gently open the valve on the chamber. Pull the vacuum for 3 min.
4. Gradually turn off the valve.
5. Turn off the house vacuum.
6. Gently disconnect the hose from the top of the chamber.
7. Slowly open the valve on the chamber to let air to gently bleed back into the chamber.
8. Turn the valve off.

9. Reconnect the vacuum hose.
10. Turn the house vacuum on.
11. Gently open the valve on the chamber and leave for 1 min.
12. Gently turn off the valve.
13. Turn off the house vacuum.
14. Gently disconnect the hose from the top of the chamber.
15. Slowly open the valve on the chamber to let air to gently bleed back into the chamber.
16. Remove all the remaining drops of the macromolecule mix with a Gilson pipette.
17. Repeat the entire process (steps 1-16) using a new rooted leaf disc with ~3 to 8-hr-old eggs and the macromolecule mix rescued in step 16.
18. After delivery of the macromolecules, rooted discs with their agar are transferred to 6-cm phytoagar plates.
19. The injected disc is covered with a plastic led with a mesh top, sealed with parafilm and placed upside down in a 28°C incubator at approximately 60% RH and a 16:8 D:N cycle.
20. If protein is to be analyzed by protein blot or fluorescence, 50-60 eggs are harvested at 24, 48 or 72 hr post injection.
21. Alternatively, insects develop to maturity on discs.
22. Discs are removed for inspection and curated daily (if needed) to control fungi growth on the agar substrate until adults eclose.
23. Nymphs and adults are scored for phenotypic changes.
24. Molecular and sequencing analysis of the gRNA target region follows standard techniques.

F. Plasmid rescue experiments demonstrate plasmid DNAs enter the embryo

[0072] The following method can be used to confirm the presence of plasmid DNA in the embryo.

1. Using a blunt dissecting pin, collect 30-60 eggs (embryos) from the leaf disc 72 hr post injection.
2. Extraction of DNA from eggs using the manufacturer's protocols for the Qiagen microDNA kit.
3. Add 50 ng of DNA from step 2 to chemically competent JM109 cells following manufacturer's protocols (Zymo).
4. Plate cells on ampicillin (100 μg/ml) LB-agar plates.
5. Pick cells from the center of a well-isolated colonies for colony PCR. Perform PCR using primers specific to the plasmid.

Example 2. Benefits of the present methods.

[0073] The *in planta* injections and vacuum infiltration methods for delivery of macromolecules into the whitefly embryo is based on the surprising discovery that macromolecules (plasmid DNA, protein and RNAs) can be taken up from the leaf and are active. This indirect delivery system has several advantages. We compare several parameters in **Table 2**.

[0074] One advantage of the present methods is that there is limited (if any) damage to the embryo, because macromolecules are delivered indirectly in the *in planta* injection and vacuum filtration methods. This is estimated from egg to adult survival rates, which are 45-65% for the indirect methods and ~ 30-35% for embryo microinjection.

[0075] Another advantage is that there is limited damage to the leaf with vacuum infiltration. We have found that there is minor leaf damage after some *in planta* injections, but eggs still mature to adults with high efficiency.

[0076] Another advantage is that the present methods provide a significant time savings in comparison to other methods. For example, it takes >1 hr to microinject 50 whitefly embryos. In contrast, it takes less than 30 min to do *in planta* injections and only 15 min for the vacuum infiltration method for 50 eggs.

[0077] An additional advantage is that the technological complexity of these three methods declines from embryo microinjections to *in planta* injection to vacuum infiltration. Highly trained personnel and equipment are needed for embryo microinjections. In contrast, vacuum

infiltration requires no needles and only a house vacuum. This method is inexpensive and easy for anyone to perform.

Table 2. Leaf injection, leaf infiltration and embryo injection parameters

	In planta injection	Vacuum infiltration	Embryo microinjection
Damage to insect egg	None	None	Significant
Damage to leaf	Minor, from injection	None	None
Volume delivered	Approx. 500 nl/injection 5 µl total (for 50 eggs)	Approx. 200 µl per disc (for 50 eggs, reused once)	Approx. 0.1 nl/egg injection*
Concentrations of macromolecules injected or infiltrated	200 ng/µl Cas9 protein 300 ng/µl gRNA 500 ng/µl plasmid DNA	50 ng/µl plasmid	200 ng/µl Cas9 protein 300 ng/µl gRNA 500 ng/µl plasmid DNA
Time per disc*	< 30 min	< 15 min	> 1 h
Plasmids delivered	Yes	Yes	Yes
gRNA delivered	Yes	Not tested ($$$)	Yes
Protein delivered	Yes	Not tested ($$$)	Yes

ODN delivered[A]	TBD	TBD	Yes
Equipment needed	Low tech injection	Vacuum chamber	High tech injection
[A] For delivery of ODNs (oligonucleotides) to assess the frequency of homologous recombination for the production of gain-of-function mutants, a concentration of 200 ng/µl is used.			

[0078] An additional benefit is that the vacuum infiltration method is also less expensive because the infiltration macromolecule mix can be reused (once).

[0079] To avoid the potentially high cost of infiltration of Cas9 protein, plasmids encoding Cas9 can be delivered by infiltration are transcribed and their RNAs translated.

[0080] Further, all three methods addressed in **Table 2** have one advantage over the ovary injection methods reported by Heu et al (2020). In particular, all can deliver larger oligonucleotides (ODNs) to facilitate generation of gain-of-function mutants by homologous recombination.

[0081] In addition, the efficiencies of loss-of-function mutants using the *in planta* injection method are higher. From our first two experiments, 20 insects were collected that had developmental defects and eyes with mosaic, sectored patterns. Of these, two have been confirmed as mutants by T7 endonuclease assays and DNA sequencing.

Example 3. Conclusions drawn from the above-described results

[0082] All experiments have been replicated several times, providing confidence in their outcomes and our conclusions, which include:

(1) Uptake of colored food dye or phenol red by whitefly embryos following *in planta* injection of the dye or vacuum infiltration of the dye into leaves demonstrates that there is transfer of molecules from the plant to the insect egg (see, **FIGS. 2A-2B**).

(2) Plasmids that are introduced into leaf discs by *via in planta* injection or infiltration enter *B. tabaci* embryos, as evidenced by plasmid rescue experiments (**FIG. 2C**).

(3) Cas9 protein that is introduced into leaf discs by *in planta* injections move into *B. tabaci* embryos and can be detected by immunoblots (**FIG. 2D**).

(4) Plasmids delivered to the leaf disc by *in planta* injection or infiltration enter the whitefly embryo and are transcribed and translated to produce a Cas9-T2A-mCherry fusion protein. mCherry fluorescence is detected at 24 hr (not shown), 48 hr and 72 hr post injection or infiltration (**FIGS. 2E-2F**).

(5) Multiple CRISPR/Cas9-edited *B. tabaci* have been recovered following *in planta* injection of Cas9 protein and the *white2* gRNA into leaves. The insects have mosaic eyes and severe developmental defects (**FIGS. 2G-2H**).

(6) Molecular evidence of editing in the *white* mutants produced by *in planta* injections was provided by T7 endonuclease assays and cloning and sequencing of the *white* gene region (**FIGS. 2I-2J**).

Example 4. Additional experiments

[0083] Obtain CRISPR-mediated homology-directed repair knock-in mutagenesis of *B. tabaci* following *in planta* microinjections of Cas9 protein, gRNA, and a long single-stranded oligonucleotide DNA (ODN) molecules, which will introduce a gene that expresses mCherry using the constitutive polyubiquitin promoter (**Table 1**). These gain-of-function mutants are assessed by mCherry fluorescence and at the molecular level of gene cassette insertion into the whitefly genome.

[0084] Generate gain-of-function mutants using the CRISPaint, a non-homologous end joining method of gene insertion. We introduce Cas9 protein, gRNAs and plasmid DNA using *in planta* injection of leaf discs.

[0085] Both the *in planta* injection technique and the vacuum infiltration are further optimized to determine optimal concentrations of Cas9 protein, gRNA and plasmid DNA.

[0086] As many insect eggs have intimate contact with plant leaves and take up water from plants, we believe that the present methods can be used to deliver macromolecules to these other insect pests. We test using insects (hemipteran/orthopteran) that use pedicels or hydropyles for water homeostasis in their eggs

[0087] The plant-pedicel continuum is used to develop a new generation of insect-proof crops. We generate transgenic plants that produce both Cas9 protein and gRNAs (or hairpin dsRNAs) in the epidermal cells of the leaf. Without being bound to the following theory, we believe that exosomes (small vesicles known to transport proteins and RNAs across plant cell membranes) may deliver these macromolecules to the egg pedicel for transport into embryos.

[0088] The gRNAs or dsRNAs can be specific, e.g., to *B. tabaci* target genes that are essential for nymph development and can be designed to highly conserved regions of each gene. Creating a null mutant using Cas9 and gRNAs or down-regulating target genes using dsRNA can significantly impair, and likely prevent, whitefly nymph development. This is a powerful tool for control of insects as nymphs will not be produced and insect feeding damage will be avoided. In addition, insect populations will not be able to expand.

Example 5.

[0089] The whitefly egg is secured to plant leaves via a small stalk called a pedicel, which is known to transport water and small molecules (inulin and acetate) into the whitefly embryo. We have shown that proteins, RNAs, DNAs, and dyes injected or vacuum infiltrated into the leaf by the base of the egg will be transferred from the plant via the egg pedicel into the whitefly eggs and its embryos. These RNAs, plasmid DNAs and proteins are biologically active within the whitefly egg. Most significantly, *in planta* injection of Cas9 and a gRNA to the *white* gene resulted recovery of a whitefly with mosaic eyes and targeted editing of the *white* gene (FIG. 3).

[0090] This plant cell-pedicel-insect embryo continuum provides us a novel and technically simple method for delivering macromolecules from the plant to the whitefly embryo to allow gene editing by CRISPR/Cas technologies or double-stranded RNA-mediated gene silencing. New precise strategies for insect control can now be developed.

[0091] We provide two strategies for the control of *Bemisia tabaci* (a whitefly) on host plants (FIG. 4). These strategies can be extended for the control of any insect that has its eggs in close contact with plant cells (leaves, roots, stems). We will construct transgenic plants that express double-stranded RNAs (dsRNAs) (Strategy 1; FIGS. 5 and 6) or express a Cas nuclease and sgRNAs (Strategy 2, FIGS. 7 and 8) that will silence or obliterate expression of genes that are essential for whitefly embryo development, respectively. These macromolecules will be transported from the transgenic plant cell through the egg's pedicel into the whitefly embryo where they are biologically active. By interfering with the expression of essential embryonic genes, embryo development will be terminated. This will prevent nymph (immature whitefly) and adult emergence and, therefore, subsequent population expansion. Without population expansion, the damage caused by whitefly feeding (plant stunting due to photosynthate depletion, fungal growth due to honeydew deposition and the vectoring of viruses) is curtailed. We will have generated an important set of

whitefly-proof plants. While adults in a population might colonize these whitefly-proof plants, it is a population dead-end.

[0092] To assure that there is a high level of embryo lethality, each transgenic plant constructed for Strategy 1 or Strategy 2 will target multiple essential genes simultaneously (FIGS. 6 and 8). This allows for functional redundancy and will assure that few, if any, embryos hatch and develop into nymphs or adults. This stacked target gene approach will enable tight control of insect development and assure success.

Strategy 1: Deliver dsRNAs to essential whitefly genes to block embryo development via the plant-pedicel continuum

[0093] When double-stranded RNAs (dsRNAs) are produced within eukaryotic cells, they are processed by the dicer machinery to produce small single-stranded RNAs (sRNAs); some sRNAs bind to a target mRNA to degrade or block translation of the target RNA. This process is called RNA interference (RNAi). sRNAs regulate myriad cellular functions within eukaryotic cells and the bidirectional trans-kingdom exchange of dsRNAs between a plant and fungal pathogen has been established. Multiple types of transgenes have been used to cause RNAi. We use hairpin RNAs (hpRNAs) as an example of this strategy.

[0094] In Strategy 1, we make transgenic plants that express hpRNA constructs that produce sRNAs that target essential whitefly embryonic genes (FIG. 5). hpRNAs or the sRNAs made within the plant cell will be delivered to the embryo via the plant-pedicel continuum. When sRNAs enter the embryo, they will silence their target gene. When hpRNAs are transferred to the insect egg, they will be processed by the insect's dicer machinery to produce sRNAs within the embryo, which will silence the target gene in developing whitefly embryos.

[0095] For our target genes, we will first use highly conserved gap and pair-rule developmental genes that are zygotically expressed and essential for insect body-plan development. Since Hemiptera are hemimetabolous insects and hatch with essentially the entire adult body plan, we can also select conserved genes essential for the development of the nervous system, other organs and the endocrine system. Using this functionally redundant approach will ensure that insect embryos will not develop or hatch on infested transgenic plants expressing these hpRNAs. We have completed a study documenting gene expression during five phases of whitefly embryonic development. This study is enabling the choice of genes **(IP protected)** for this strategy.

[0096] As the in vivo efficiency of silencing of each hpRNA is difficult to predict, we will express four or more hpRNAs that target essential whitefly embryonic genes in each transgenic plant using strong constitutive or epidermal cell-specific plant promoters to make each hpRNA (FIG. 6). One example is that each transgenic plant will express hpRNAs to silence one gap, one neural, one organogenesis, and one endocrine system gene. The use of four genes targeting very different but essential functions provides functional redundancy to our control strategy. One or more of the hpRNAs will silence their targets. A second cassette with four different gap, neural, organogenesis and endocrine function genes will also be produced. Transgenic plants expressing cassette 1 and cassette 2 will be generated (FIG. 6); these plants can be crossed to generate plant lines that express eight hpRNA genes to assure even tighter control of whitefly embryo development.

[0097] Construction and characterization of transgenic lines: The hpRNA cassettes will be placed in an Agrobacterium binary vector between the right and left borders for integration into the plant genome. Each hpRNA construct will have a plant selectable marker. T0 plants with all four hpRNA genes will be identified using PCR and expression levels of each hpRNA and sRNAs determined by qRT-PCR and RNA blots, respectively. We will identify T0 plants that harbor a single intact copy of the transgene cassette to avoid gene silencing in future generations. T1 and T2 generation plants will be generated and characterized. Transgenic plants expressing cassette 1 and cassette 2 will be generated (FIG. 6); these plants will be crossed.

[0098] T2 homozygous lines (with cassette 1, cassette 2 or both cassettes) and wild-type (WT) control plants will be used to test the efficiency of whitefly target gene silencing in whitefly infestation studies of Brassica leaf discs. Whitefly females will lay 50-100 eggs per disc. We will follow egg development daily, as egg pigmentation reflects viability and development. Eggs are uniformly cream color at the time of egg deposition. Viable, maturing eggs become slightly brown with 1-2 days and eye spots become visible later in embryonic development. Following the strategies described herein, eyes will not be detected and we will see a high percentage of dead eggs, which will be uniformly deep brown. We will collect 100 eggs on day 1, 2, 3, 4 and 5 after oviposition on T2 or WT plants. RNAs will be extracted. qRT-PCR will be performed to assess the expression of the target genes and presence of hpRNAs in the whitefly egg. RNA blots or the SplintR ligase methods will be used to detect plant sRNAs in embryos. As a result, target RNAs will not be detected (or at lower levels), hpRNAs and sRNAs will be detected, and few embryos will be viable.

[0099] We will also determine the percentage of eggs that hatch, egg to adult survival and nymph to adult survival in the T2 and WT plant leaf discs. We will look for any phenotypic abnormalities in developing nymphs and adults. Following the strategies described herein, there will be a low % of egg hatch, few nymphs will develop and few adults will emerge on the T2 discs relative to WT controls.

[0100] We will confirm the results observed by the leaf disc method using intact plants. We will infest T2 or WT leaves with 100 whiteflies. After three days, adults and mesh bags will be removed and the number of eggs deposited will be counted (~750 per plant). We will monitor plants daily beginning at day 15 for adult emergence. Most insects will emerge synchronously over a 3-4 day period. We will determine the % egg to adult on the T2 plants vs WT plants. We will look for any phenotypic abnormalities in adults. Following the strategies described herein, there will be a low % of egg hatch, few nymphs will develop and few adults will emerge on the T2 plants relative to WT controls.

Strategy 2: Engineering the plant to deliver the CRISPR/Cas9 machinery to the whitefly embryo

[0101] We will engineer whitefly host plants to deliver the components of CRISPR technology to the whitefly embryo. Editing of essential genes will immediately lead to the death of the embryo prior to hatch. This approach is insect species-specific (and even target pest population-specific).

[0102] To deliver Cas9 to the whitefly embryo, we will express Cas9 in the cytosol and/or Golgi to encourage incorporation into vesicles for transport through the pedicel and into the whitefly embryo (FIG. 8). Cas9 genes will be expressed using a strong constitutive promoter (i.e., 35S promoter) or an epidermal-specific promoter. In the same T-DNA cassette, we will place eight U6:sgRNA genes (FIG. 7). There will be two sgRNAs for each target gene to enhance the probability of deletions and null mutations. These sgRNA genes will target the exons of genes essential for embryonic development (see Strategy 1). When desired, the sgRNA sequences will be designed to match exactly their target sites in the local insect pest population (called private alleles); this will be useful when an invasive insect is being targeted and its native population needs to be retained. We will ensure that there are no target sequences for these sgRNAs in the host plant genome. As in Strategy 1, we will create two different transgene cassettes. We will compare the ability of transgenic plants expressing cassette 1, cassette 2 or both cassettes for their ability to block embryo development.

[0103] Transgenic plants expressing 35S:Cas9-cytosol (or 35S: Cas9-Golgi) and eight U6:gRNA genes specific to each whitefly target site gene will be constructed. Cas9 protein and the sgRNAs will be taken up by the egg through the pedicel pathway (FIG. 8). Once in the embryo, Cas9 and the sgRNAs will edit the four essential genes rendering them dysfunctional. This will result in embryonic death before egg hatch. Since there are no cells and nuclear membranes during the stages of mitosis before cellular blastoderm, cytosolic Cas9 protein and sgRNAs will have full access to the whitefly chromosomes for editing.

[0104] T0, T1, and T2 transgenic plants will be generated and characterized as described in Strategy 1. We will also generate plants expressing only Cas9 or only the U6:gRNA genes; these will serve as negative controls. We will characterize the expression of the four target genes in developing embryos by qRT-PCR (see Strategy 1). We will assess the viability of whitefly eggs on rooted leaf discs and intact plants as described in Strategy 1. We will also determine egg hatch, egg to nymph survival and egg to adult survival as described in Strategy 1.

[0105] Following the strategies described here, few (if any) eggs will develop or hatch on T2 plants; few (if any) nymphs will survival to adult hood. Given the functional redundancy of the plants expressing cassette 1 and cassette 2, plants expressing both cassettes may confer the most powerful resistance to whiteflies. Hence, embryo development will be abolished.

[0106] One advantage of Strategies 1 and 2 is that they do not require the generation or the release of transgenic insects. Rather the gene editing in the field pest population is achieved through the interaction of the insect egg with the host plant through the pedicel. In a sense, the Cas9 protein and gRNAs achieve maximum exposure to the pest insects through the planting and growing of the transgenic plants. An additional benefit is that, in the case of a species complex, as is the case with *B. tabaci*, these approaches can simultaneously target the same gRNA sites in many species within the complex. Another advantage is that the gRNAs are designed to the target sequences in the local pest population. This helps to confine the targeting to the geographical area of concern and is important from a biocontainment perspective. Finally, for both approaches, transgenic lines of crop plants can be generated in anticipation of pest insect invasion into a region and stored as seeds. Seed banks of essential crop species could be established for several invasive insect pest scenarios.

REFERENCES:

Buckner JS, Freeman TP, Ruud RL, Chu C-C, Henneberry TJ (2002) Characterization of the whitefly egg pedicel. Archives of Insect Biochemistry and Physiology 49:22-33

Byrne DN, Cohen AC, Draeger EA (1990) Water-uptake from plant-tissue by the egg pedicel of the greenhouse-whitefly, *Trialeurodes vaporariorum* (Westwood) (Homoptera, Aleyrodidae). Canadian Journal of Zoology-Revue Canadienne De Zoologie 68 (6):1193-1195. doi:10.1139/z90-177

Cai Q, He B, Weiberg A, Buck AH, Jin H (2019) Small RNAs and extracellular vesicles: New mechanisms of cross-species communication and innovative tools for disease control. PLOS Pathogens 15 (12):e1008090. doi:10.1371/journal.ppat.1008090

Hartley JC (1965) The structure and function of the egg-shell of *Deraeocoris ruber* L. (Heteroptera, Miridae). Journal of Insect Physiology 11 (1):103-109. doi:10.1016/0022-1910(65)90135-6

Heu CC, McCullough FM, Luan JB, Rasgon JL (2020) CRISPR-Cas9-based genome editing in the silverleaf whitefly (*Bemisia tabaci*). CRISPR Journal 3 (2):89-96. doi:10.1089/crispr.2019.0067

Hinton HE (1981) Biology of Insect Eggs. Volumes I-III. Elsevier

Johnson CG (1934) On the eggs of Notostira erratica L. (Hemiptera, Capsidae). Trans Sot Brit Ent 1:1-32

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by pierid butterflies triggers defense responses in Arabidopsis. Plant Physiology 143 (2):784-800. doi:10.1104/pp.106.090837

Morgan JM (1984) Osmoregulation and water stress in higher plants. Annual Review of Plant Physiology 35 (1):299-319. doi:10.1146/annurev.pp.35.060184.001503

Paniagua Voirol LR, Valsamakis G, Lortzing V, Weinhold A, Johnston PR, Fatouros NE, Kunze R, Hilker M (2020) Plant responses to insect eggs are not induced by egg-associated microbes, but by a secretion attached to the eggs. Plant, Cell & Environment 43 (8):1815-1826. doi:10.1111/pce.13746

Réaumur R-AFd (1734) Mémoires pour servir à l'histoire des insectes / Par m. de Réaumur. De l'Imprimerie royale, Paris

Regente M, Pinedo M, San Clemente H, Balliau T, Jamet E, De La Canal L (2017) Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth. Journal of Experimental Botany 68 (20):5485-5495. doi:10.1093/jxb/erx355

Rutter BD, Innes RW (2017) Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. Plant Physiology 173 (1):728-741. doi:10.1104/pp.16.01253

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from *Pieris brassicae* eggs activate an immune response in Arabidopsis. eLife 9. doi:10.7554/elife.60293

Taylor GS (1992) The structure of the eggs of some Australian Psylloidea (Hemiptera). Australian Journal of Entomology 31 (2):109-117. doi:10.1111/j.1440-6055.1992.tb00467.x

Weber H (1931) Lebensweise und umweltbeziehungen von *Trialeurodes vaporariorum* (Aleurodina). Z Morph Cykol Tiere 23:575-753

Whangbo JS, Hunter CP (2008) Environmental RNA interference. Trends in Genetics 24 (6):297-305. doi:10.1016/j.tig.2008.03.007

White TCR (1968) Uptake of water by eggs of *Cardiaspina densitexta* (Homoptera: Psyllidae) from leaf of host plant. Journal of Insect Physiology 14 (11):1669-1683. doi:10.1016/0022-1910(68)90100-5

Wigglesworth VB (1965) The Principles of Insect Physiology. 6th edn. Cambridge University Press

[0107] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

WHAT IS CLAIMED IS:

1. A method of introducing a macromolecule into an egg of a sap-feeding insect, the method comprising providing a leaf of a host plant upon which the egg is present, and introducing the macromolecule into the leaf in the proximity of the egg, wherein the egg comprises a pedicel and wherein the macromolecule is transferred from the leaf into the egg through the pedicel.

2. The method of claim 1, wherein the insect is a whitefly.

3. The method of claim 2, wherein the species of the whitefly is *Bemisia tabaci*.

4. The method of any one of claims 1 to 3, wherein the leaf upon which the egg is present is a rooted leaf disc.

5. The method of any one of claims 1 to 4, wherein the plant is a Brassica plant.

6. The method of any one of claims 1 to 5, wherein the macromolecule is introduced into the leaf by *in planta* injection.

7. The method of any one of claims 1 to 5, wherein the macromolecule is introduced into the leaf by vacuum infiltration.

8. The method of any one of claim 1 to 7, wherein the macromolecule is a protein.

9. The method of claim 8, wherein the protein is Cas9.

10. The method of claim 8 or 9, wherein protein is subsequently detectable in the egg.

11. The method of any one of claims 8 to 10, wherein the introduction of the macromolecule into the leaf results in a phenotypic change in a nymph or adult developing from the egg.

12. The method of any one of claims 1 to 7, wherein the macromolecule is a nucleic acid.

13. The method of claim 12, wherein the nucleic acid is RNA.

14. The method of claim 13, wherein the RNA is selected from the group consisting of a guide RNA, double stranded RNA (dsRNA), miRNA, siRNA, and shRNA.

15. The method of claim 14, wherein the introduction of the RNA into the leaf results in an alteration in expression of a target gene and/or a phenotypic change in a nymph or adult developing from the egg.

16. The method of claim 15, wherein the target gene is a gene involved in an essential whitefly embryonic function.

17. The method of claim 16, wherein the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function.

18. The method of claim 12, wherein the nucleic acid is DNA.

19. The method of claim 18, wherein the DNA is a plasmid.

20. The method of claim 19, wherein the plasmid comprises a coding sequence for Cas9 and/or a guide RNA, operably linked to a promoter.

21. The method of claim 19 or 20, wherein the plasmid comprises a coding sequence for a dsRNA, miRNA, siRNA, or shRNA, operably linked to a promoter.

22. The method of any one of claims 18 to 21, wherein the introduction of the DNA into the leaf results in an alteration in expression of a target gene and/or a phenotypic change in a nymph or adult developing from the egg, and/or the detectable expression of a protein or RNA encoded by the plasmid.

23. The method of claim 22, wherein the target gene is a gene involved in an essential whitefly embryonic function.

24. The method of claim 23, wherein the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function.

25. The method of claim 1, wherein the macromolecule is introduced into the insect by genetically modifying the host plant, such that the macromolecule is expressed in the host plant leaf and transferred to the egg through the pedicel.

26. The method of claim 25, wherein the macromolecule is a Cas9 protein and/or a guide RNA.

27. The method of claim 25, wherein the macromolecule is an inhibitory RNA molecule selected from the group consisting of dsRNA, miRNA, siRNA, and shRNA.

28. A method of manipulating the genome of a sap-feeding insect, comprising providing a leaf of a host plant upon which an egg of the insect is present and introducing a macromolecule into the leaf in the proximity of the egg,

wherein the egg comprises a pedicel,

wherein the macromolecule is transferred from the leaf into the egg through the pedicel,

wherein the macromolecule is or encodes a component of a CRISPR-Cas system targeting a sequence within the insect genome, and

wherein the CRISPR-Cas system introduces a modification of the insect genome at or in proximity to the targeted sequence.

29. The method of claim 28, wherein the macromolecule is a Cas9 protein and/or a guide RNA, or a plasmid encoding a Cas9 protein and/or a guide RNA, wherein the guide RNA targets the sequence within the insect genome.

30. The method of claim 28 or 29, wherein the modification is a deletion, insertion, or nucleotide substitution at the targeted sequence.

31. The method of any one of claims 28 to 30, wherein the modification alters the expression of a gene in the sap-feeding insect.

32. The method of claim 31, wherein the gene is a gene involved in an essential whitefly embryonic function.

33. The method of claim 32, wherein the essential whitefly embryonic function is segmentation, neural development, organogenesis, or endocrine system function.

34. The method of any one of claims 28 to 33, wherein the modification results in a phenotypic change in a nymph or adult developing from the egg.

35. The method of any one of claims 28 to 34, wherein the sap-feeding insect is a whitefly.

36. The method of claim 35, wherein the whitefly is *Bemisia tabaci*.

37. The method of any one of claims 28 to 36, wherein the leaf is a rooted leaf disc.

38. The method of any one of claims 28 to 37, wherein the plant is a Brassica plant.

39. The method of any one of claims 28 to 38, wherein the macromolecule is introduced into the leaf by *in planta* injection.

40. The method of any one of claims 28 to 38, wherein the macromolecule is introduced into the leaf by vacuum infiltration.

41. The method of any one of claims 28 to 36, wherein the macromolecule is introduced into the insect by genetically modifying the host plant, such that the macromolecule is expressed in the host plant leaf and transferred to the egg through the pedicel.

FIG. 1A

Infiltration into leave disc

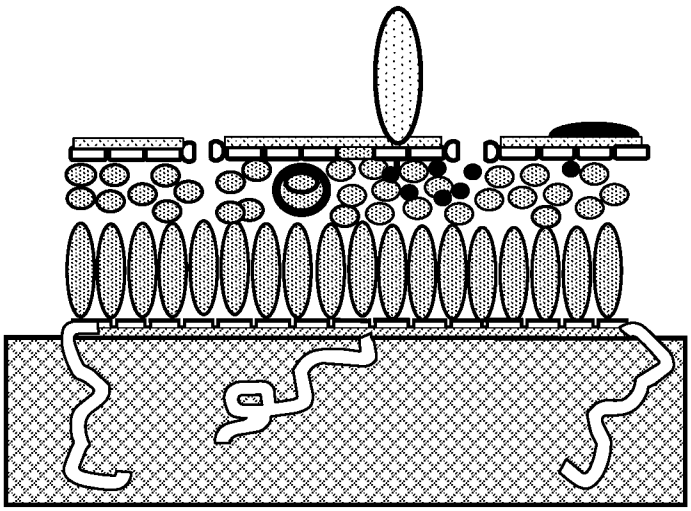


FIG. 1B

Injection into leaf disc

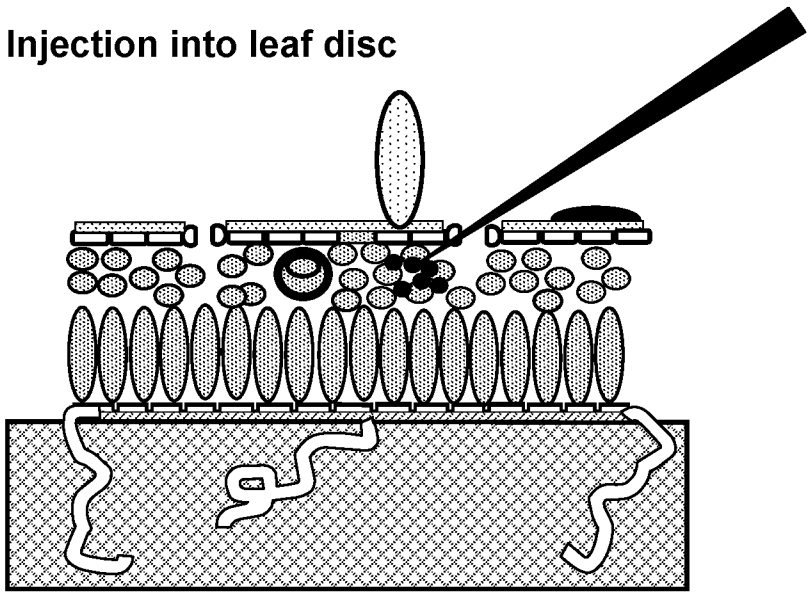


FIG. 2A

Vacuum infiltration
Red dye and 3xP3:eGFP

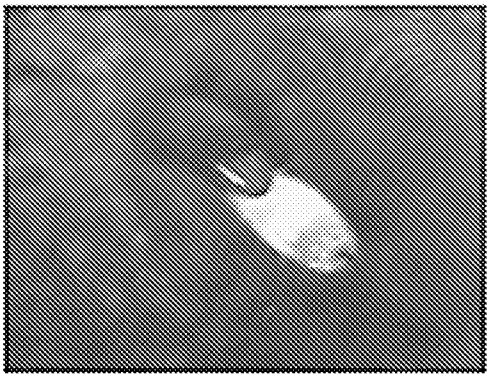


FIG. 2B

In planta injection
Phenol red

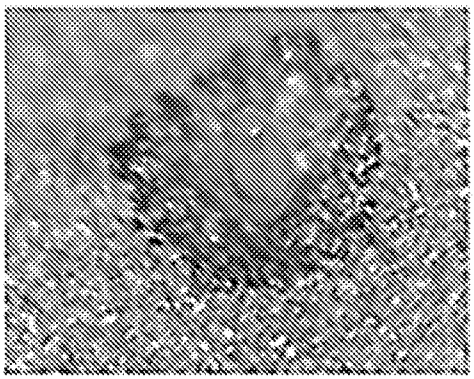


FIG. 2C

In planta injection
plasmids

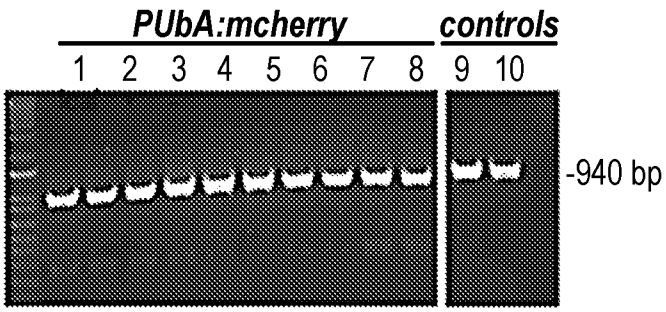


FIG. 2D

In planta injection
Cas9 protein

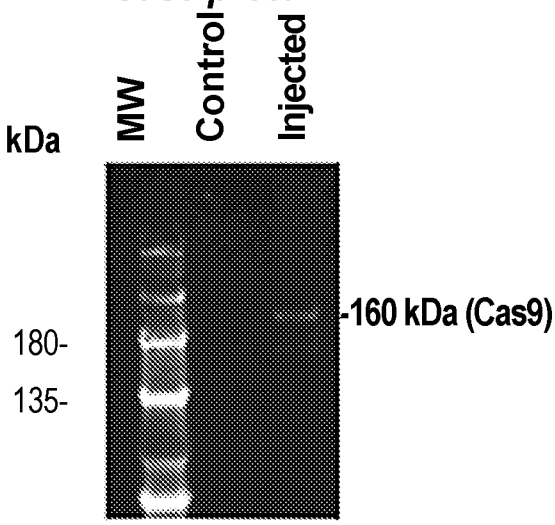


FIG. 2E

In planta injection – confocal imaging
exu:Cas9-T2A-mCherry (72 hr)

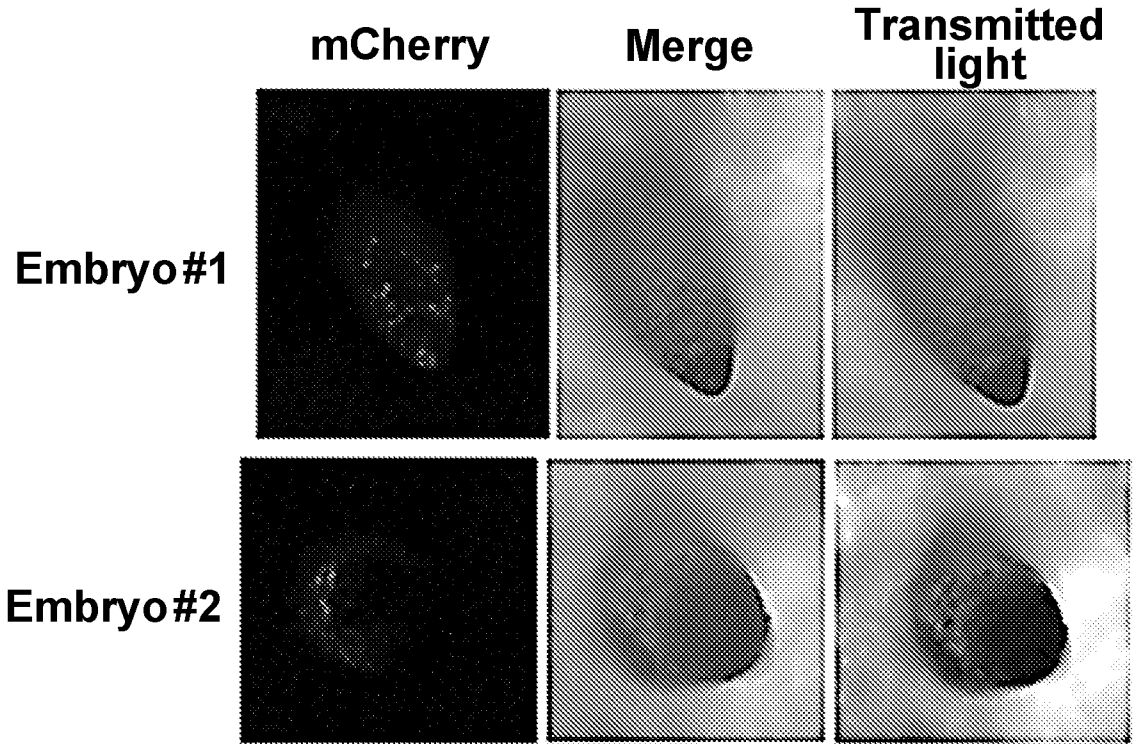


FIG. 2F

Vacuum infiltration (48 hr)

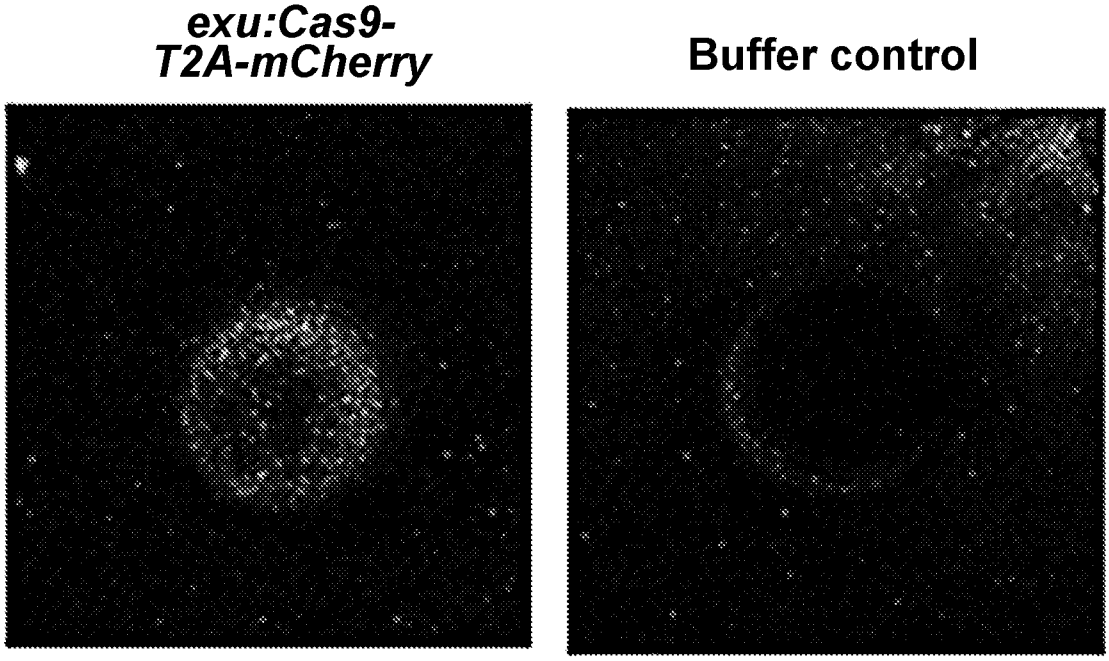


FIG. 2G

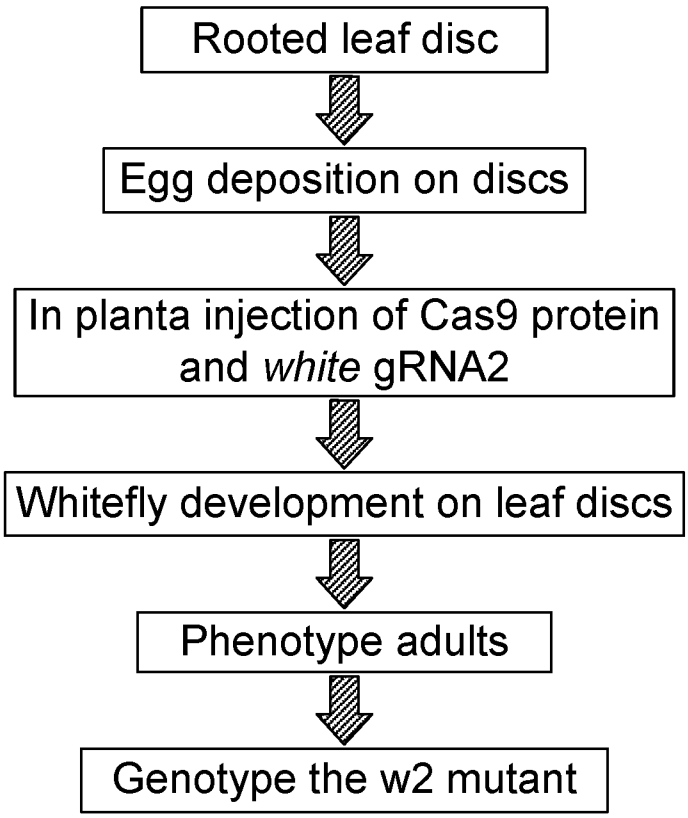


FIG. 2H

***w2* mosaic mutant**

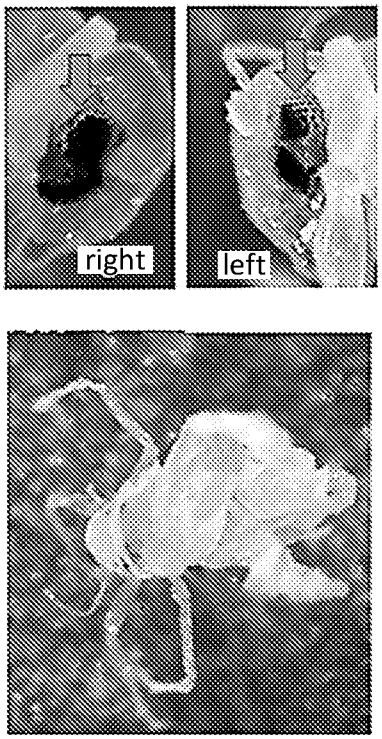
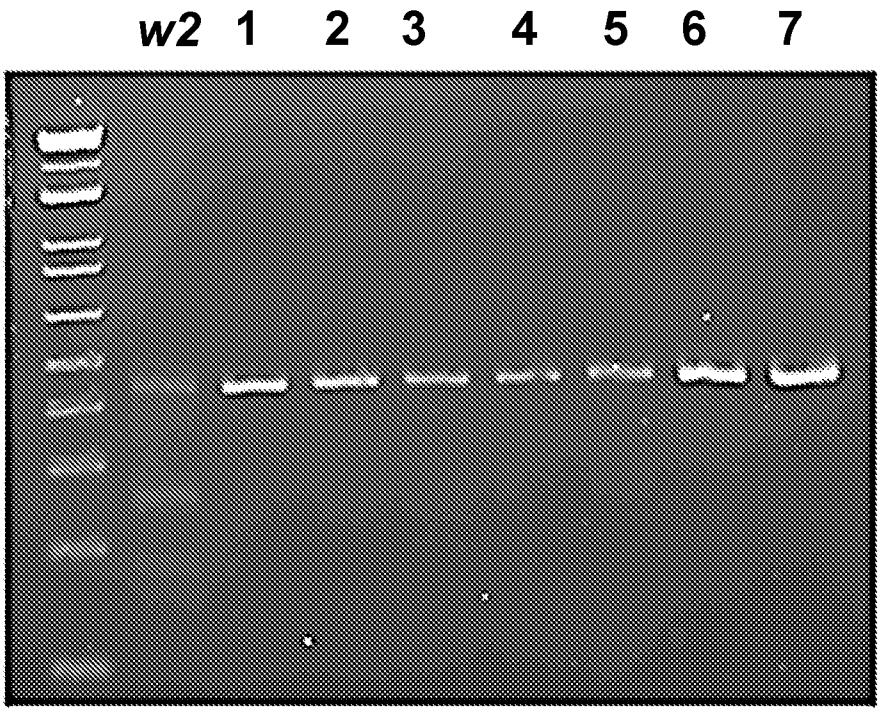


FIG. 2I



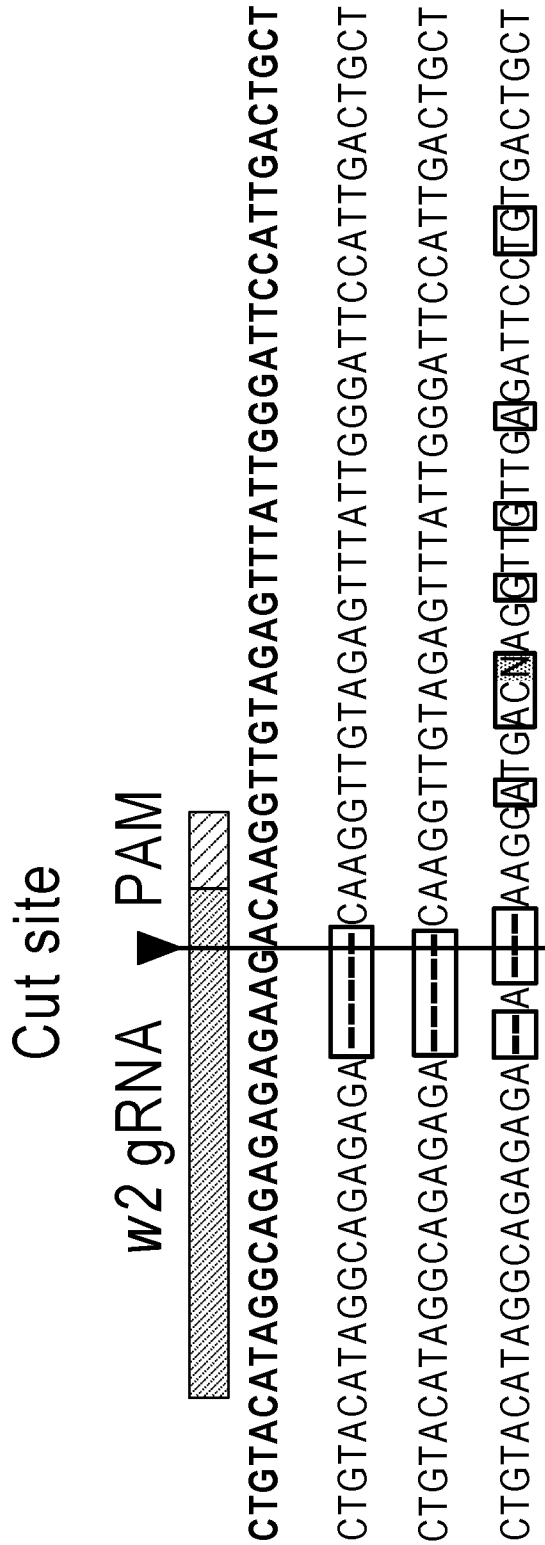

Cut site
w2 gRNA
PAM
CTGTACATAGGCAGAGAGAAGACAAGGTTGTAGAGTTTATTGGGATTCCATTGACTGCT
CTGTACATAGGCAGAGAGA----CAAGGTTGTAGAGTTTATTGGGATTCCATTGACTGCT
CTGTACATAGGCAGAGAGA----CAAGGTTGTAGAGTTTATTGGGATTCCATTGACTGCT
CTGTACATAGGCAGAGAGA--A----AAGGATTGTAGACNAGGTTTGTTGGATTCCTGTGACTGCT

FIG. 2J

FIG. 3

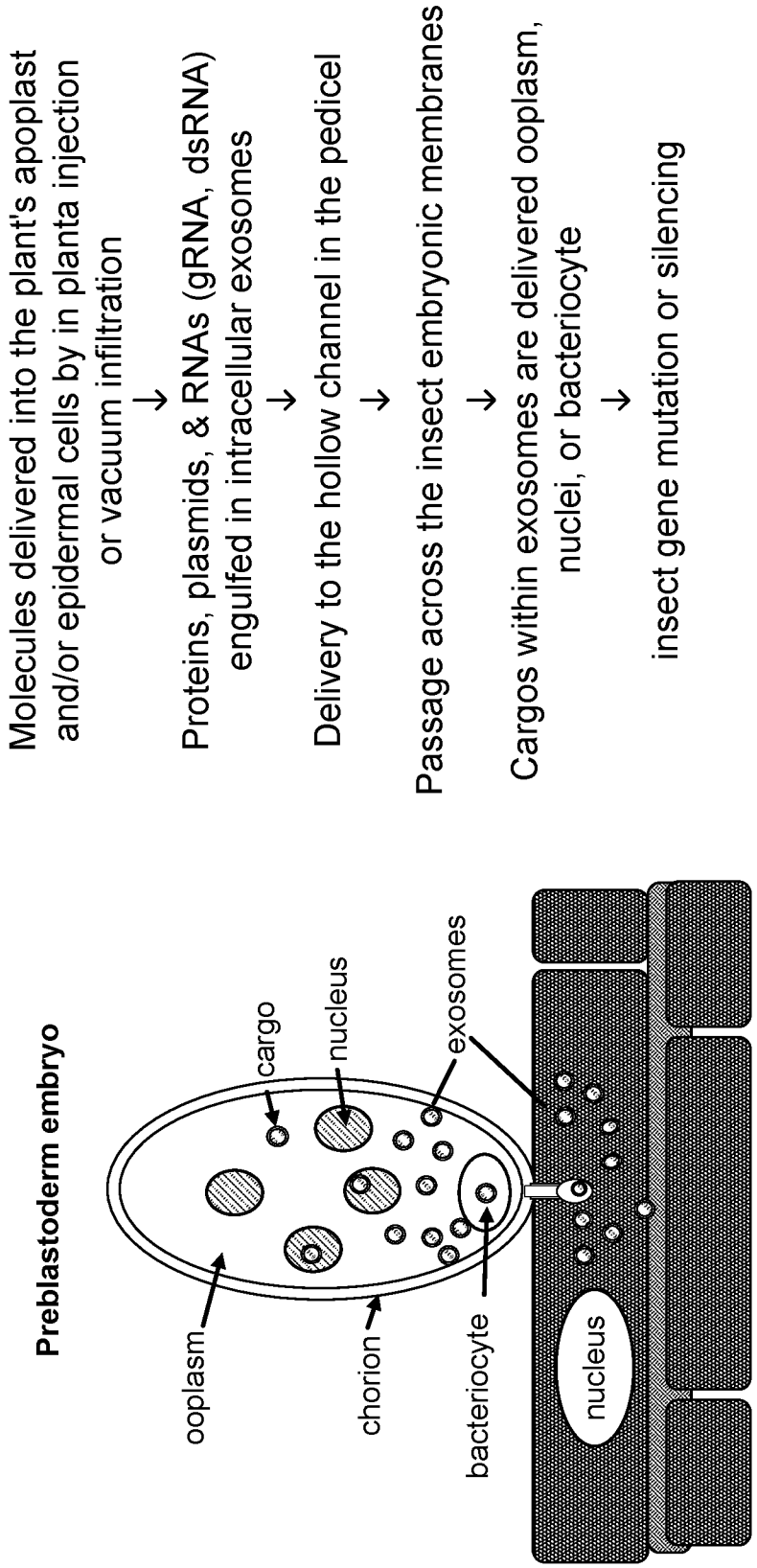

Molecules delivered into the plant's apoplast and/or epidermal cells by in planta injection or vacuum infiltration
Proteins, plasmids, & RNAs (gRNA, dsRNA) engulfed in intracellular exosomes
Delivery to the hollow channel in the pedicel
Passage across the insect embryonic membranes
Cargos within exosomes are delivered ooplasm, nuclei, or bacteriocyte
insect gene mutation or silencing
Preblastoderm embryo
cargo
nucleus
exosomes
ooplasm
chorion
bacteriocyte
nucleus

FIG. 4

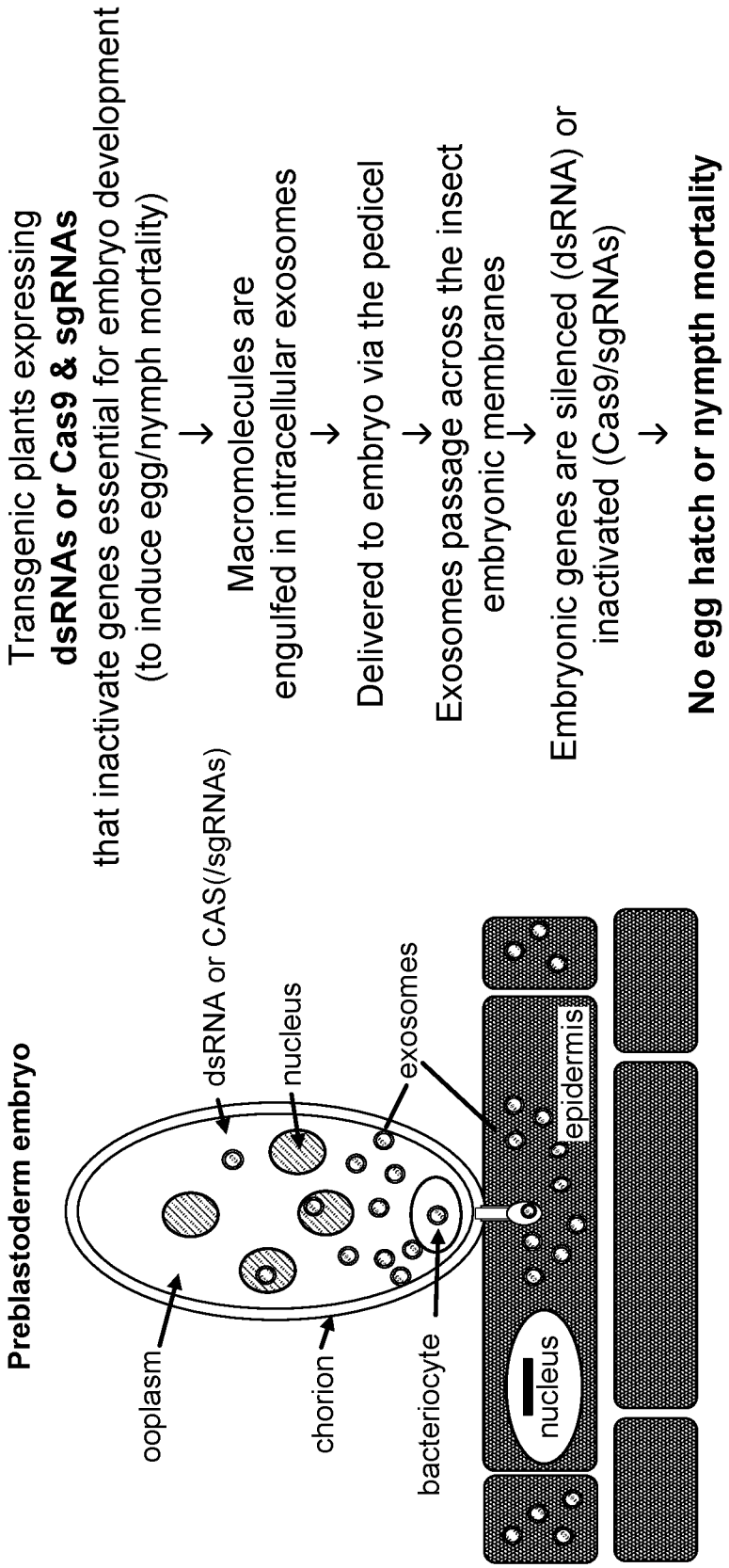

Transgenic plants expressing
dsRNAs or Cas9 & sgRNAs
that inactivate genes essential for embryo development
(to induce egg/nymph mortality)
→
Macromolecules are
engulfed in intracellular exosomes
→
Delivered to embryo via the pedicel
→
Exosomes passage across the insect
embryonic membranes
→
Embryonic genes are silenced (dsRNA) or
inactivated (Cas9/sgRNAs)
→
No egg hatch or nympth mortality
Preblastoderm embryo
dsRNA or CAS(/sgRNAs)
nucleus
exosomes
ooplasm
chorion
bacteriocyte
epidermis
nucleus

FIG. 5

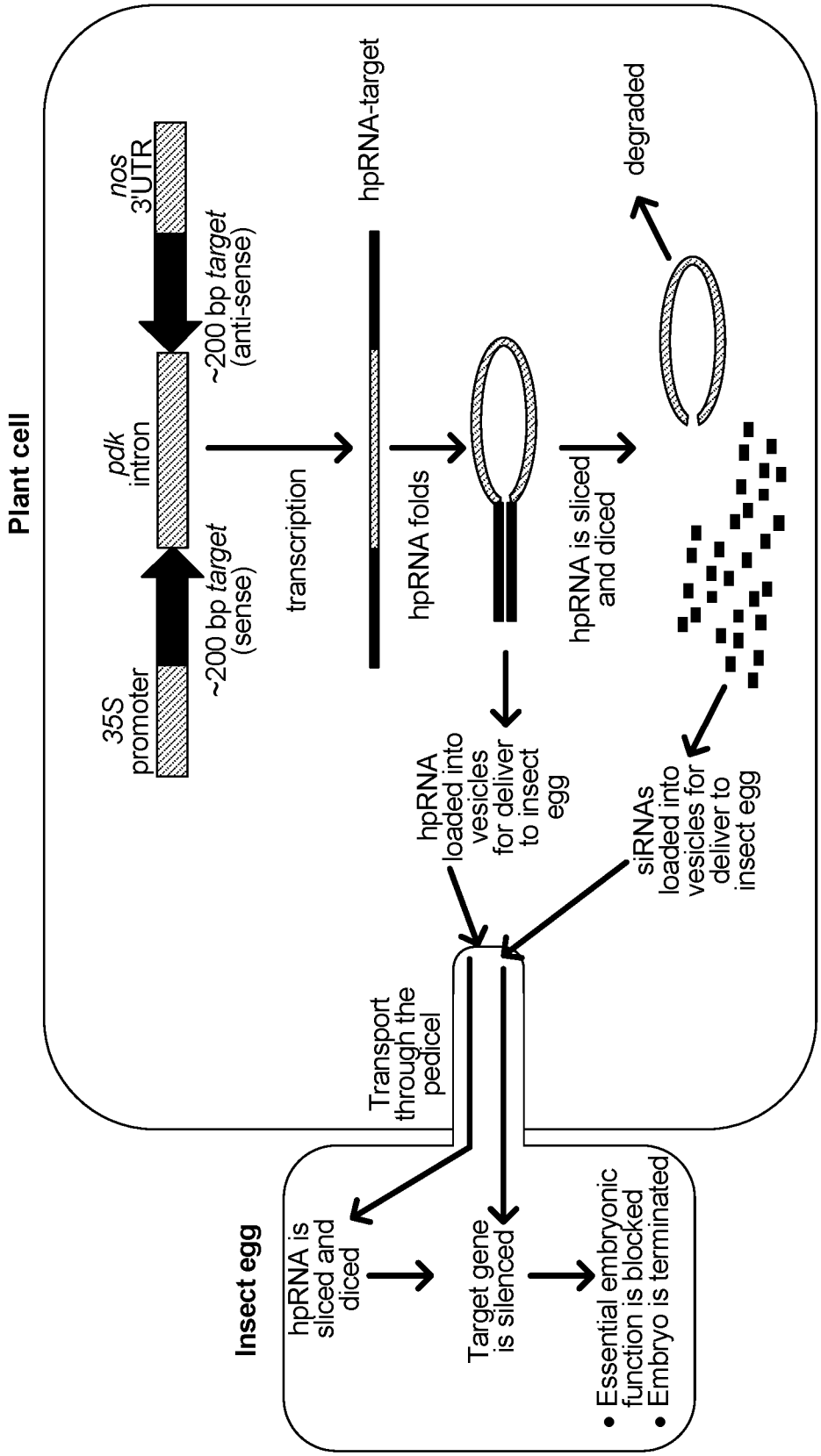

Plant cell
35S promoter
~200 bp target (sense)
pdk intron
~200 bp target (anti-sense)
nos 3'UTR
transcription
hpRNA-target
hpRNA folds
hpRNA is sliced and diced
degraded
hpRNA loaded into vesicles for deliver to insect egg
siRNAs loaded into vesicles for deliver to insect egg
Transport through the pedicel
Insect egg
hpRNA is sliced and diced
Target gene is silenced
• Essential embryonic function is blocked
• Embryo is terminated

FIG. 6

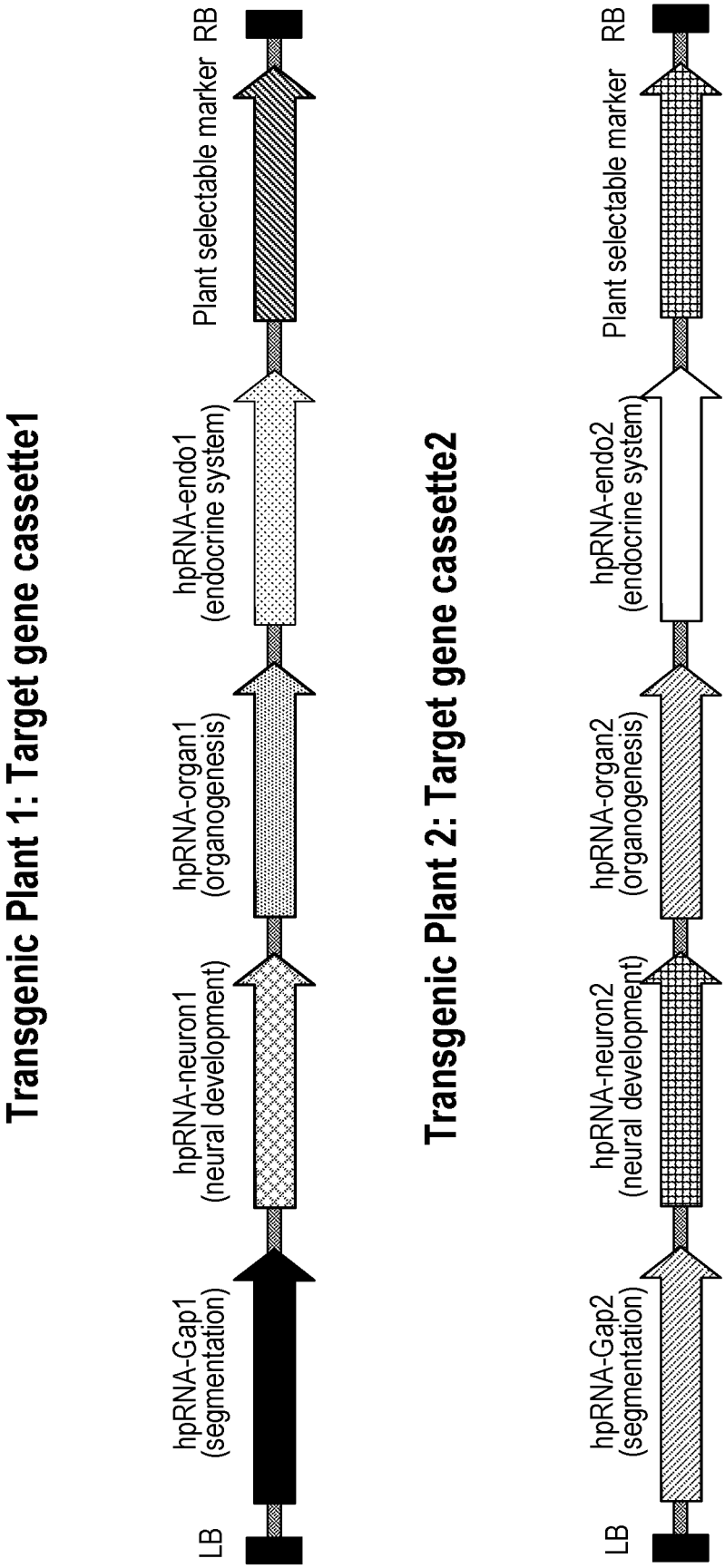

Transgenic Plant 1: Target gene cassette1
LB
hpRNA-Gap1 (segmentation)
hpRNA-neuron1 (neural development)
hpRNA-organ1 (organogenesis)
hpRNA-endo1 (endocrine system)
Plant selectable marker
RB
Transgenic Plant 2: Target gene cassette2
LB
hpRNA-Gap2 (segmentation)
hpRNA-neuron2 (neural development)
hpRNA-organ2 (organogenesis)
hpRNA-endo2 (endocrine system)
Plant selectable marker
RB

FIG. 7

- Made transgenic plant expressing an endomembrane-targeted or cytosolic Cas9 using an **constitutive or epidermal cell-specific promoter** (Fig. 5)
- Introduce sgRNA target gene cassette into this Cas9 cassette.
  - Two *U6.sgRNAs* per gene – to increase deletions that cause null mutations.
  - Four genes targeting different embryonic biological processes provide durability.
- The sgRNA cassette will provide a quadruple layer of protection that will account for differences in the ability of a sgRNA to edit a gene and for population sequence drift.
- Transgenic plants 1 and 2 can be crossed to target 8 genes at a time.

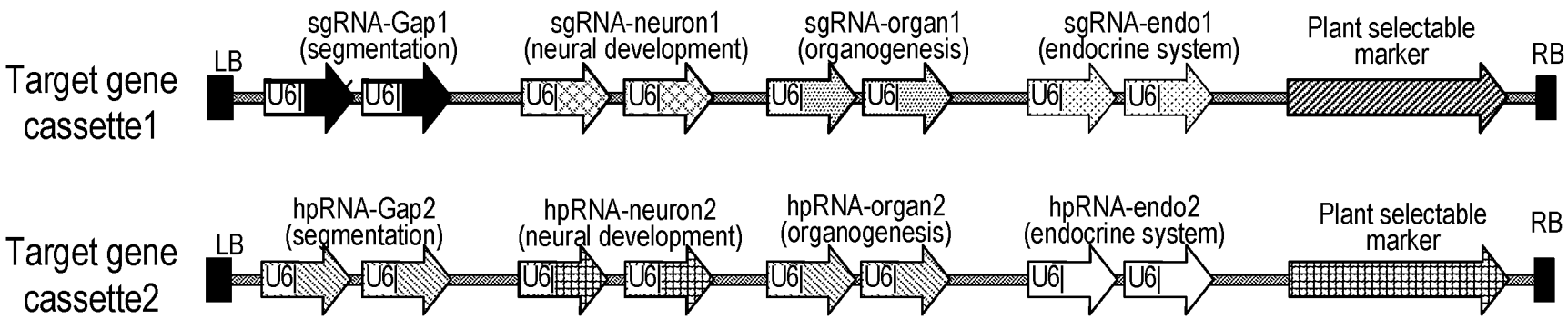
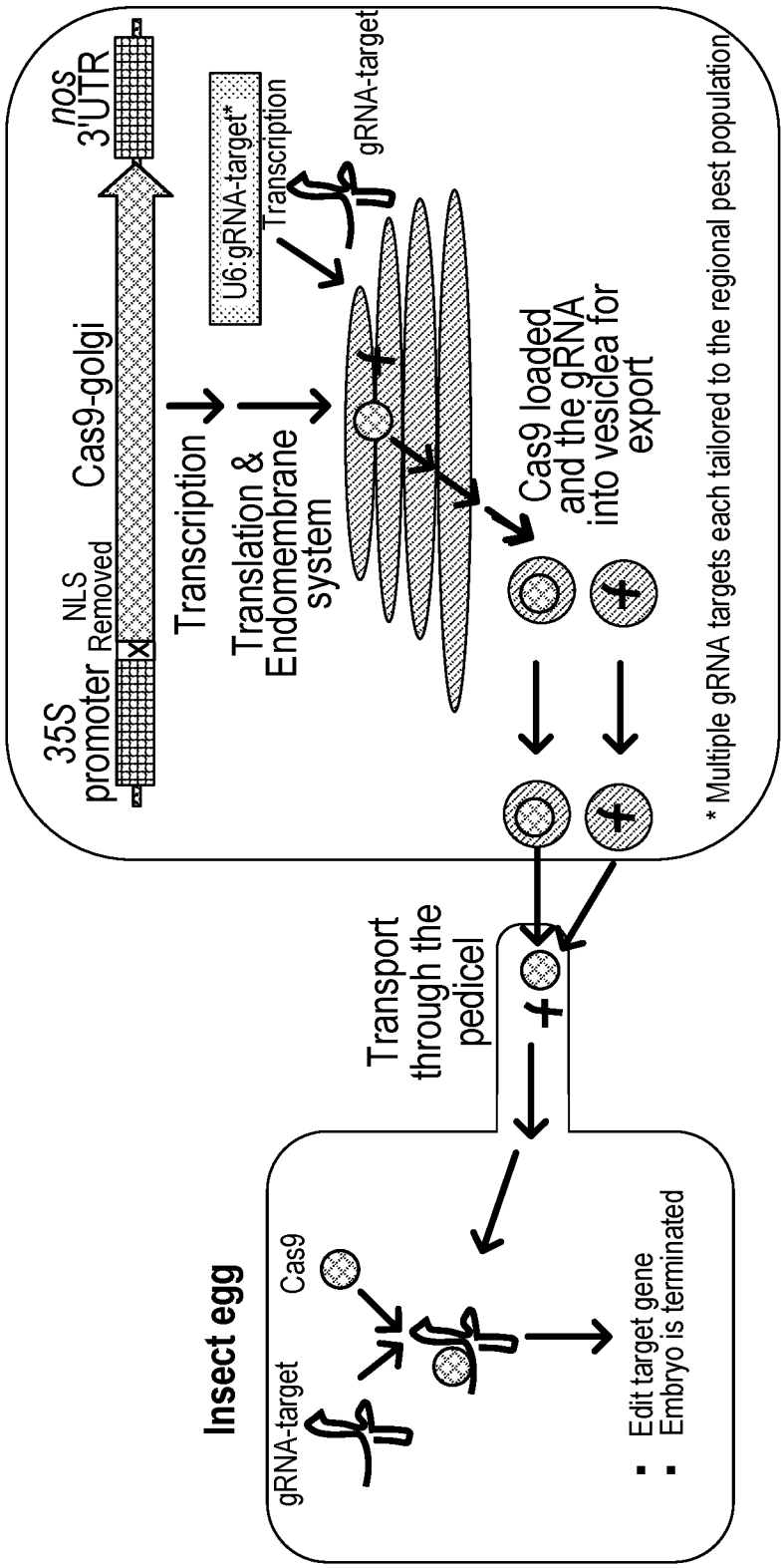


FIG. 8


CRISPR-delivered resistance to insect infestation
Plant cell
35S promoter
NLS Removed
Cas9-golgi
nos 3'UTR
Transcription
Translation & Endomembrane system
U6:gRNA-target*
Transcription
gRNA-target
Cas9 loaded and the gRNA into vesiclea for export
* Multiple gRNA targets each tailored to the regional pest population
Transport through the pedicel
Insect egg
gRNA-target
Cas9
Edit target gene
Embryo is terminated

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 23/85330

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12N 15/11 (2024.01)
ADD. C12N 15/09, C12N 15/63, C12N 15/87 (2024.01)

CPC - INV. C12N 15/102
ADD. C12N 15/111, C12N 15/113, C12N 2310/20

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.
Y	US 2021/0105986 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 15 April 2021 (15.04.2021) para [0007]; [0036]; [0044]; [0048]; [0077]; [0079]; [0081]; [0082]; [0084]; [0095]	1-4, 25-30
Y	BUCKNER et al. Characterization and functions of the whitefly egg pedicel. Archives of Insect Biochemistry and Physiology, 18 December 2001, Vol. 49, No. 1, pgs. 22-33, abstract	1-4, 25-30
Y	US 2009/0188008 A1 (LASSNER) 23 July 2009 (23.07.2009) para [0025]; [0113]; [0118]	25-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"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
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 March 2024 (01.03.2024)	APR 29 2024
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 23/85330

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-24, 31-41
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.