Composition and Methods for Pest Control Management

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COMPOSITIONS AND METHODS FOR PEST CONTROL MANAGEMENT

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References Cited
U.S. PATENT DOCUMENTS
7,261,586 B2 8/2007 Wu et al.

FOREIGN PATENT DOCUMENTS

OTHER PUBLICATIONS

Disclosed are genetically modified nudiviruses capable of being sexually transmitted by an insect useful for controlling pest populations. The genetically modified nudiviruses are capable of causing sterility in a target population of insects. Also disclosed are insects infected with the disclosed genetically modified nudiviruses, methods of making the genetically modified nudiviruses, and methods of using the genetically modified nudiviruses to control an insect pest population.
OTHER PUBLICATIONS


FIG 1.

Wild-type HzNV-2, 231,621 bp

Direct Repeats:

ORF2
ORF1
ORF7, juvenile hormone esterase

ORF55
ORF56
ORF78
ORF90
ORF92
ORF113

Wild-type
KS-3
KS-45
KS-51
FIG 2.

[Bar chart showing the percentage of gonadal F1 female progeny for different viruses used to infect female moths. The x-axis represents the virus used, and the y-axis represents the percentage of progeny. The chart compares OviD2 and OviD3.]
FIG 3.

- Virus used to infect female moths

- Number of eggs laid by F1 female progeny


- OviD2

- OviD3
Females were infected and eggs were counted 1-4 days later.
FIG 5.

[Bar chart showing percent of gonadal females for Syringe and Pin inoculation methods.]

- KS-3
- WT
- yfp HzNV-2
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COMPOSITIONS AND METHODS FOR PEST CONTROL MANAGEMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Application Ser. No. 62/161,674, filed on May 14, 2015, entitled “Mutant Nudivirus and Method for Using Same for Insect Control,” the contents of which are incorporated herein in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under 1338775 by the National Science Foundation. The government has certain rights in the invention.

BACKGROUND

Insect pests cause crop damage worldwide resulting in significant losses to food and fiber crops and increased production costs that target control of such pests. For example, the Heliothine complex of lepidopteran moths cause in excess of 2 B dollars in damage and cost of control in the United States annually. While all crops are susceptible to similar pest pressure, transgenic expression of Bacillus thuringiensis (Bt) toxins was developed to control the lepidopteran pests and has become a major tool for control of these and other insect pests. Since the commercial introduction of Bt crops in 1996, they have been adopted around the world and have been grown on more than one billion acres worldwide. In the US, 81% of corn and 84% of cotton express one or more Bt toxins. (http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx, 2015 report.) Unfortunately, due to the remarkable ability of insects to adapt to insecticides, resistance to Bt toxins was predicted and reports of field-evolved resistance and reduced efficacy are increasing. Such resistance is a threat to the sustainability of important Bt crops, in the US and elsewhere. Thus, there is a continuing need to develop new methods to control insect pests. For example, Helicoverpa zea (H. zea, commonly known as the corn earworm), is a major polyphagous moth pest in the Heliothine complex in the United States and causes millions of dollars of damage to corn and cotton plants each year.

A number of pests in the Heliothine complex of moths, notably H. zea H. armigera and Heliothis virescens are highly polyphagous and cause economically significant damage to many crops. Crops commonly damaged by H. zea include cotton, corn, soybean, sunflowers, tomato, sorghum, strawberry, peppers, beans, aubergine, okra, peas, millet, cucumber, melon, lettuce, cauliflower, and cabbage. Because H. zea attacks a wide variety of plants and, in many instances, is developing resistance to Bt crops, farmers rely heavily on pesticides to control this pest insect.

The need for pest management, such as in field, fruit and vegetable crops, is a need in the art, which will only become more critical as resistance to Bt expands. Further, there is a need for pest management that does not involve the use of conventional pesticides or transgenic technologies such as in organic cropping systems. The instant invention addresses one or more aforementioned needs in the art.

BRIEF SUMMARY

Disclosed are genetically modified nudiviruses capable of being sexually transmitted by an insect useful for controlling pest populations. The genetically modified nudiviruses are capable of causing sterility in a target population of insects. Also disclosed are insects infected with the disclosed genetically modified nudiviruses, methods of making the genetically modified nudiviruses, and methods of using the genetically modified nudiviruses to control an insect pest population.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic of nudivirus genome showing genes known to be involved or implicated in sterilizing mutation.

FIG. 2. Percentage of agonadal female F1 progeny. Wild-type (WT) and mutant HzNV-2 generated by chemical mutagenesis (KS-3, KS-38, KS-39, KS-45, KS-51, and KS-52) were injected into adult female moths on the day of emergence, and eggs were collected on oviposition days 2 (OviD2) and 3 (OviD3). Female F1 progeny were reared to adult moths and evaluated for the presence of a viral plug indicating an agonadal pathology (OviD2-WT n=17, KS-3 n=24, KS-38 n=23, KS-39 n=26, KS-45 n=19, KS-51 n=14, KS-52 n=18; OviD3-WT n=15, KS-3 n=16, KS-38 n=22, KS-39 n=16, KS-45 n=19, KS-51 n=18, KS-52 n=23).

FIG. 3. Occurrence of complete sterility in agonadal female F1 progeny. Wild-type (WT) and mutant HzNV-2 (KS-3, KS-38, KS-39, KS-45, KS-51, and KS-52) were injected into adult female moths on the day of emergence, and offspring eggs were collected on oviposition days 2 (OviD2) and 3 (OviD3). Female F1 progeny were reared to adult moths and evaluated for ability to lay viable eggs. Failure to lay eggs or lay viable eggs indicates complete sterility (OviD2-WT n=17, KS-3 n=24, KS-38 n=23, KS-39 n=26, KS-45 n=19, KS-51 n=14, KS-52 n=18; OviD3-WT n=15, KS-3 n=16, KS-38 n=22, KS-39 n=16, KS-45 n=19, KS-51 n=18, KS-52 n=23). This illustrates that these mutant HzNV-2 causes decreased egg production and sterility.

FIG. 4. Effect of viral titer on H. zea egg production. Female adult moths (7) were injected with 100 µl wt HzNV-2 (high dose: 4x10^4 pfu/ml; low dose: 40 pfu/ml) or yfp recombinant HzNV-2 (high dose: 1x10^7 pfu/ml; low dose: 1x10^6 pfu/ml) isolated from cell culture and mated with uninfected male moths (9). Eggs were collected on oviposition days 2-5 and counted. Uninfected females were mated with uninfected males as a control.

FIG. 5. Direct inoculation of insect larvae causes high numbers of agonadal moths. Wild-type (WT) HzNV-2 and mutant KS3 were amplified in S0 insect cell cultures and injected into third instar larvae via an insulin syringe. WT HzNV-2 and mutant yfp HzNV-2 virus isolated from viral plugs of agonadal female moths were used to infect third instar larvae via a pre-sterilized pin. After both types of infections, larvae were reared to adult moths, and females were examined for the presence of a viral plug, an indicator of virus infection and agonadal pathology (Syringe, WT n=20, KS3 n=25; Pin, WT n=20, yfp HzNV-2 n=21).

FIG. 6. 1.5% agarose gel showing PCR results that yfp primers were used to amplify hypothetical gene ORF78 from HzNV-2. Also disclosed are insects infected with the disclosed genetically modified nudiviruses, methods of making the genetically modified nudiviruses, and methods of using the genetically modified nudiviruses to control an insect pest population.

DETAILED DESCRIPTION

As used herein and in the appended claims, the singular forms “a,” “and,” and “the” include plural referents unless
the context clearly dictates otherwise. Thus, for example, reference to “a method” includes a plurality of such methods and reference to “a dose” includes reference to one or more doses and equivalents thereof known to those skilled in the art, and so forth.

The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, e.g., the limitations of the measurement system. For example, “about” can mean within 1 or more than 1 standard deviations, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, or up to 10%, or up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term “about” meaning within an acceptable error range for the particular value should be assumed.

The term “closely related” as used herein, with respect to the term insect and/or moth, means a species so closely related so as to support replication of the HzNV-2 virus.

The terms “express” and “expression” mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an “expression product” such as a protein. The expression product itself, e.g., the resulting protein, may also be said to be “expressed”. An expression product can be characterized as intracellular, extracellular or secreted. The term “intracellular” means something that is inside a cell. The term “extracellular” means something that is outside a cell. A substance is “secreted” by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

The term “gene”, also called a “structural gene” means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include introns and regulatory DNA sequences, such as promoter sequences, 5′-untranslated region, or 3′-untranslated region which affect for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription.

By “genetically modified” is meant a gene that is altered from its native state. The term “genetically modified,” as used herein, includes a sequence (a virus, for example) that contains genetic material from more than one organism. The term further includes a sequence that is modified from its native state, for example, via a deletion or insertion, and which does not include genetic material from more than one organism. The latter may be referred to as a “mutant” as used herein.

The instant disclosure addresses one or more needs in the art as described above. In one aspect, the present disclosure addresses the globally important need for new methods to control insect pests in crops threatened by such pests. In a further aspect, the disclosure addresses an increasingly important issue, Bt resistance, that threatens the sustainability of insect-resistant transgenic crops.

A sexually transmitted insect virus, Helicoverpa zea nudivirus-2 (HzNV-2, accession number NC_004156.), is known to cause approximately 33% of infected H. zea to be sterile. (Raina 1995). Wildtype (WT) HzNV-2, however, is not a potential biological control agent due to the high proportion of asymptomatic carrier moths. Applicant has found that HzNV-2 can be modified so that extremely high percentages, for example, up to 100%, or greater than about 90%, of the infected H. zea become sterile. As such, the mutant HzNV-2 may be an important tool in controlling various insect pests by causing collapse in the target insect population. In turn, this modified virus can be used to infect a target insect, and control an insect population without the use of traditional pesticides, or, alternatively, can be used in combination with traditional pesticides such that the amount of the pesticide used is minimized. Such a technology may have particular utility in control of populations of Bt resistant insects and invasive insect populations for which traditional pesticides are ineffective. Applicant’s approach allows for pest control via release of insects infected with a sexually transmitted virus that can be transmitted by mating in the targeted farming area. The approach developed by Applicant is effective for both transgenic and/or non-transgenic crops and is capable of targeting pest species in which the virus replicates and is sexually transmitted.

Attempts to control insect populations via genetic manipulation of crops is currently limited due to the ability of insects to rapidly develop resistance to the genetically added toxins, and is further limited by the costs to producers to use such modified crops. For example, crops expressing the Bacillus thuringiensis (Bt) toxins were introduced twenty years ago to control caterpillar pests. Since then, they have been adopted worldwide, planted on more than one billion acres and have become one of the most successful and rapidly adopted agricultural technologies since the ‘green revolution’ of the mid-20th century (James, 2012). As of 2015 in the US, 81% of corn and 84% of cotton express one or more Bt toxins. However, the widespread adoption of Bt technology carries the significant risk that overuse will inevitably lead to development of insect resistance to Bt toxins and crop failures, which threatens the technology’s continued viability (Carriere et al., 2010, Tabashnik et al., 2013; Tabashnik et al., 2009). This risk, which always has been recognized by regulators, industry, and researchers, has been managed by resistance monitoring and the use of refuge strategies to delay resistance. These refuge strategies, which have been mandated by the EPA in the USA with similar mandates in other countries, entail the planting of nearby non-transgenic plants to maintain susceptible insect populations (EPA, 1998; Huang et al., 2011). Unfortunately, this practice is not always followed due to cost to producers, and is not always effective because of the remarkable ability of insects to evolve resistance to insecticides. Increasing insect resistance to Bt plants is reported, and some insects exhibit resistance traits that are genetically dominant (Campagne et al., 2013). To summarize, Bt-resistant insects represent an ongoing and increasingly important threat to the continued efficacy of Bt crops, and to food and fiber production in the US and worldwide.

One insect pest threatening Bt crops is the corn earworm, Helicoverpa zea, a lepidopteran moth. H. zea is found throughout North America, for example, where it is the second most costly crop pest (Fit, 1989), and is also found in Central America, the Caribbean, and South America. H. zea, which feeds on many different plants and has several common names (e.g., corn earworm, cotton budworm, tomato fruitworm) has some strains that are 1000-times...
more resistant to Bt toxin than susceptible insects (Ali and Luttrell, 2007; Ali et al., 2006).

Applicant has developed a new approach to suppress insect pest populations. In a further aspect, Applicant has developed a new approach to managing Bt resistance, which relies upon engineering or mutating a sexually-transmitted insect virus that sterilizes infected insects (including complete or partial sterility). Insects containing the mutant virus may be released in areas where Bt resistance is present in *H. zea* populations, thereby suppressing these targeted populations and preserving the utility of the Bt transgenic plants and/or non-transgenic plants. Similarly, the susceptible pest insects are commonly invasive across the world and the disclosed methods may be used to reduce and eliminate the invasive pest populations. The viruses developed by Applicant are mutant and recombinant forms of a naturally-occurring (i.e., wild-type) virus, *Helicoverpa zea* nudivirus 2 (HzNV-2), which infects *H. zea*. HzNV-2 is the only lepidopteran insect virus which has been shown to be sexually transmitted and causes sterility in both males and females. In one aspect, the infected insect may have partial sterility, defined as when a female *H. zea* moth lays less than 30 viable eggs each day due to damage to her reproductive organs. In one aspect, the infected insect may have complete sterility, defined as the inability of a female moth to lay viable eggs due to damage to her reproductive organs.

In one aspect, disclosed is a genetically modified nudivirus of a wild type nudivirus. The genetically modified nudivirus contemplated herein is generally capable of being sexually transmitted by an insect and capable of causing sterility in an insect at a rate of greater than about 50%, or from about 50% to about 100%, or from about 80% to about 95% or from about 90% to about 100% following infection of said insect with said nudivirus comprising a genetic mutation.

In one aspect, the wild type nudivirus has at least about 80% sequence identity to *Helicoverpa zea* nudivirus 2 (HzNV-2). The wild type nudivirus may be characterized in that it has a latent phase, has about 80% or greater sequence identity to *Helicoverpa zea* nudivirus 2 (HzNV-2, also known as *Heliothis zea* nudivirus or gonad specific virus) virus, and is capable of replicating in one or more moths.

In one aspect, the genetically modified nudivirus may contain a disruption in a latent phase of said wild type nudivirus.

In one aspect, the insect may be a lepidopteran moth in the family noctuidae which supports replication of the HzNV-2 virus in reproductive tissues sufficient to cause sterility at a rate of 50% or greater. The insect may be selected from the *Helicoverpa zea* (H. zea) *H. armigera*, *H. assulta*, *Heliothis virescens*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua* or a closely-related moth, for example, a closely related moth, or noctuid moths. The insect may be a female or a male.

In one aspect, the genetically modified nudivirus may be a mutation in one or more genes selected from the persistence-associated gene (pagl) (which encodes PAT1, or the persistence associated transcript (SEQ ID NO: 7), ORF90 (SEQ ID NO: 4), ORF92 (SEQ ID NO: 5), ORF2 (SEQ ID NO: 3), or combinations thereof, such that the modified virus is sufficient to disrupt expression of one or more of such genes, for example, wherein said disruption reduces expression or is a functional knockout. In one aspect, the genetic modification may be a mutation in the persistence associated gene (pagl) sufficient to disrupt expression of the pagl gene. In one aspect, the genetic modification may be a mutation in the PAT1 gene (SEQ ID NO: 7), which is the persistently-associated transcript and has been shown to be involved in the establishment of latent infections of HzNV-1. In one aspect, the genetic modification may be a mutation in one or more sequences selected from dr1 (atgaactgaggtatcatgcac, SEQ ID NO: 14), dr2 (gaacactctaaactaaggggcctgtaaccaag, SEQ ID NO: 15), dr3 (atgaanaagctggcaggaagagggagaagagctga, SEQ ID NO: 16), dr4 (atacactgtaactaagctgagactgctgctgcaaaagccaaagctg, SEQ ID NO: 18), dr6 (agctgcaactaagggcagaggtcctgctgcaaaagccaaagctg, SEQ ID NO: 19), or a combination thereof. In one aspect, the genetic modification may be a mutation in dr3 (SEQ ID NO: 16), for example, KS-3 in which there is a 4 bp insertion at 175,550 and KS-45, 80 bp insertion at 175,650. In one aspect, the genetic modification may be a mutation in dr6 (SEQ ID NO: 19), for example, KS-51, having a 29 bp deletion at 180,270-180,299.

In a further aspect, the genetic modification is one in which an increase in activity of a viral regulatory gene results from the modification, wherein said viral regulatory gene is hhi-1 (SEQ ID NO: 8). In certain aspects, the identification of a genetic modification of interest can be determined via detection of increased hhi-1 activity.

In one aspect, the genetically modified nudivirus may be obtained via chemical mutagenesis. In another aspect, the genetically modified nudivirus may be obtained via recombinant DNA technology. Exemplary, non-limiting methods are disclosed herein. In a further aspect, the genetically modified nudivirus may be obtained using gene editing technology as is known in the art.

In one aspect, a method of reducing a population of lepidopteran moths is disclosed. The method may comprise the step of introducing an insect infected with a genetically modified nudivirus as disclosed herein into the population of interest. In one aspect, infected insects of a single sex may be introduced into a target population, for example an all-male or all-female population of insects. In another aspect, a mixed population of infected insects may be introduced.

In one aspect, an insect infected with a virus as described above is disclosed. The insect may be a lepidopteran moth. In further aspects, the insect may be *Helicoverpa zea* (H. zea) *H. armigera*, *H. assulta*, *Heliothis virescens*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua* or a closely-related moth, for example, a closely related moth, or noctuid moths. The insect may be a female or a male.

In one aspect, a method of making an insect capable of transmitting a genetically modified nudivirus as disclosed herein to a population of insects is disclosed. The method may comprise the step of infecting an insect with a genetically modified nudivirus as described herein. In one aspect, the insect is a lepidopteran moth. The insect may be *Helicoverpa zea* (H. zea) *H. armigera*, *H. assulta*, *Heliothis virescens*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua* or a closely related moth or noctuid moth. The method may utilize male insects, female insects, or both. In one aspect, the genetically modified nudivirus may be derived from a viral plug. The genetically modified nudivirus may be administered orally to the insect. In other aspects, the genetically modified nudivirus may be administered to an insect via direct inoculation of insect larvae or adult moths by puncturing the cuticle of the insect with a pin containing viral inoculum derived from a viral plug. In a further aspect, the genetically modified nudivirus may be administered to the insect via direct hypodermic injection into third instar larvae or moths.
In one aspect, a method of protecting a crop susceptible to a moth pest from moth pest damage is disclosed. In this aspect, the method may comprise the step of introducing insects infected with a genetically modified nudivirus as described herein, into a crop of interest. The crop may be any crop threatened by the pest, and may include, for example, the following non-limiting list of crops: corn, cotton, soybeans, tomatoes, sorghum, artichoke, asparagus, cabbage, cantaloupe, collard, cowpea, cucumber, eggplant, lettuce, lime bean, melon, okra, pea, pepper, potato, pumpkin, snap bean, spinach, squash, sweet potato, and watermelon, alfalfa, clover, cotton, flax, oat, millet, rice, sorghum, soybean, sugar cane, sunflower, tobacco, vetch, and wheat, avocado, grape, peaches, pear, plum, raspberry, strawberry, carnation, geranium, gladiolus, nasturtium, rose, snapdragon, zinnia, and combinations thereof (see http://edis.ifas.ufl.edu/in302). In certain aspects, the crop may be a Bacillus thuringiensis (Bt) toxin producing crop. The insect used may be any insect as described above.

In a further aspect, a method of sterilizing an insect population is disclosed. The method may include the step of introducing a genetically modified nudivirus as described herein into a target insect population. This may include an invasive insect population, and may further include Heliocoverpa zeae (H. zeae), H. armigera, Heliothis virescens, Agrotis ipsilon, Spodoptera frugiperda, Spodoptera exigua and closely related moths or noctuid moths.

In one aspect, a method of making a genetically modified nudivirus via chemical modification is disclosed. The method may comprise the steps of:

a) incubating a population of insect cells infected with a virus with about 0.05 mM to about 0.1 mM 1,3-butadiene diepoxide (or 1,2,3,4-Diepoxybutane or “DEB”) for at least 1 hour and up to five hours at a temperature range of about 26 to about 28°C, wherein the population of infected insect cells may comprise an Sf9 insect cell, for example, further wherein the insect cell may be infected with a virus having at least 80% identity, or at least 85% identity, or at least 90% identity, or at least 95% identity, or at least 99% identity to a wild-type HzNV-2 virus (SEQ ID NO: 1), and wherein the incubation is sufficient to induce one or more mutations in the HzNV-2 virus;

b) purifying the virus, wherein the purifying step includes the steps of:

i. culturing the population of insect cells infected with a virus in a DEB-free media, wherein the population of infected insect cells are isolated and washed prior to the culturing step;

ii. collecting a supernatant from DEB-free media to obtain a DEB-exposed virus population;

iii. amplifying and collecting the mutated virus from the DEB-exposed virus population, wherein the collection step may comprise selecting virus from a plaque having a large plaque phenotype.

Exemplary methods making a genetically modified nudivirus via chemical modification are provided below.

EXAMPLES

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All references, publications, patents, patent applications, and commercial materials mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the materials and/or methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Method—Generating a Recombinant HzNV-2 Virus

To generate a yfp insert mutant virus using recombinant DNA technology, Applicant replaced the pagl gene with a gene encoding yellow fluorescent protein (yfp) by homologous recombination. pagl expresses a microRNA that suppresses the expression of the viral transcription factor, hhi-1, an RNA intermediate necessary to maintain latency in HzNV-1 (Chao, 1998; Wu and Wu, 2011). To inactivate the pagl gene, a pUC57-based transfer vector, yfp-pUC57, was designed and synthesized with the yfp gene controlled by the Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus immediate 2 (OpIE2) promoter, and flanked by 1.2 kb of viral HzNV-2 sequences upstream and downstream of pagl.

The yfp HzNV-2 recombinant virus was generated by homologous recombination of yfp/pagl-pUC57 plasmid with HzNV-2 genomic DNA after transfection into Sf9 insect cells. The mutant virus was plaque purified, screened for YFP fluorescence using a Zeiss observer A1 fluorescent microscope and the AxioVision Rel. 4.6 program, and amplified in Sf9 cells in nudivirus media (lx Supplemented Grace’s Media, 7% FBS, 1% Penicillin/streptomycin) in 25 cm² tissue culture flasks. Viral DNA was isolated from the cell culture supernatant using DNAzol (ThermoFisher Scientific #4398881). PCR results confirmed deletion of pagl and presence of the yfp gene using internal pagl (F 5'-GGGTCTGTTGCGACCTAAAGGTCTA(SEQ ID NO: 13)) and yfp (F 5'-CGAAGAGCTCTTCACTGGCGTGGT-3') primers, respectively (FIG. 6). Subsequent PCR reactions detected pagl DNA indicating that the yfp HzNV-2 virus is a yfp insertional mutant rather than a complete replacement of this gene. Nevertheless, inactivation of pagl was sufficient to produce a virus that caused sterility in up to 100% of infected insects. Primers to the HzNV-2 open-reading frame ORF78 (F 5'-GGGGTTCTGGGTGACCCCTAAGGCTTGAAGTCTA(SEQ ID NO: 11)) and yfp (F 5'-GGGCTCTCTGATGCAGTATCGGCGCTATCGAGATCGTGTTAATCAGT-3') primers, respectively (FIG. 6) were used as a control for detecting the HzNV-2 Genome.

Method of Targeting Mutations in a Nudivirus Genome

Applicant has developed a novel method using diepoxide (DEB) to produce deletions in nudivirus genomes by chemical mutagenesis. DEB is known to crosslink DNA and lead to deletions of multiple bases (from ~ 50 bases to several kilobases), often within a single gene (Reardon et al., 1987; Wijen et al., 2001). DEB tends to cause mutations within regions of DNA that are actively transcribed. Nevertheless, published protocols for DEB mutagenesis do not teach mutating a nudivirus as it infects an insect cell so that genes involved in establishing viral lysogeny are preferentially mutated. In the literature, DEB mutagenesis usually involves feeding DEB to insects (Reardon et al., 1987; Genetics 115:323-331; Kimble et al., 1990 Genetics. 1990 December; 126(4):991-1005; Olsen and Green 1982 Mutat Res. 1982
DEB mutagenesis was surprisingly efficient; 36% of the 30 viruses caused more agonalad females than the WT virus in the in vivo screen. Random mutation of the HzNV-2 genome would be expected to rarely affect latency because only two of more than 113 viral genes are known to have a role in establishing the latency. In the described method, however, approximately 1/6 of mutants appeared to alter or eliminate the latent phase.

A detailed exemplary method is as follows:

**Culture conditions.** Seed SF9 insect cells at 2.5x10^6 cells/ml in 2 mL with nudivirus media (1x Supplemented Grace’s Media, 7% FBS, 1% Penicillin/streptomycin) in a 25 cm² tissue culture flask. Then 3 mL wild-type HzNV-2 virus previously amplified in tissue culture at an estimated MOI of 1 is added.

WT HzNV-2 virus was amplified by first seeding SF9 insect cells in all wells of a 6-well culture dish at 8x10^5 cells/ml in 2 mL with nudivirus media. After a 1 hr incubation at 27°C, 50 µl of filtered WT HzNV-2 obtained from a viral plug of agonadal female moth (acquired the same day) was added to each well using a large bore tip. Plates were incubated for 2 days at 27°C. Viral supernatants were then collected, cells and debris were removed by centrifugation (9000g, 10 min, 4°C), and supernatants from all wells were filter sterilized using a 0.22 µm filter and combined. The approximate viral titer from the procedure is 1.5x10^6 pfu/mL.

To isolate the WT HzNV-2 from infected female moths, the viral plug from an infected female moth is first extracted from the body and moved to a 1.5 ml-microcentrifuge tube. 100 µl 1xPBS is added and the plug is homogenized manually with a pipette tip to release the virus. The large fragments of insect cuticle and tissue are then removed. This viral solution is termed unfiltered viral plug extract (UVPE). The filtered viral plug extract (FVPE) is a filtered solution (with a 0.22 µm filter) of 1x supplemented Grace’s media, 2% unfiltered viral plug extract, and 5% penicillin/streptomycin antibiotics.

Controls for this mutagenesis are performed in parallel. Controls included uninfected SF9 culture, uninfected SF9 culture treated with DEB, and virus-infected SF9 culture. These cultures are prepared the same way and at the same time as the mutagenized culture described herein.

The infected SF9 insect culture is incubated at 27°C for 1.5 hours.

**Chemical Mutagenesis.**

In a fume hood, DEB (other names 1,3-butadiene diepoxide or 1,2,3,4-Diepoxybutane) is added at a 0.1 mM final concentration to the culture. The culture is then incubated at 27°C for 3 hours.

After the 3 h incubation, the culture is moved to the fume hood. A cell scraper is used to detach cells from the 25 cm² flask. The cells and supernatant are moved to a 50 ml-conical tube and centrifuged at 9000g for 10 min at 4°C. The supernatant is removed to a specified waste container. Cells are washed with 10 ml PBS, incubated in fume hood for 5 min, then spun down at 9000g for 10 min at 4°C.

The wash supernatant is removed to a specified waste container. The cell pellet is then resuspended in 5 ml nudivirus media and moved to a new 25 cm² tissue culture flask, which is now considered DEB-free. Culture is incubated at 27°C for 2 d.

After 2 days, the cell culture medium containing DEB-exposed HzNV-2 is collected after culture centrifugation at 9000g for 10 min and filter sterilization using a 0.22 µm filter. 2 ml of the virus stock is added to a new 25 cm² tissue culture flask containing SF9 insect cells that were seeded at 1x10^6 cells/ml in a 5 ml total volume with nudivirus media. The virus-infected culture is incubated at 27°C for 7 d.
To purify and amplify the virus to a suitable volume for insect infection, the virus-containing medium was collected after centrifugation (3000 rpm, 10 min, 4°C), filter sterilized using a 0.22 µm filter, and stored in 1 ml aliquots at -80°C. The titer of the virus is approximately 1 x 10^6 pfu/ml. While HzNV-2 has been shown to infect several lepidopteran cell lines including SF-9 and TN-368 cells, Applicant found that it is difficult to pass the virus in insect cells due to the virus causing quick cellular lysis. The disclosed methods allow for amplification of the virus to a volume that allows for both amounts sufficient virus for storage and also virus suitable for insect infection.

Viruses were isolated using a traditional plaque assay, described in, for example, Anderson, D., Harris, R., Polayes, D., Ciccarone, V., Donahue, R., Gerard, G., and Jessee, J. (1996) Rapid Generation of Recombinant Baculoviruses and Expression of Foreign Genes Using the Bac-To-Bac® Baculovirus Expression System. Focus 17, 53-58. Large plaques, referred to as the p0 viruses, were further amplified.

For p0 to p1 amplification, Sf9 insect cells were seeded at 5 x 10^6 cells/ml in a final 2 ml volume with nivudirus media in 12-well culture plates and incubated at 27°C for 1 h. Afterwards, viral plaques were picked using a large bore pipette tip and transferred to one well. Plates were incubated at 27°C for 5 days. Medium containing DEB-treated HzNV-2 virus is collected after culture centrifugation (900xg for 10 min at 4°C) and filter sterilized using a 0.22 µm filter.

For p1 to p2 amplification, Sf9 insect cells were seeded at 8 x 10^6 cells/ml in a final 2 ml volume with nivudirus media in 6-well culture plates and incubated at 27°C for 1 h. Afterwards, 600 ml p1 virus was added to one well using a serological pipet. Plates were incubated at 27°C for 4 days. Medium containing DEB-mutated HzNV-2 was then collected after culture centrifugation (900xg for 10 min at 4°C).

For p2 to p3 amplification, Sf9 insect cells were seeded at 1 x 10^7 cells/ml in 5 ml with nivudirus media in 25 cm^2 tissue culture flask with no incubation. ~1.5 ml (or all) p2 virus was added to one flask using a serological pipet. Flasks were incubated at 27°C for 4 days. Medium containing DEB-mutated HzNV-2 is then collected after culture centrifugation (900xg for 10 min at 4°C) and filter sterilization using a 0.22 µm filter. p3 virus is stored in 1 ml aliquots at -80°C.

p3 virus was used to infect 3rd instar larvae via the direct inoculation method. Each mutant virus caused an infection in the insect leading to formation of a viral plug found in agonadal female moths. Virus from these viral plugs may be utilized in subsequent experiments.

Confirming Sterilizing Activity of Recombinant and Chemically Mutated HzNV-2 Virus

Taken together, the data demonstrate that recombinant and mutant HzNV-2 viruses can be effectively achieved using one or more of the above-described methods. Recombinant and mutant HzNV-2 viruses having a disrupted latency phase may be produced in which genes that affect the latency phase are disrupted or structural genetic elements required to establish or break latency are altered. The resulting genetically modified mutant HzNV-2 viruses cause elevated levels of sterility in infected H. zea moths. In one aspect, the genes that are disrupted include one or more of pag1, ORF90, and ORF92. It is noted that 100% sterile phenotypes may be produced by mutating different viral genes and regions of the viral genome.

The sterilizing activity of the resulting mutants were assessed through many experiments in which virus was injected into H. zea adults or 3rd instar larvae. In a typical experiment, WT-HzNV-2, the recombinant yfp HzNV-2, and mutant KS3 viruses were collected and purified from viral plugs found in virus-infected female moths and then injected into newly healthy female moths 2 days after emergence. The eggs laid on oviposition day 3 were collected and the F1 progeny female adults were analyzed for sterility as indicated by the presence of a viral plug and number of eggs laid (Table 2). Both female groups infected with mutants yfp HzNV-2 and KS-3 laid few or no eggs and had a much higher percentage of agonadal females than the WT-infected group. Thus, the viruses obtained using the described methods are suited for use in sterilizing populations of pests susceptible to infection by the described viruses, and may be utilized to control pest populations.

### Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plugs</th>
<th>Eggs laid</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT HzNV-2</td>
<td>34% many</td>
<td></td>
</tr>
<tr>
<td>KS3</td>
<td>95% a few</td>
<td></td>
</tr>
<tr>
<td>yfp HzNV-2</td>
<td>100% none</td>
<td></td>
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</table>

In a similar experiment, WT and 9 chemical mutant viruses were evaluated for the ability to cause agonadal moths. Briefly, adult female moths were injected with 100 µl of ~ 10^8 pfu/ml of virus isolated from viral plugs on the day of emergence. Eggs laid on oviposition days 2 and 3 were collected and reared to adult moths. The F1 progeny female moths were evaluated for the ability to lay eggs and the presence of a viral plug. Four mutants (KS-3, KS-45, KS-52, KS-51) caused viral plug formation in 100% of the F1 female progeny (FIG. 2). No eggs were laid by F1 female progeny of female moths infected with the three mutants (indicative of an agonadal phenotype (KS3, KS45, KS51) (FIG. 3). F1 female progeny of female moths infected with another mutant KS52, laid fewer eggs than F1 female progeny of female moths infected with WT virus on oviposition day 2 and no eggs on oviposition day 3.

The functional mutations defined by the applicant, KS-3, KS-45 and

### Table 1

Locations of mutations in the chemical mutants relative to HzNV-2. Wild-type (WT) HzNV-2 virus was mutated with DEB to form HzNV-2 chemical mutants. Mutants KS-3, KS-45, and KS-51 were sequenced and gene deletions were identified. It is notable that the three mutants have mutations in a region that is not present in the HzNV-2 genome.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>WT base pairs affected</th>
<th>WT genes affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>yfp HzNV-2</td>
<td>210,631-214,753 bp</td>
<td>pag1, ORF90, hypothetical protein</td>
</tr>
<tr>
<td>KS-3</td>
<td>45 bp insertion at bp 175,550</td>
<td>ORF90, hypothetical protein</td>
</tr>
<tr>
<td>KS-45</td>
<td>50 bp insertion at bp 175,650</td>
<td>ORF90, hypothetical protein</td>
</tr>
<tr>
<td>KS-51</td>
<td>180,270-180,299 deletion</td>
<td>ORF92, hypothetical protein</td>
</tr>
<tr>
<td></td>
<td>77 bp insertion at bp</td>
<td>Intergenic DNA between hypothetical proteins ORF55 and ORF56</td>
</tr>
</tbody>
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In another experiment, WT and 9 chemical mutant viruses were evaluated for the ability to cause agonadal moths. Briefly, adult female moths were injected with 100 µl of ~10^8 pfu/ml of virus isolated from viral plugs on the day of emergence. Eggs laid on oviposition days 2 and 3 were collected and reared to adult moths. The F1 progeny female moths were evaluated for the ability to lay eggs and the presence of a viral plug. Four mutants (KS-3, KS-45, KS-52, KS-51) caused viral plug formation in 100% of the F1 female progeny (FIG. 2). No eggs were laid by F1 female progeny of female moths infected with the three mutants (indicative of an agonadal phenotype (KS3, KS45, KS51) (FIG. 3). F1 female progeny of female moths infected with another mutant KS52, laid fewer eggs than F1 female progeny of female moths infected with WT virus on oviposition day 2 and no eggs on oviposition day 3.

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### Table 2

Injection of female moths with mutant HzNV-2 KS3, recombinant mutant yfp HzNV-2, or wild-type (WT) HzNV-2 and analyses of their female offspring for sterility as determined by the presence of a viral plug and production of eggs.

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<td></td>
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<tr>
<td>yfp HzNV-2</td>
<td>100% none</td>
<td></td>
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</table>
KS-51, identify and localize to ORFs that have several unrelated direct repeated sequences ranging from 24 to 81 bp in size and having these sequence repeated from 4 to 12 times. These repeated sequences were identified by Burand et al. (2012). Such repeated sequences may have structural as well as coding roles and with some functions of repeated sequences involving recognition sites for DNA proteins and directing conformational changes of DNA that can promote DNA replication, DNA recombination and/or RNA transcription as examples. A similar repeated sequence exists in ORF 2 (direct repeat 1; Table 2; Burand et al., 2012) and is claimed herein as an identified sequence, region and ORF that is susceptible to mutation and such mutations are likely to impact DNA replication and recombination and the function of the virus relative to its effects on viral lysogeny and increased sterility among H. zea infected with mutations that alter ORF2 (SEQ ID NO: 3). It is notable that the 3 mutations defined by the applicant localize to ORFs 90 and 92 which contain 4 of the 6 repeated sequences in the HzNV2 genome. ORF 2 and ORF 91 contain the only other large repeated sequences in the viral genome and are thus obvious candidates for mutagenesis with an expectation that it would impact HzNV2 replication and recombination.

Table 2 shows additional data that compare genetically engineered and chemical mutant viruses. Progeny female moths developing from female moths infected with the genetically engineered recombinant virus, yfp HzNV-2 did not lay eggs and all F1 female progeny had viral plugs indicating their sterility. Similarly, essentially all F1 progeny of female moths infected with the chemical mutant viruses KS3, KS45, KS51, and KS52 had viral plugs and exhibited sterility (Table 2, FIGS. 2 and 3). By contrast, much smaller percentages (~33%) of insects infected with WT virus were agonadal (had plugs) with most moths infected with the WT HzNV-2 being fertile and laying many eggs (Table 2, FIG. 3). Similar results are seen on other oviposition days, although the number of female sterile moths in the F1 progeny of female moths infected with WT HzNV-2 increases at later oviposition days as reported in the literature (Hamm et al., 1996; Burand 2013). These results support the hypothesis that inactivation of several nudivirus genes, for example, pag1 (SEQ ID NO: 6), ORF90 (SEQ ID NO: 4), and ORF92 (SEQ ID NO: 5) can cause sterility in essentially 100% of infected insects.

While it is to be recognized that viruses capable of being sexually transmitted among an insect pest species, can be mutated and selected using the described protocols without knowledge of specific genes involved in the phenotype of the resulting virus (for example, using the DEB protocol described above), some genes associated with increased sterility following viral infection have been identified by the Applicant. For example, the pag1, ORF 90, and ORF92 genes have been found by Applicant to be associated with increased sterility in virus-infected insects.

Infection of an Insect Using Modified HzNV-2

Published nudivirus literature describes various techniques for infecting and sterilizing H. zea moths with WT HzNV-2 but are lacking in various aspects such that a meaningful protocol can be carried out. Applicant has developed protocols that efficiently 1) infect the adult moth, 2) infect the moth offspring, and 3) mimic natural infections of moth populations in the field.

Method of Sterilizing the Offspring of Adult Moths. A Protocol for Producing Agonadal Infections with WT HzNV-2

To infect adult moths, the virus is injected into the abdomen of adult moths using an insulin syringe. In such experiments, WT virus collected from a viral plug effectively infected female adult moths and F1 progeny mimicking natural infected moth populations (i.e. 20-50% agonadal) (Table 3). Virus is collected by removing the plug from the abdominal area and suspending the plug in 100 µl PBS; this is called the unfiltered viral plug extract (UVPE). The virus is then diluted to 2% in 1x Grace's media and filtered through a 0.22 micron filter to sterilize; this is called the filtered viral plug extract (FVPE). Applicant determined there was no difference in the percentage of agonadal F1 progeny when using either UVPE or FVPE for injections. (Table 3.) Applicant used FVPE for most experimentation.

<table>
<thead>
<tr>
<th>Oviposition day</th>
<th>Virus type</th>
<th># Females evaluated for plug</th>
<th>F1 progeny females with plug</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>UVPE</td>
<td>30</td>
<td>33%</td>
</tr>
<tr>
<td>3</td>
<td>FVPE</td>
<td>25</td>
<td>36%</td>
</tr>
<tr>
<td>3</td>
<td>UVPE and FVPE</td>
<td>35</td>
<td>74%</td>
</tr>
</tbody>
</table>

Developing the Protocol to Generate a High Number of Agonal Moths

Subsequent experimentation determined that injecting virus extracted from viral plugs results in a higher percentage of agonadal F1 progeny than injecting virus produced from infections of Sf9 cells in culture. Briefly, adult female moths were infected with 50-60 µl of either FVPE or virus amplified in cell culture. Eggs were collected on oviposition day 3 and reared to adults. F1 female progeny were evaluated for the presence of a plug. Almost all of the F1 female progeny developed a plug if F0 female moths were injected with viral plug extract, whereas only 10% of F1 progeny developed a plug if injected with virus collected from infected Sf9 cells. (Table 4).

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Virus strain</th>
<th># Females evaluated for a plug</th>
<th>F1 female progeny with plug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture</td>
<td>yfp HzNV-2</td>
<td>11</td>
<td>9%</td>
</tr>
<tr>
<td>Cell culture</td>
<td>KS-3</td>
<td>20</td>
<td>10%</td>
</tr>
<tr>
<td>Plug virus</td>
<td>yfp HzNV-2</td>
<td>26</td>
<td>100%</td>
</tr>
<tr>
<td>Plug virus</td>
<td>KS-3</td>
<td>20</td>
<td>95%</td>
</tr>
</tbody>
</table>

Thus, the titer of virus amplified in cell culture used for infection of adult moths was not optimal. Titer is an important factor in developing a method for creating high volumes of sterile insects. The optimal viral titer will be as low as possible but sufficient to cause agonadal adults. To assess the effects of virus titer, adult female moths were injected with either 10^2, 10^3 or 10^4 pfu/ml virus (WT or yfp HzNV-2) and mated with uninfected males. Eggs were collected on oviposition days 3-5, and reared to the adult stage. Moths were evaluated for the presence of a viral plug and egg production. Virus titer of 10^4 pfu/ml led to a high number of agonadal F1 progeny on oviposition day 5 (Table 5) indi-
cating that the virus replication in the host moth was important for effective transmission of the virus to the offspring eggs.

**Table 5**

<table>
<thead>
<tr>
<th>Virus group</th>
<th>F1 progeny collected on oviposition day</th>
<th># females evaluated</th>
<th># Agonadal females with plugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>Day 3 7 200 10%</td>
<td>Day 4 6 100 100%</td>
<td>Day 5 2 0 0%</td>
</tr>
<tr>
<td>WT-infected</td>
<td>Day 3 9 5 5%</td>
<td>Day 4 5 100 100%</td>
<td>Day 5 10 0 0%</td>
</tr>
<tr>
<td>yip-HzNV-2 infected</td>
<td>Day 3 4 0 0%</td>
<td>Day 4 4 0 0%</td>
<td>Day 5 10 0 70%</td>
</tr>
<tr>
<td>yip-HzNV-2 infected</td>
<td>Day 3 9 100 100%</td>
<td>Day 4 9 100 100%</td>
<td>Day 5 10 0 70%</td>
</tr>
</tbody>
</table>

Injection of the recombinant virus into naïve male moths, followed by mating with healthy females, does not significantly reduce the number of eggs laid by female moths although the infection can be transmitted in this manner. However, when newly emerged female moths were injected with recombinant yip HzNV-2 and mated with healthy males, the egg number was dramatically reduced (FIG. 4). This effect was dose-dependent as a viral dose of 1x10^6 pfu exhibited fewer eggs compared to a viral dose of 1x10^7 pfu. The total number of eggs laid by female moths injected with WT HzNV-2 was not reduced relative to media injected controls; this effect is only observed with the mutant and recombinant viruses.

Methods of Sterilizing Adult Moths by Injecting the Insects at the Larval Stage

An alternative protocol for inducing sterility is to inject virus into 3rd instar larvae such that the injected insects exhibit the sterile pathology as adults. While this is not the normal mode of transmission, larval injections are much faster, amenable to automation and likely mimic the activation of viral replication (Rallis et al., 2002-a). Applicant has created two protocols for inducing sterility in 3rd instar larvae: syringe injection and direct inoculation.

Infection Using Syringe Injection

Injecting supernatants from cultures of virus-infected cells into adult moths does not produce high numbers of agonadal offspring (Table 4). To determine if virus produced from infected, cultured insect cells effectively initiated productive virus infections when injected into larvae, Applicant injected virus-containing tissue culture medium into 3rd instar larvae. 3rd instar larvae (83 larvae per group) were injected using an insulin syringe with either WT HzNV-2, yip HzNV-2, or KS-3 virus (25-50 μl) or not injected. Larvae were reared to adults. Surprisingly, none of the yip HzNV-2-infected larvae pupated and eventually died (Table 6). However, ~95% of female larvae infected with either WT HzNV-2 or KS-3 virus produced a viral plug as adults (FIG. 5). Applicant concluded that while injecting virus amplified in tissue culture into adult moths does not produce many agonadal offspring, injecting virus amplified in tissue culture into 3rd instar larvae was an effective method as almost all moths became agonadal.

**Table 6**

<table>
<thead>
<tr>
<th>Group</th>
<th>Insects survived injection and pupated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>100%</td>
</tr>
<tr>
<td>W T-infected</td>
<td>60%</td>
</tr>
<tr>
<td>KS3-infected</td>
<td>73%</td>
</tr>
<tr>
<td>yip HzNV-2-infected</td>
<td>0%</td>
</tr>
</tbody>
</table>

Injection of viral plug extract into 3rd instar larvae with an insulin syringe was also evaluated. Briefly, WT HzNV-2 and yip HzNV-2 FVPE was diluted to 7x10^6 pfu/ml and 2.5x10^9 pfu/ml and 25 μl was injected into 3rd instar larvae. Larvae were reared to adults and female moths were evaluated for the presence of a plug. Female moths that were injected as larvae with a titer of 10^6 pfu/ml developed a viral plug, whereas only ~80% of those injected with 10^5 pfu/ml developed a viral plug (Table 7). Applicant concluded that injecting 3rd instar larvae with viral plug extract was also highly effective at developing agonadal adults, but titer should be around 10^5 pfu/ml.

**Table 7**

<table>
<thead>
<tr>
<th>Infection group</th>
<th>Virus titer (pfu/ml)</th>
<th>Females with plug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>W T-infected</td>
<td>1 x 10^6</td>
<td>50%</td>
</tr>
<tr>
<td>KS3-infected</td>
<td>1 x 10^6</td>
<td>100%</td>
</tr>
<tr>
<td>yip HzNV-2-infected</td>
<td>4 x 10^6</td>
<td>70%</td>
</tr>
<tr>
<td>yip HzNV-2-infected</td>
<td>8 x 10^6</td>
<td>100%</td>
</tr>
</tbody>
</table>

Infection Using Direct Inoculation Using a Pin

Direct injection of third instar larvae or moths are efficient methods for infecting HzNV-2 in the laboratory, but feeding is a preferred method.

Infecting H. zea with WT HzNV-2 via an oral route of infection is possible. Raina et al. (2006) fed WT HzNV-2 to 1st instar larvae for 1-3 days and found that 9-17% (varies based on gender and duration of feeding) of adults became agonadal. Hamm et al. (1996) fed WT HzNV-2 to adult moths in an aqueous solution and from 60-100% of the offspring adults were agonadal. The HzNV-2 genome also encodes genes related to four baculovirus genes (p74, pif-1, pif-2, and pif-3) whose protein products are involved in viral entry per os (Burund, Kim, Afonso et al. 2012). Although the natural route of infection for HzNV-2 is through mating and/or transovarial transmission, other methods for infecting insects include direct inoculation and feeding of both larvae and adults.

A direct inoculation method for transmitting the virus to 3rd instar larvae (Table 8) was developed and may be amenable to automation. This method is similar to that used by Hamm et al., (1996) to infect 1st instar larvae with viral
plug extract with 9 of 10 larvae becoming agonadal as adults. Direct inoculation is a rapid means to introduce virus
into H. zea larvae in which a sterile pin is dipped into the
viral solution and then used to prick larvae between the head
capsule and abdomen with sufficient force to penetrate the
cuticle and enter the insect’s body cavity. WT HzNV-2 (A,
B) virus obtained from two different cell culture infections
A and B) were used for direct inoculation. The pricked
larvae were reared to adult moths, and female moths were
evaluated for the presence of a plug. 57% (WT A) and 16%
(WT B) of moths were agonadal.

To test virus isolated from plugs, ~10^8 pfu/ml of WT
HzNV-2 or recombinant yfp HzNV-2 viruses were isolated,
filter sterilized and used in direct inoculation experiments.
The inoculated larvae were reared to adult moths and mated.
All female moths developing from larvae inoculated with
recombinant yfp HzNV-2 were sterile (no eggs laid; plugs in
100% of females). Upon dissection, all male moths exam-
ined were found to be agonadal.

For larvae inoculated with WT HzNV-2, 90% of female
moths had a viral plug with the number of eggs laid
commensurately reduced relative to controls (Table 8). In
summary, the direct inoculation method not only was as
efficient as injecting the virus into larvae, it also was much
faster.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Female moths with plugs</th>
<th>State of reproductive tracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpricked</td>
<td>High</td>
<td>0% agonadal</td>
</tr>
<tr>
<td>Medium control</td>
<td>High</td>
<td>0% agonadal</td>
</tr>
<tr>
<td>WT HzNV-2</td>
<td>Low</td>
<td>0% agonadal</td>
</tr>
<tr>
<td>yfp HzNV2</td>
<td>None</td>
<td>100% agonadal</td>
</tr>
</tbody>
</table>

**TABLE 8**

Effects of direct inoculation of 3rd instar larvae with wild-type (WT) and yfp recombinant HzNV2 purified from viral plugs on moth sterility.

In accordance with the instant disclosure, three different
methods for infecting large numbers of larvae may be used
as follows:

1. Virus Feeding. The protocol used by Raina and Lupiani
(2006) may be used. Briefly, newly hatched H. zea larvae
may be placed in a 100x15 mm Petri dish containing a diet
with 1000 pfu of mutant HzNV-2. To increase the efficient
uptake of the virus, the fluorescent brightener Blankophor
may be added to the diet (Martinez et al., 2009). The larvae
may be allowed to feed for 48 hrs then placed in diet-
containing cups. H. zea is very cannibalistic and so must be
housed individually at a young instar. The pupae will be
sexed and emerged females will be analyzed for viral plugs,
the ability to lay fertile eggs after mating, and the presence
of intact reproductive organs.

The males may then be dissected and their reproductive
organs examined to determine if they are agonadal. The
presence of mutant HzNV-2 may be confirmed by PCR, as
described above.

2. Virus Aerosols. The virus may be delivered as a
lyophilized powder (Kirkpatrick et al., 1994) or as an
aqueous mist (U.S. Pat. No. 7,261,886). 3rd instar H. zea
larvae may be anesthetized with CO2 and placed in a test
chamber. The lyophilized mutant virus may be placed in the
chamber at different doses and dispersed continuously
throughout the chamber by a gentle stream of air, for a total
exposure time of 30 min. Each insect may be sexed after
they pupate, and after emergence, each moth may be ana-
lyzed for sterility as described above. For the aqueous mist
delivery, a Potter precision laboratory spray tower (Burkard
Scientific) may be used, and 3rd instar larvae may receive
doses of mutant HzNV-2 from 10^4 to 10^6 pfu/ml. Agonadal
pathology may be assessed in adult moths.

3. Immersion. H. zea larvae may be submerged in a
HzNV-2 solution as described by Lu et al. (2011). This
method involves first stressing the insects at 47°C for
15 hours before soaking the 3rd instar larvae in different
concentrations of mutant HzNV-2 (10^4-10^6 pfu/ml) for 1 hr.
Sterility in adult moths may be determined as described
above.

Method of Protecting a Crop from Pest Insects

The instant disclosure addresses a method for control of
lepidopteran pest moths by rendering them sterile from
infection with mutant or transgenic HzNV-2. The delivery of
HzNV-2 or a mutant form thereof in accordance with the
disclosed methods and compositions to the targeted popula-
tion may be through established methods for release of
moshs for sterile insect control. In one aspect, the moths or
other pest infected with a mutant virus as disclosed herein,
are released at point locations and permitted to disperse over
a range. The range may be, for example, about 800 meters
from the release site or released aerially from planes, heli-
copters or drones. Moths infected with mutant or recombi-
nant HzNV-2 may be released after infection using one or
more of the disclosed methods at ratios from 0.1 infected
moshs/WT moth in the field population moth up to 10
infected moshs/WT moth in the field population. Targeted
release at lower ratios may rely on generational transmission
of the infection for control and may require supplemental
release on virus-infected adult moths.

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What is claimed is:

1. A genetically modified *Helicoverpa zea* nudivirus 2 (HzNV-2) virus, having a mutation in a region selected from Open Reading Frame 90 (ORF90), Open Reading Frame 92 (ORF92), Persistence-associated Gene (pag1), or a combination thereof, to disrupt expression of the gene product encoded by said mutant region, wherein said mutation increases the rate of an agonadal phenotype in a progeny of a *Helicoverpa zea* (*H. zea*) moth infected with said genetically modified HzNV-2 as compared to a progeny of a *H. zea* moth infected with a wild-type HzNV-2 virus.

2. The genetically modified nudivirus of claim 1, wherein said genetically modified HzNV-2 virus is achieved via chemical mutagenesis.

3. The genetically modified nudivirus of claim 1, wherein said genetically modified HzNV-2 virus is achieved via recombinant DNA technology.

4. The genetically modified nudivirus of claim 1, wherein said genetically modified HzNV-2 virus is achieved via gene editing technology.

5. An insect infected with the virus of claim 1.

6. The insect of claim 5, wherein said insect is *H. zea*.

7. A method of protecting a crop susceptible to *H. zea* moth crop damage, comprising the step of introducing an *H. zea* moth infected with the genetically modified nudivirus of claim 1 into said crop.

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