Recombinant Baculovirus-Based Insecticides

Cross-Reference to Related Applications

This application claims priority pursuant to 35 U.S.C. 119 based upon Provisional Application Serial No. 60/084,705 filed May 8, 1998, the entire disclosure of which is hereby incorporated by reference.

Field of the Invention

The present invention relates to improved *Plutella xylostella* baculoviruses for use as biological insecticides. The invention provides recombinant baculoviruses that have been genetically engineered to incorporate genes encoding insecticidal toxins.

Background of the Invention

Baculoviruses are insect viruses which are useful as biological insecticides. Over 400 baculovirus isolates have been described. The *Autographa californica* nuclear polyhedrosis virus (AcMNPV), the prototype virus of the family Baculoviridae, was originally isolated from *Autographa californica*, a lepidopteran noctuid commonly known as the alfalfa looper. This virus infects 12 families and more than 30 species within the order of Lepidopteran insects (Granados *et al.*, *The Biology of Baculoviruses*, I, 99 (1986)). AcMNPV is not known to infect productively any species outside this order.

The life cycle of baculoviruses, as exemplified by AcMNPV, includes two stages. Each stage of the life cycle is represented by a specific form of the virus: budded virions (BV) which are nonoccluded, and occlusion bodies (OB).

In the naturally occurring insect-infectious form, multiple virions are found embedded in a paracrystalline protein matrix known as an occlusion body (OB), which is also referred to as a polyhedral inclusion body (PIB). The proteinaceous viral occlusions are referred to as polyhedra. A polyhedrin protein having molecular weight of 29 kD is the major viral-encoded structural protein of the viral occlusions (U.S. Patent Number 4,745,051).

The viral occlusions are an important part of the natural baculovirus life cycle, providing the means for horizontal (insect-to-insect) transmission among susceptible insect species. In the environment, a susceptible insect (usually in the larval stage) ingests the viral occlusions from a contaminated food source, such as a plant. The crystalline occlusions dissociate in the gut of susceptible insects to release the infectious viral particles. These occlusion-derived viruses (ODV) replicate in the cells of the midgut tissue.

It is believed that virus particles enter the cell by endocytosis or fusion, and the viral DNA is uncoated at the nuclear pore or in the nucleus. Viral DNA replication is detected within six hours. By 10-12 hours post-infection (p.i.), secondary infection spreads to other insect tissues by the budding of the extracellular virus (BV) from the surface of the cell. The BV form of the virus is responsible for cell-to-cell spread of the virus within an individual infected insect, as well as for transmitting infection in cell culture.

Late in the infection cycle (12 hours p.i.), polyhedrin protein can be detected in infected cells. It is not until 18-24 hours p.i. that the polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions. Viral occlusions accumulate to large numbers over 4-5 days as cells lyse. These polyhedra have no active role in the spread of infection in the larva. BVs in the haemolymph multiply and spread, leading to the death of the larva.

When infected larvae die, millions of polyhedra remain in the decomposing tissue, while the BVs are degraded. When other larvae are exposed to the polyhedra by ingestion of, e.g., contaminated plants or other food material, the cycle is repeated.

In summary, the occluded form of the virus is responsible for the initial infection of the insect through the gut, as well as for the environmental stability of the virus. ODVs are essentially non-infectious when administered by injection, but are highly infectious when ingested orally. The non-occluded form of the virus (i.e., BV) is

responsible for secondary and cell-to-cell infection. BVs are highly infectious for cells in culture or internal insect tissues by injection, but are not infectious when ingested.

A major impediment to the widespread use of insecticidal baculoviruses in agriculture is the time lag between initial infection of the insect and its death. This time lag can range from days to weeks. During this period, the insect continues to feed, causing further damage to the plant. To shorten this lag time, recombinant baculoviruses have been constructed that express an insect-controlling or modifying substance, such as a toxin, neuropeptide, hormone, or enzyme (Tomalski, M. D. *et al.*, *Nature*, 352:82-85 (1991); Federici, *In Vitro*, 28:50A (1992); Martens *et al.*, *App. & Envir. Microbiology*, 56:2764-2770 (1990); Menn *et al.*, *Agric. Food Chem.*, 37:271-278 (1989); Eldridge *et al.*, *Insect Biochem.*, 21:341-351 (1992); Hammock *et al.*, *Nature*, 344:458-461 (1990)).

A second major impediment to the use of insecticidal baculoviruses is the limited host range of the viruses. While the limited host range of baculoviruses makes them safe to non-target organisms, it has also meant that they are unable to control the variety of lepidopteran pests present in the field. This is particularly true when the complex of lepidopteran insects that needs to be controlled comprises insects that are either non-permissive or semi-permissive for infection by the particular insecticidal baculovirus being used. An insect that is non-permissive for infection is one that cannot be productively infected at any dose; an insect that is semi-permissive for infection is one that can only be productively infected when exposed to an amount of the virus that is at least one, but more generally two or more, orders of magnitude greater than that required for productive infection in susceptible insects, i.e., permissive hosts. Prior to the present invention, no baculovirus of the genus Nucleopolyhedrovirus had been identified for which the diamondback moth, *Plutella xylostella*, is a permissive host. This insect is an important pest in many vegetable crops and has developed resistance to both chemical and biological insecticides.

Thus, there is a need in the art for biological insecticides that exhibit (i) improved efficacy and shorter lag times between infection and mortality and (ii) exhibit improved host range, allowing them to control a broad complement of lepidopteran insect pests, including, e.g., *Plutella xylostella*.

Summary of the Invention

The present invention provides recombinant *Plutella xylostella* baculoviruses (PxNPVs), which exhibit superior insecticidal activity against *Plutella xylostella* insects relative to other wild-type and recombinant baculoviruses.

Recombinant PxNPVs according to the invention are PxNPVs containing one or more genetic alterations relative to wild-type PxNPV. Genetic alterations include without limitation introduction or deletion of one or more restriction sites; modification, deletion, or duplication of one or more viral-encoded genes; and introduction of one or more genes encoding heterologous proteins, i.e., proteins that are non-virally encoded or are encoded by a different virus.

Preferably, recombinant PxNPVs have incorporated within their genomes a nucleic acid sequence encoding an insect-modifying substance, such as, e.g., an insecticidal toxin, peptide hormone, enzyme, or receptor, which is operably linked to a promoter capable of activating transcription in the target insect. Most preferably, the insect-modifying substance is an insecticidal toxin. It is believed that a recombinant baculovirus encoding an insecticidal toxin in the context of the PxNPV genomic background provides significantly improved insecticidal benefits.

Non-limiting examples of insecticidal toxins include AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins. In some embodiments, the native secretion signal peptide, i.e., the signal peptide associated with the toxin, is employed; in other embodiments, a heterologous signal peptide is employed to promote secretion of the toxin from infected insect cells. Non-limiting examples of useful heterologous signal peptides include those derived from the pBMHPC-12 signal sequence from *Bombyx mori*; the adipokinetic hormone signal sequence from *Manduca sexta*; the apolipophorin signal sequence from *Manduca sexta*; the chorion signal sequence from *Bombyx mori*; the cuticle signal sequence from *Drosophila melanogaster*; the esterase-6 signal sequence from *Drosophila melanogaster*; and the sex-specific signal sequence from *Bombyx* mori.

The invention also encompasses direct ligation vectors, which are designed to facilitate the construction of recombinant PxNPV genomes by DNA fragment ligation

in vitro. Introduction of the DNA resulting from such a ligation into an appropriate host cell results in the production of recombinant PxNPVs.

In another aspect, the invention provides expression cassettes encoding insecticidal toxins. The expression cassettes comprise a promoter sequence, preferably derived from a *Drosophila melanogaster* hsp70 promoter, which is operably linked to a nucleic acid sequence encoding a toxin. The expression cassettes of the invention may also be incorporated into plasmid vectors, which are designated modular expression vectors.

In yet another aspect, the invention provides codon-optimised genes encoding insecticidal toxins, such as, e.g., those shown in Figures 10 and 11 below. Codon-optimised genes are those in which particular codons present in the native toxin-encoding sequence have been substituted with alternative codons that are more efficiently utilized by the insect cell protein-synthesizing machinery.

In yet another aspect, the invention provides insecticidal compositions and formulations comprising at least one recombinant PxNPV as described above and an agriculturally acceptable carrier.

In yet another aspect, the invention provides methods for killing insect pests and for reducing insect infestation, which comprise administering to a desired locus an insecticidal-effective amount of PxNPV-containing insecticidal compositions or formulations.

Brief Description of the Drawings

Figure 1 is a schematic illustration of the procedures used to construct the pmd 205.1, pmd 216.1, and pmd 220.2 vectors for the production of recombinant PxNPVs deleted for the viral ecdysteroid glucosyl transferase (egt) gene.

Figure 2 is a schematic illustration of the procedures used to construct the LAB 50.2 vector.

Figure 3 is a schematic illustration of the structure of pMEV modular expression vectors.

Figure 4 is a schematic illustration of pMEV vectors containing different promoters.

Figure 5 is a schematic illustration of the cloning of sequences into the modular expression vectors.

Figure 6 is an illustration of the *D. melanogaster* hsp70 promoter module in pMEV5 and the amplification primers used to isolate the sequence.

Figure 7 is an illustration of the *D. melanogaster* hsp70 promoter module in pMEV6 and the amplification primers used to isolate the sequence.

Figure 8 is an illustration of a codon-optimized DNA sequence encoding AaIT and the oligonucleotides and amplification primers that were used to synthesize the sequence.

Figure 9 is a schematic illustration of the structure of the pMEV/ADK modular expression vectors.

Figure 10 is an illustration of a codon-optimized DNA sequence encoding the LqhIT2 toxin and the oligonucleotides and amplification primers that were used to synthesize the sequence.

Figure 11 is an illustration of a codon-optimized DNA sequence encoding ω-ACTX-HV1 toxin and the oligonucleotides and amplification primers that were used to synthesize the sequence.

Detailed Description of the Invention

In practicing the present invention, many techniques in molecular biology, microbiology, recombinant DNA, protein biochemistry, and insect virology known to those skilled in the art may be used, such as those explained fully in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); Ausubel *et al.*, *Current Protocols in Molecular Biology*, 1997 (John Wiley & Sons); the series, *Methods in Enzymology* (Academic Press, Inc.); *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.); Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station Bulletin No. 1555, 1987; and O'Reilly et al., *Baculovirus Expression Vectors: A Laboratory Manual*, 1994 (Oxford Univ. Press, N.Y.)

The present invention provides isolated, purified recombinant *Plutella xylostella* baculoviruses (PxNPVs) which are useful as biological insecticides. PxNPV as used herein refers to an isolate of baculovirus of the genus Nucleopolyhedrovirus (NPV), the wild-type version of which was isolated from infected *Plutella xylostella* larvae and

which is genetically distinct from other known baculoviruses and exhibits higher infectivity for *Plutella xylostella* larvae relative to other baculoviruses. The genomic sequences of recombinant PxNPVs according to the present invention, excluding heterologous sequences, exhibit at least about 90% sequence identity, and preferably at least about 95% sequence identity, with the genomic sequence of PxNPV deposited as ATCC VR-2607. PxNPVs encompassed by the present invention may also be identified by:

(i) Measurement of infectivity for *Plutella xylostella* larvae. PxNPVs of the invention typically exhibit infectivity for *Plutella xylostella* larvae that is at least about two orders of magnitude greater than that exhibited by the V8 strain of AcMNPV deposted as ATCC VR-2465.

(ii) Digestion of viral genomic DNA with HindIII, XhoI, and/or PstI and comparison of the resulting restriction fragment pattern with patterns produced by digestion of genomic DNA derived from PxNPV and those derived from non-PxNPV baculoviruses. PxNPVs of the invention, excluding fragments resulting from genetic alterations, exhibit restriction fragments characteristic of PxNPV, i.e., that are present in PxNPV digests and absent from the digests produced by other baculoviral DNAs.

The present inventors have discovered that the recombinant PxNPVs of the present invention exhibit superior insecticidal activity on *Plutella xylostella* larvae relative to wild-type PxNPVs and/or recombinant NPVs derived from other species of baculoviruses. Recombinant PxNPVs according to the present invention are PxNPVs in which one or more genetic alterations have been introduced relative to wild-type PxNPV. "Genetic alteration" refers to any change in the sequence of the PxNPV genome including, without limitation, introduction of one or more restriction sites; deletion of one or more restriction sites; modification, deletion, or duplication of one or more viral-encoded genes; and introduction of one or more genes encoding heterologous proteins, i.e., non-virally encoded proteins or proteins encoded by a different virus. For example, modified PxNPVs whose genomes contain restriction sites not present in wild-type PxNPV or, conversely, are lacking one or more restriction sites present in wild-type PxNPV are encompassed by the present invention. The term "restriction site" refers to a nucleic acid sequence that is a recognition site for a restriction endonuclease. Typically, addition and/or deletion of one or more restriction sites is performed to create a cloning site not present in wild-type PxNPV, i.e., a sequence comprising at least one unique restriction site for incorporation

of a heterologous gene into the PxNPV genome. Preferably, recombinant PxNPVs have incorporated within their genome at least one heterologous gene, including without limitation genes encoding an insect-modifying substance such as, e.g., an insecticidal toxin, a hormone, an enzyme, or a receptor.

Most preferably, recombinant PxNPVs contain a heterologous gene encoding an insecticidal toxin. Suitable insecticidal toxins include without limitation those listed in the Table below:

Toxin	Reference
AaIT, AaH IT1, AaH IT2	Darbon et al., *Int. J. Peptide Protein Res.* 20: 320-330, 1982; Loret et al., *Biochem.* 29: 1492-1501, 1990
LqhIT2,	Zlotkin et al., *Biochem.* 30: 4814-4821, 1991; Zlotkin et al., European Patent Application EP 0374753 A2, 1990
LqqIT1, LqqIT2	Zlotkin et al., *Arch. of Biochem & Biophys.* 240: 877-887, 1985; Zlotkin et al., European Patent Application EP 0374753 A2, 1990
BjIT1, BjIT2	Lester et al., *Biochem. Biophys. Acta* 701: 370-387, 1982; Zlotkin et al., European Patent Application EP 0374753 A2, 1990
LqhP35	Zlotkin et al, European Patent Application EP 0374753 A2, 1990
LqhαIT	Eitan et al., *Biochem.* 29: 5941-5947, 1990
SmpIT2	Zlotkin et al., European Patent Application EP 0374753 A2, 1990
SmpCT2	Zlotkin et al., European Patent Application EP 0374753 A2, 1990

SmpCT3	Zlotkin et al., European Patent Application EP 0374753 A2, 1990
SmpMT	Zlotkin et al., European Patent Application EP 0374753 A2, 1990
DK9.2	Krapcho et al., International Patent Application WO 0374753 A2, 1990
DK11	Krapcho et al., International Patent Application WO 92/15195, 1992
DK12	Krapcho et al., International Patent Application WO 92/15195, 1992
μ-agatoxin	Skinner et al., *J. Biol. Chem.* 264: 2150-2155, 1989; Adams et al., *J. Biol. Chem.* 265: 861-867, 1990
King Kong toxin	Hillyard et al., *Biochem.* 28: 358-361, 1989
Pt6	Leisy et al., European Patent Application EP 0556160 A2, 1993
NPS-326	Krapcho et al., International Patent Application WO 93/15192, 1993
NPS-331	Kraphco et al., International Patent Application WO 93/15192, 1993
NPS-373	Krapcho et al., International Patent Application WO 93/15192, 1993
Tx4(6-1)	Figueriredo et al., *Toxicon* 33: 83-93, 1995
TxP-1	Tomalski et al., *Toxicon* 27: 1151-1167, 1989
ω-atracotoxins	Atkinson et al., International Patent Application WO 93/15108, 1993
α-conotoxins	Gray et al., *J. Biol. Chem.* 256: 4734-4740, 1981; Gray et al., *Biochem.* 23: 2796-2802, 1984

μ-conotoxins	Cruz et al., *J. Biol. Chem.* 260: 9280-9288, 1989; Cruz et al., *Biochem.* 28: 3437-3442, 1989
chlorotoxin	Debin et al., *Am. J. Physiol.* 264: 361-369, 1993
ω-conotoxins	Olivera et al., *Biochem.* 23: 5087-5090, 1984; Rivier et al., *J. Biol. Chem.* 262: 1194-1198, 1987

The toxin-encoding sequence is operably linked to a promoter, i.e., a promoter sequence is placed upstream of the toxin-encoding sequence so that expression of the toxin is under the control of the promoter. The promoter may be a baculovirus-derived promoter, such as, e.g., DA26, 35K, 6.9K, and polyhedrin (polh) promoters (O'Reilly *et al.*, *J. Gen. Virol.* 71:1029 (1990); Friesen *et al.*, *J. Virol.* 61:2264, 1987; Wilson *et al.*, *J. Virol.* 61:661-666, 1987; Hooft van Iddekinger *et al.*, *Virol.* 131:561, 1983; and *see*, generally, Miller, ed., *The Baculoviruses*, Plenum Press, New York, 1997). Alternatively, a host cell promoter may be used, such as, e.g., an insect-derived hsp70 promoter or actin promoter. Any native or synthetic promoter active in promoting transcription in target insect cells may be used.

Furthermore, the DNA sequence encoding the toxin may comprise the native upstream sequence encoding the signal peptide which, in its cell of origin, directs secretion of the toxin. Alternatively, the toxin-encoding sequence may be fused in-frame with an upstream DNA sequence encoding a heterologous signal sequence, i.e., a sequence derived from another source, including without limitation sequences derived from the pBMHPC-12 signal sequence from *Bombyx mori*, the adipokinetic hormone signal sequence from *Manduca sexta*, the apolipophorin signal sequence from *Manduca sexta*, the chorion signal sequence from *Bombyx mori*, the cuticle signal sequence from *Drosophila melanogaster*, the esterase-6 signal sequence from *Drosophila melanogaster*, and the sex-specific signal sequence from *Bombyx* mori, all of which are disclosed in U.S. Patent No. 5,547,871.

In some embodiments, the recombinant PxNPVs of the invention have incorporated within their genome a gene encoding wild-type or mutant juvenile hormone esterase (JHE), expression of which can cause irreversible termination of the feeding stage and pupation and thus result in death of the target insect. See, e.g., WO 94/03588.

Recombinant PxNPVs may also be produced by genetically altering a wild-type or pre-existing recombinant PxNPV strain in a manner that results in the modification of one or more viral-encoded functions. For example, one or more viral genes, such as, e.g., those encoding the viral polyhedrin protein, ecdysteroid glucosyl transferase (EGT), or p10 protein may be modified, deleted, or duplicated. Furthermore, viral-encoded sequences derived from other baculoviruses may also be introduced, such as, e.g., the region from the V8 strain of AcMNPV which carries a determinant that results in a faster killing phenotype, as disclosed in U.S. Patent No. 5,662,897.

Non-limiting examples of recombinant PxNPVs according to the invention include those having ATCC deposit numbers VR-2607,VR-2608, and VR-2609.

The present invention provides methods and compositions for the construction of recombinant PxNPVs. Any method known in the art may be used to construct recombinant PxNPVs. For example, co-infection of an appropriate host cell with two strains of PxNPV may result in homologous recombination *in vivo* between related sequences, resulting in the formation of a recombinant PxNPV. Similarly, homologous recombination can occur *in vivo* in cells co-transfected with purified PxNPV viral genomic DNA and a second nucleic acid containing PxNPV sequences. Alternatively, recombinant PxNPVs may be produced by introducing into a cell isolated viral genomic DNA which had been previously modified *in vitro*.

In one series of embodiments, recombinant PxNPVs are formed by the use of direct ligation vectors and modular expression vectors. These components, and methods for using them to form recombinant PxNPVs, are described below.

PxNPV-Derived Direct Ligation Vectors

Direct ligation virus vectors comprise purified PxNPV viral genomic DNAs which can be used to construct recombinant PxNPV genomes by DNA ligation *in vitro*. Direct ligation vectors direct the production of recombinant PxNPV virions when introduced into an appropriate host cell. In some embodiments, PxNPV direct ligation vectors comprise PxNPV genomic DNA that has been modified to incorporate at least one cloning site not present in wild-type PxNPV. A cloning site comprises one or more restriction sites which are either absent from wild-type PxNPV genome or are not found within the nucleic acid encoding or regulating an essential PxNPV viral function. In the

latter case, the direct ligation vectors of the invention are engineered to delete any such restriction sites which lie outside the cloning site, so that the cloning site comprises at least one unique restriction site. Digestion of a direct ligation vector using one or more restriction enzymes specified by the cloning site thus produces a DNA preparation into which a heterologous nucleic acid segment can be introduced, usually in a single ligation step, without disrupting an essential viral function. Following ligation of a heterologous nucleic acid into a direct ligation vector, the resulting DNA preparation may be introduced into an appropriate host cell for propagation of recombinant PxNPV. Direct ligation vectors simplify the production of recombinant PxNPVs by obviating the dependence on *in vivo* recombination events to form recombinant viruses. See, e.g., International Patent Application WO 94/28114.

Cloning sites for use in PxNPV direct ligation vectors are designed by first selecting one or more restriction enzymes that either (i) do not digest PxNPV DNA at all or (ii) recognize a small number of sites that do not lie within the nucleic acid encoding or regulating an essential PxNPV viral function. The selection is performed by (i) searching the PxNPV DNA sequence computationally or (ii) subjecting PxNPV DNA to digestion with the enzyme(s) and detecting the presence or absence of digestion products. If the enzyme recognizes a small number of sites, these sites may be disrupted, using conventional techniques (such as, e.g., restriction enzyme digestion followed by blunt-ending and re-ligation) to produce PxNPV DNA that lacks the sites but supports viral replication and infectivity.

After selection of one or more restriction sites, the cloning site is introduced into PxNPV DNA (whether wild-type or modified as described above to inactivate one or more restriction sites) by any appropriate means, including homologous recombination *in vivo* or ligation *in vitro*. Preferably, the cloning site includes at least non-overlapping restriction sites to allow (i) directional cloning of an insert nucleic acid and/or (ii) independent insertion of multiple insert nucleic acids.

To form direct ligation vectors from PxNPV, other modifications may be introduced, including without limitation those that result in inactivation of a viral gene, such as, e.g., that encoding polyhedrin, ecdysteroid glucosyl transferase (EGT) or p10 protein. In some embodiments, the cloning site is introduced in a location that results in such inactivation.

Modular Expression Vectors and Expression Cassettes:

Modular expression vectors for use in the present invention are plasmid vectors containing an expression cassette which can be excised from the modular expression vector and ligated into the PxNPV direct ligation vectors described above. Typically, the expression cassette comprises, in a 5' - to - 3' direction: a promoter sequence operably linked to a 5' untranslated region (UTR) which includes the transcription start site; a sequence containing one or more restriction sites to facilitate insertion of a heterologous gene (this sequence is designated an "insertion site"); and a 3' UTR sequence containing at least a site for 3' terminal mRNA processing and polyadenylation. The expression cassette is flanked on either end by appropriate restriction sites compatible with a PxNPV direct ligation vector.

Suitable promoters for use in modular expression vectors include baculovirus promoters and host cell promoters. Suitable baculovirus promoters include, without limitation, DA26, 35K, 6.9K, and polyhedrin (polh) promoters. Suitable host cell promoters include without limitation hsp70 and actin promoters, preferably derived from an insect species. Sequences "derived from" a promoter sequence encompass modifications, including deletions, insertions, substitutions and duplications, of native promoter sequences. The only requirement is that the final promoter function effectively in a target insect cell to direct the expression of the heterologous gene to which it is operably linked.

Expression cassettes may also include sequences encoding signal sequences, which direct the secretion of the heterologous protein. The signal sequences may be those associated with the heterologous protein or may be derived from a different protein. Suitable signal sequences include, without limitation, those derived from the pBMHPC-12 signal sequence from *Bombyx mori*; the adipokinetic hormone signal sequence from *Manduca sexta*; the apolipophorin signal sequence from *Manduca sexta*; the chorion signal sequence from *Bombyx mori*; the cuticle signal sequence from *Drosophila melanogaster*; the esterase-6 signal sequence from *Drosophila melanogaster*; and the sex-specific signal sequence from *Bombyx* mori. A nucleic acid sequence encoding the signal peptide is inserted between the 5-UTR and the start of the mature heterologous protein. The junction between the 3' terminus of the signal peptide-encoding sequence and the start of the mature heterologous protein is designed so that insertion of the heterologous sequence results in an

in-frame fusion protein between the signal peptide and the heterologous sequence. Sequences "derived from" a known signal sequence encompass modifications, including deletions, insertions, and substitutions, of amino acid residues within the signal sequence. The only requirement is that the resulting sequence function effectively in a target insect cell to direct the secretion from the insect cell of the heterologous sequence to which it is linked.

Heterologous sequences for use in the present invention include, without limitation, those encoding insect-modifying substances, such as, e.g., insecticidal toxins, hormones, enzymes, and receptors. Suitable insecticidal toxins include without limitation AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

The nucleic acid sequences encoding the insect-modifying substances and/or the signal peptides may correspond to the native nucleic acid sequences encoding these peptides. Alternatively, the sequences may be altered to take into account the optimal codon usage for known genes in either the virus vector (or closely related strains) and/or in the insects that are the targets of the insecticidal viruses of the invention. Codon optimized sequences, i.e., sequences in which the nucleic acid sequence encoding a particular amino acid has been modified without changing the amino acid encoded at that position, may be designed using methods well-known in the art, such as, for example, by comparing codon usage in known gene sequences in the virus and/or in the target insect and in the nucleic acid sequences encoding the signal peptides and insect-modifying substances of the invention. Preferably, codon usage in the sequences encoding the signal peptides and insect-modifying substances reflects the codon usage of the virus vector or the target insect. Examples of codon-optimized toxin sequences are shown in Figures 10 and 11.

Production of Recombinant PxNPVs

Recombinant PxNPVs may be produced by either (i) co-transfecting PxNPV DNA and a heterologous sequence into an appropriate host cell, to allow for homologous recombination *in vivo* or (ii) ligation *in vitro* of a heterologous sequence into a direct ligation vector, followed by introduction of the construct into an appropriate host cell to

allow for viral propagation. Appropriate host cells are any cells that support baculovirus replication, including without limitation Sf9 cells, Sf21 cells, and High Five™ cells (Invitrogen, Carlsbad CA). An isolated virus according to the invention is one which has been cloned through plaque purification in tissue culture, for example, or otherwise prepared from a single viral genotype.

Typically, a modular expression vector is constructed to contain an expression cassette in which a suitable promoter sequence is operably linked to a sequence encoding a heterologous protein, i.e., expression of the heterologous protein is placed under the control of the promoter. The expression cassette is excised from the modular expression vector and inserted into a PxNPV direct ligation vector by DNA ligation *in vitro*. The ligation mixture is then transfected into an appropriate host cell. Recombinant PxNPVs are recovered from the growth medium and characterized for LC_{50} and LT_{50} using any conventional method, including without limitation diet overlay assays, diet incorporation assays, and leaf dip assays.

LC_{50} is the concentration of virus at which 50% of infected larvae are dead within the duration of the test period. LT_{50} is the time after infection when 50% of the infected larvae are dead when exposed to a specified dose of virus.

Preferably, PxNPVs according to the invention exhibit an LC_{50} of about 1 x 10^5 OBs/ 16cm^2 or less on *Plutella xylostella* larvae when measured using the standard diet overlay assay as described in Example 6 below. Other baculovirus isolates typically exhibit higher LC_{50}s on *Plutella xylostella* larvae, i.e., they are less efficacious, relative to their infectivity for other insect species.

Insecticidal Compositions and Formulations

The present invention provides insecticidal compositions and formulations that include one or more recombinant PxNPVs. Preferably, the recombinant PxNPVs of the invention kill *Plutella xylostella* larvae more effectively than wild-type PxNPV or recombinant versions of other baculoviruses (see, e.g., Example 11 below).

An insecticidal composition according to the invention includes at least one recombinant PxNPV. An insecticidal formulation comprises at least one recombinant PxNPV in an insecticidally effective amount and an agriculturally suitable carrier. An insecticidally effective amount is an amount that causes a detectable reduction in the

infestation, as manifested in the number or amount of the insect pests in a given area or amount of a crop; the damage caused by the insect pests; or any other appropriate parameter of infestation. The formulations may be in the form of wettable powders, dispersible granular formulations, granules, suspensions, emulsions, solutions for aerosols, baits, and other conventional insecticide preparations. Suitable carriers are, without limitation, water, alcohol, hydrocarbons or other organic solvents, or a mineral, animal, or vegetable oil, or a powder such as talc, clay, silicate, or kieselguhr. Wetting agents, coating agents, UV protectants, dispersants, and sticking agents may also be included. A nutrient such as a sugar may be added to increase feeding behavior and/or attract insects. Flow agents such as, for example, clay-based flow agents, may be added to minimize caking of wettable powders or other dry preparations. The compositions may be formulated as coated particles or as microencapsulated material. The formulations must be non-phytotoxic and not detrimental to the integrity of the recombinant PxNPV contained therein, nor should any components significantly deter insect feeding or any viral functions. Exemplary formulations are disclosed in EP published application 0697 170 A1; PCT application WA 92/19102; and U.S. Patent No. 4,948,586.

The insecticidal formulations of the invention may also include one or more chemical insecticides and/or one or more non-PxNPV biological control agents. Chemical insecticides include without limitation pyrethroids, pyrazolines, organophosphates, carbamates, formadines, and pyrroles, all of which are well-known in the art. Exemplary compounds are disclosed in PCT applications 96/03048, 96/01055, and 95/95741. Biological control agents include, e.g., non-PxNPV baculoviruses (native or recombinant); *Bacillus thuringiensis*; *Nosema polyvora*; *M. grandis*; *Bracon mellitor*; entomopathogenic fungi; and nematodes.

The present invention also provides methods for killing insect pests. The methods comprise contacting the insects with an insecticide-effective amount of the compositions or formulations of the invention. The invention also provides methods for reducing insect infestation of, e.g., a crop, which comprise administering to a desired locus an insecticidally effective amount of the compositions or formulations of the invention. The insecticidal formulations are administered using conventional techniques, such as, e.g., spraying or dusting crops. Typically, the formulations are administered at dosages of between about 2.4×10^8 and about 2.4×10^{12} OBs/hectare (OBs are occlusion bodies).

Effective dosages depend on, for example, the insect target, the recombinant PxNPV used, and the plant crop being treated. The dosages comprising an insecticidally effective amount can be determined by those of ordinary skill in the art using conventional methods.

Description of the Preferred Embodiments

The following examples illustrate the invention without limitation.

Example 1: Construction of Egt-Deleted β-Galactosidase-Containing Recombinant PxNPV

The following experiments were performed to produce a recombinant PxNPV in which the viral ecdysteroid glucosyl transferase (egt) gene had been deleted and replaced with an *E. coli* β-galactosidase (β-gal) marker gene.

A stock of wild-type PxNPV that had been passaged through insects was plaque purified using conventional procedures as described in O'Reilly *et al.* (*Baculovirus Expression Vectors: A Laboratory Manual*, Oxford University Press, New York, NY, 1994) to produce a clonal stock of virus for genetic engineering. The integrity of this stock, designated PxNPV-3, was confirmed by comparison of restriction enzyme patterns to the parental stock of PxNPV. Bioassays against a panel of insect species also confirmed that its virulence matched the uncloned stock.

Figure 1 illustrates the procedure used to construct a transfer vector for use in disrupting the egt gene in PxNPV by insertion of a β-galactosidase gene. The close similarity of the restriction enzyme patterns of AcNPV and PxNPV indicated homology at the DNA level which enabled the use of transfer vectors based on the V8 strain of AcNPV, which is disclosed in U.S. Patent No. 5,662,897. A vector comprising the β-gal cassette, designated pmd 216.1, was constructed as follows. A Bam HI-to-Xba I fragment containing the β-gal gene under control of the *Drosophila melanogaster* hsp 70 promoter was isolated from pAcDz1 (Zuidema *et al.*, *J. Gen. Virol.* 71:2201 (1990)). This fragment was then subcloned into pBluescript™ SK$^+$ (Stratagene, La Jolla, CA) between the Bam HI and Xba I sites of the polylinker. The Pst "G" fragment of AcNPV strain V8vEGTDEL, which contains the egt gene and surrounding region (*see*, U.S. Patent No. 5,662,897), was then subcloned into the polylinker of pUC 9 at the Pst I site. The resulting plasmid was designated pmd 205.1.

The final transfer vector was constructed by performing a four-fragment ligation between (i) pBluescript™ SK$^+$ digested with Bam HI and Pst I which had been dephosphorylated with calf intestinal phosphatase; (ii) the 0.975 kb Bgl II-to-Pvu II fragment of pmd 205.1; (iii) the 3.86 kb Sma I-to-Xba I β-gal fragment of pmd 216.1; and (iv) the 1.6 kb Xba I-to-Pst I fragment of pmd 205.1. The resulting transfer vector, designated pmd 220.2, contains an hsp 70-driven β-gal marker gene cloned into the site of the egt deletion.

An egt-deleted β-gal marked PxNPV was constructed by homologous recombination between pmd 220.2 and PxNPV-3 DNA cotransfected into cultured Sf-9 cells. 1 μg of pmd 220.2 DNA and 250 ng of PxNPV-3 DNA were mixed with 25 μg of Lipofectin (Gibco BRL, Gaithersburg MD) in a final volume of 200 μl of TNM-FH (JRH Biosciences, Lenexa KS). After incubation at room temperature for 15 minutes, the mixture was brought to a final volume of 2.0 ml with TNM-FH complete media (TNM-FH plus 10% fetal bovine serum and 1% pluronic F68 (Gibco BRL, Gaithersburg, MD)). The mixture was overlaid on 2.75 x 10^5 Sf-9 cells which had been plated in a well of a standard 6-well tissue culture plate. Cells were refed with fresh media at 24 hours and budded virus was harvested after an additional 96 hours. Recombinant viruses were occlusion body positive (occ+) and expressed the β-gal gene. Recombinants (occ+/blue plaques) were identified by three rounds of plaque purification in the presence of 100 μg/ml 5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The resulting virus was designated T96-19.1.1.1.

Example 2: **Construction of an Egt-Deleted Direct Ligation PxNPV Viral Vector**

The following experiments were performed to construct a direct ligation vector derived from PxNPV (see, e.g., International Patent Application US 94/06079). This vector contains a polylinker (designated "Bsu-Sse linker", see below) inserted into the PxNPV genome at the egt site.

Two oligonucleotides were synthesized to form the Bsu-Sse linker, having the sequences:

5'- CCTCAGGGCAGCTTAAGGCAGCGGACCGGCAGCCTGCAGG -3' (Oligo 32) and

5'- CCTGCAGGCTGCCGGTCCGCTGCCTTAAGCTGCCCTGAGG -3' (Oligo 33)

Once annealed, these two oligomers encode several restriction endonuclease sites, including Bsu 36I and Sse 8387I sites, useful in the construction of recombinant viruses. Each was diluted to a concentration of 30 pmol/μl into an annealing buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM EDTA. The mixture was heated to 95°C for 10 minutes and then slowly cooled to room temperature over several hours.

The annealed oligonucleotides were inserted into the pmd 205.1 transfer vector as follows. The pmd 205.1 vector was digested with Spe I, which cuts at a single site located just upstream of the egt deletion (Figure 2). The ends of the digested plasmid were filled in using the Klenow fragment of DNA polymerase I in the presence of all four nucleotides. 100 ng of the linearized plasmid were then ligated to 15 pmol of the double stranded linker with T4 DNA ligase in a total volume of 10 μl. After the ligation reaction was complete, the T4 ligase was heat inactivated and the mixture was treated with polynucleotide kinase. The 8.8 kb DNA band was purified by electrophoresis in a 1% low melt preparative grade agarose gel (BioRad, Richmond VA). The gel slice containing the 8.8 kb linear DNA was melted at 65°C and an in-gel ligation using approximately 1/10 of the gel slice was used to recircularize the DNA, which was then used to transform *E. coli* DH5α cells.

The resulting plasmids were screened using polymerase chain reaction (PCR) to determine the orientation of the Bsu-Sse linker relative to the egt gene. Oligos 32 and 33 were separately paired with oligomer EGT 1 (5'-GCGGCCAATATATTGGCCGTGTTT-3'), which is specific for the region of the egt gene 5' to the deletion. The orientation of the Bsu-Sse linker in LAB 50.2 is indicated in Figure 2.

An egt-deleted direct ligation PxNPV vector was constructed by homologous recombination between LAB 50.2 and T96-19.1.1.1 PxNPV viral DNA that had been co-transfected into cultured Sf9 cells as described in Example 1, using 1 μg of LAB 50.2 and 250 ng of T96-19.1.1.1 viral DNA. In this case, recombinant viruses were occlusion body positive and did not express the β-gal gene. Recombinant (occ+/white) viruses were identified by three rounds of plaque purification in the presence of 100 μg/ml X-gal. The resulting virus was named T97-8.1.1.1 (ATCC deposit no. VR-2608).

Example 3: Construction of Modular Expression Vectors Useful for Production of Recombinant PxNPVs

A. pMEV 1-4

The design, construction and usage of vectors pMEV1, pMEV2, pMEV3 and pMEV4 for the expression of foreign genes under the control of baculoviral promoters are disclosed in International Patent Application US 94/06079. These vectors have the general structure shown in Figure 3. Each vector was derived from the pBluescript SK+ plasmid (Stratagene, La Jolla CA) by substituting the region between the Sst I and Xho I sites in the pBluescript polylinker with an expression cassette composed of the following elements: (i) a short synthetic linker DNA with recognition sites for restriction enzymes Sst I, Sse 8387 I and Stu I; (ii) a promoter module, which consists of the promoter and complete 5' untranslated region (UTR) of a selected AcMNPV viral gene; (iii) a polylinker module, to facilitate insertion of a protein coding region of interest; (iv) a 3' UTR module, which consists of the complete 3'-untranslated region of the AcMNPV 6.9K (basic protein) gene; and (v) a short synthetic linker DNA with recognition sites for restriction enzymes Stu I, Bsu 36 I and Xho I.

As shown in Figure 4, the promoter modules in vectors pMEV1 through pMEV4 are derived from genes that are expressed at different stages in the virus life cycle. The DA26 gene promoter module in pMEV1 and the 35K gene promoter module in pMEV4 are derived from genes that are expressed early in the virus life cycle; that is, before the onset of DNA synthesis. The 6.9K gene promoter module in pMEV2 is derived from a "late" structural gene, which is expressed after the onset of DNA synthesis, and the polyhedrin (polh) gene promoter module in pMEV3 is from a "very late" gene that encodes the major structural component of viral occlusion bodies.

The polylinker module is designed to allow placement of a protein coding region immediately adjacent to the 5' UTR of the promoter module without the introduction of extraneous linker sequences. As shown in Figure 5, the polylinker contains an Esp3 I site which is positioned so that digestion of the vector with Esp3 I cuts between positions -4 and -5 in the top strand of the promoter module and at the junction between the promoter and polylinker modules in the bottom strand. Treatment of Esp3 I-digested DNA with DNA polymerase in the presence of the four standard 2'-deoxynucleoside triphosphates (dNTPs) creates a linearized vector that is blunted at the exact 3' terminus of the promoter

module. This segment can be joined to a 5' blunt-ended fragment whose sequence begins with the ATG initiation codon of the desired protein coding region. To facilitate directional cloning of the protein coding fragment, the 3' terminus of the fragment is constructed so that it contains a recognition site for one of the enzymes that cleave within the polylinker module (illustrated with BamH I in Figure 5) and both the protein coding fragment and vector are digested with this enzyme prior to ligation.

A key feature of these vectors is the presence of recognition sites for restriction enzymes Sse8387 I and Bsu36 I at either end of the expression cassette. This allows for the excision of inserted sequences from the modular expression vector and their ligation into the direct ligation PxNPV vectors of the present invention (see, e.g., Example 2 above).

B. pMEV5 and pMEV6

Two vectors, pMEV5 and pMEV6, were constructed to incorporate a *D. melanogaster* hsp70 (major heat shock) gene promoter. pMEV5 contains a 724 bp segment of the *D. melanogaster* hsp70 promoter/5' UTR (designated hsp70Bam in Figure 4) extending from position -493 to position +231 with respect to the transcription start site of the hsp70 gene. pMEV6 contains a 475 bp segment of the same promoter/5' UTR (designated hsp70Xba in Figure PD2), extending from position -244 to position +231.

The promoter modules used to construct pMEV5 and pMEV6 were synthesized by PCR amplification using plasmid phcHSP70PL (Morris *et al.*, *J. Virol.* 66:7397 (1992)) as the template. The oligonucleotide primers for each reaction and the sequence of the amplified product are shown in Figures 6 and 7 (for pMEV5 and pMEV6, respectively). Each of the primers has a bipartite structure. The 5' portion has no sequence homology with the phcHSP70PL template and is used to incorporate specific restriction sites into the termini of the final PCR product. These include the Sst I, Sse8387 I and Stu I sites at the 5' end of the PCR product and the Esp3 I and Xba I sites at the 3' end of the PCR product (see Figures 6 and 7). The 3' portion of each primer contains sequences homologous to the phcHSP70PL template and defines one of the boundaries of the hps70-specific sequences in each module.

Prior to the PCR reactions, the (-) strand primer (HSP70Esp) was phosphorylated at its 5' terminus by incubating 200 pmol of primer for 30 min at 37°C in a 10 ml reaction containing 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 10 units T4 polynucleotide kinase (New England Biolabs, Beverly MA). The PCR reactions were then performed in separate MicroAmp tubes (Perkin Elmer, Norwalk CT) according to the following procedure. 50 pmol of each primer was combined with 0.25 - 1.0 ng of phcHSP70PL DNA in a 50 μl reaction containing 200 μM dNTPs, 1X cloned *Pfu* polymerase buffer (Stratagene, La Jolla CA) and 5 units of cloned *Pfu* DNA polymerase (Stratagene, La Jolla CA). The samples were placed in a Perkin-Elmer Model 9600 thermal cycler (Perkin Elmer, Norwalk CT) and subjected to 2 cycles of 1 min at 94°C (denaturation step), 1.5 min at 50°C (annealing step) and 3 min at 72°C (extension step) followed by 28 cycles of 1 min at 94°C, 1.5 min at 55°C and 3 min at 72°C. On the last cycle, the extension reaction was carried out for an additional 7 min and the reactions were brought to 4°C. The amplification products were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), adjusted to 0.3M sodium acetate, and precipitated with ethanol.

After dissolution in a suitable reaction buffer, the DNA was digested with Pst I (which cleaves at the Sse8387 I site) and the fragments containing the presumptive hsp70 promoter modules were purified by electrophoresis on a 1.2% low melt preparative grade agarose gel (BioRad, Richmond, CA). The base vector for constructing pMEV5 and pMEV6 was prepared by cleaving pMEV1 with EcoR I and filling in the ends with the Klenow fragment of DNA polymerase I in the presence of all four dNTPs. Following secondary digestion of the vector by Sse8387 I and dephosphorylation of the termini with calf intestine alkaline phosphatase, the large (3.1 kb) fragment containing the pBluescript backbone and the 3' UTR and polylinker modules was purified on a low melt agarose gel and ligated individually with the purified hsp70 promoter modules to form pMEV5 and pMEV6.

C. pMEV5/ADK-AaIT and pMEV6/ADK-AaIT

The pMEV5 and pMEV6 vectors described above were further engineered to incorporate the gene encoding AaIT, an insect-specific toxin that is expressed in the venom of the North African scorpion *Andoctuonus australis* (Hector) (Zlotkin *et al.*,

Biochimie 53:1073 (1971)). When AaIT is injected into the body cavity of an insect larva, it binds selectively to voltage-sensitive sodium channels and causes a transitory contractile paralysis. Chronic administration of the toxin, which can be achieved by infecting insect larvae with AaIT-producing baculoviruses, is associated with a prolonged state of paralysis and eventual death (Stewart *et al.*, *Nature* 352:85 (1991); Maeda *et al.*, *Virol.* 184:777 (1991); McCutchen *et al.*, *Biotechnol.* 9:848 (1991)). U. S. Patent 5,547,871 discloses a codon-optimized ADK-AaIT gene sequence in which secretion of the AaIT toxin is directed by a signal peptide from the *Manduca sexta* adipokinetic hormone (ADK) gene. The insertion of the ADK-AaIT coding region into the pMEV1 - pMEV4 vectors (to yield plasmids pMEV1/ADK-AaIT - pMEV4/ADK-AaIT) is disclosed in International Patent Application US 94/06079.

To construct the pMEV5 and pMEV6 derivatives encoding ADK-AaIT, a fragment containing the ADK-AaIT gene sequence was synthesized by PCR using pMEV3/ADK-AaIT as a template. The (+) strand primer, designated PD30, begins at the initiator ATG codon of the ADK signal peptide. The (-) strand primer, designated 69K3UT, was chosen so that it primes DNA synthesis at a location downstream of the polylinker module; that is, within the 3'UTR of pMEV3/ADK-AaIT. The sequences of the primers and the amplified DNA fragment are presented in Figure 8.

Prior to amplification, the 5' terminus of the (+) strand primer, PD30, was phosphorylated as described above for HSP70Esp. The PCR reaction was also carried out as described above, except that the amplification was performed for 25 cycles of 1 min at 94°C, 1.5 min at 52°C, and 3 min at 72°C. After synthesis, the DNA was digested with BamH I, which cuts in the polylinker module, and the 274 bp 5'-blunt/3'-BamH I fragment containing the ADK-AaIT coding region is inserted into pMEV5 and pMEV6. The structures of the resulting plasmids, pMEV5/ADK-AaIT and pMEV6/ADK-AaIT, were confirmed by restriction enzyme analysis and partial DNA sequence determination.

D. MEVS vectors containing an ADK signal peptide module

The following experiments were performed to produce a series of modular expression vectors that contain DNA encoding the ADK signal peptide (see above) that can be used to direct the secretion of any inserted sequence. A series of vectors (designated pMEV1/ADK through pMEV6/ADK) was constructed in which the 57 bp codon-optimized

sequence for the ADK signal peptide (residues 1 - 57 in Figure 8) was placed between the promoter and polylinker modules. The expression cassettes in these vectors have the general structure shown in Figure 9.

To construct the pMEVx/ADK vector series, a DNA fragment containing the promoter module and linked ADK signal peptide was recovered from the corresponding pMEVx/ADK-AaIT vector by PCR. The (+)-strand primer for each reaction was a promoter specific oligonucleotide that primes DNA synthesis at the 5' end of the promoter module and incorporates restriction sites for Sst I, Sse8387 I and Stu I into the 5' end of the amplified fragment. The templates and (+) strand primers for each reaction are listed in the following table:

Template	Primer	Sequence
pMEV1/ADK-AaIT	DA26FZ	5'- AGCAGCGAGCTCCTGCAGGCCTACGCGT AATTCGATATAGAC -3'
pMEV2/ADK-AaIT	69KFZ	5'- AGCAGCGAGCTCCTGCAGGCCTATGCCG TGTCCAATTGCAAG -3'
pMEV3/ADK-AaIT	PHF	5'- AGCAGCGAGCTCCTGCAGGCCTGACGCA CAAACTAATATCAC -3'
pMEV4/ADK-AaIT	35KPRO1	5'- AGCAGCGAGCTCCTGCAGGCCTCTTGAT GTCTCCGATTTC -3'
pMEV5/ADK-AaIT	HSP70Bam	5'- AGCAGCGAGCTCCTGCAGGCCTGATCCT TAAATTGTATCCTA -3'
pMEV6/ADK-AaIT	HSP70Xba	5'- AGCAGCGAGCTCCTGCAGGCCTAGAATC CCAAAACAAACTGG -3'

The (-) strand primer for each reaction, designated ADKRev (5'-CGGATCTAGACACGTCTCGGGCCTCAGCGATAATCACGAAGGC-3'), primes synthesis from the 3' terminus of the ADK signal peptide and incorporates sites for Esp3 I and Xba I into the 3' end of the amplified fragment. The PCR reaction was carried out as described above for pMEV5-6, except that the amplification is performed for 25 cycles of 1 min at 94°C, 1.5 min at 52°C and 3 min at 72°C. The DNA synthesized from all of the templates except pMEV5/ADK-AaIT was digested with Pst I, which cuts at the Sse8387 I site upstream of the promoter, and Xba I, which cuts downstream of the ADK signal peptide, and the fragment containing the promoter module and signal peptide was inserted

between the Pst I (Sse8387 I) and Xba I sites in one of the existing pMEVx vectors, such as pMEV1.

Because the hsp70Bam promoter module in pMEV5 contains an internal Xba I site, the hsp70Bam/ADK module must be inserted into pMEVx framework in two pieces. Accordingly, one portion of the DNA synthesized from the pMEV5/ADK-AaIT template was digested with *Pst* I and *Xho* I and a second portion was digested with Xho I and Xba I. The Pst I/Xho I fragment representing the 5' segment of the hsp70Bam/ADK module was combined with the Xho I/Xba I fragment representing the 3' segment of the hsp70Bam/ADK module and ligated into a Pst I/Xba I vector fragment prepared from pMEV1.

Each of the resulting pMEVx/ADK vectors is identical in structure to the corresponding pMEVx vector, except for the insertion of the 57 bp ADK signal peptide between the promoter and polylinker modules.

Example 4: **Construction of Modular Expression Vectors Encoding Insecticidal Toxins**

The following experiments were performed to produce modular expression vectors encoding insecticidal toxins suitable for incorporation into recombinant PxNPVs according to the present invention.

A. Txp-I

The straw itch mite toxin TxP-I is a paralytic neurotoxin isolated from the venom of the predatory straw itch mite, *Pyemotes tritici* (Tomalski *et al.*, *Toxicon* 27:1151 (1989)). The *tox34* gene encodes a precursor protein of 291 amino acids (Tomalski *et al.*, *Nature* 352:82 (1991)).

To test the efficacy of the *tox34* gene within the context of a recombinant PxNPV, three different Txp-1 constructs were prepared which differed in the aminoterminal signal sequence that directs secretion of the toxin from cells. One construct retained the native *tox34* aminoterminal sequence; one contained an ADK signal peptide in place of the aminoterminal 40 residues of the *tox34* preprotein; and one contained the ADK signal peptide in place of the aminoterminal 26 residues of the *tox34* preprotein.

Three different segments of the *tox34* gene corresponding to codons 1 - 291 (designated tox34), 27 - 291 (tox34L) and 40 - 291 (tox34S) were synthesized by PCR and

adapted for cloning into one or more of the modular expression vectors described above. The template for the amplification reactions was plasmid pHSP70tox34 (Lu *et al.*, *Biological Control* 7:320 (1996)). The plus-strand primer for each amplification is shown in the following table:

Fragment	Primer	Sequence
tox34	TOX34ATG	5' - ATGAAAATTTGTACATTTTTTATTCC -3'
tox34L	TOX34NT1	5' - GTTAAACCTTTTAGGTCTTTTAATAATATTTCC -3'
tox34S	TOX34NT2	5' - GATAATGGCAATGTCGAATCTGTA - 3'

The (-) strand primer, designated TOX34CT2, 5'-GTACCCCCGGGATCCAATTTAACACAGTCTTGAATCACTT-3', primes synthesis from the 3' end of the *tox34* coding region and incorporates restriction sites for BamH I and Xma I (Sma I) into the 3' terminus of the amplified fragment. Prior to amplification, the 5' terminus of each (+) strand primers was phosphorylated using the procedure described for primer HSP70Esp in Example 3 above. The PCR reactions were carried out as described in Example 3, except that the cycling consisted of 2 cycles of 1 min at 94°C, 1.5 min at 45°C and 3 min at 72°C followed by 28 cycles of 1 min at 94°C, 1.5 min at 55°C and 3 min at 72°C.

Following synthesis, the DNA was digested with Xma I and the 5'-blunt/3'-Xma I *tox34* coding region fragments (designated tox34, tox34L or tox34S) were purified and cloned into a MEVS vector prepared as described in Example 3, except that the polylinker was cleaved with Xma I (which cleaves at the Sma I site) rather than with BamH I.

The following table summarizes the components from which each TxP-I expressing MEVS vector was constructed:

Construct	Vector	Txp-I encoding fragment
pMEV1/Tox34	pMEV1	tox34
pMEV5/Tox34	pMEV5	tox34
pMEV6/Tox34	pMEV6	tox34
pMEV1/ADK-Tox34L	pMEV1/ADK	tox34L
pMEV1/ADK-Tox34S	pMEV1/ADK	tox34S

pMEV2/ADK-Tox34L	pMEV2/ADK	tox34L
pMEV2/ADK-Tox34S	pMEV2/ADK	tox34S

B. LqhIT2

In addition to excitatory toxins, such as AaIT, many *Buthinae* scorpion venoms contain a second type of insect-specific toxin that causes a slow, progressive flaccid paralysis (Zlotkin *et al.*, *Biochemistry* 30:4814 (1991)). The best studied member of this group is the toxin LqhIT2, which is isolated from the scorpion *Leiurus quinquestriatus hebreaeus*. The cloned cDNA for LqhIT2 reveals a 21 amino acid signal peptide and three C-terminal amino acids (Gly-Lys-Lys) that are removed from the toxin post-translationally (Zlotkin *et al.*, *Arch. Insect Biochem. Physiol.* 22:55 (1993)).

To investigate the ability of the LqhIT2 toxin to improve the insecticidal activity of PxNPV, a DNA sequence encoding the mature LqhIT2 toxin was assembled and cloned into four different pMEVx/ADK expression vectors: pMEV1/ADK, pMEV2/ADK, pMEV5/ADK and pMEV6/ADK. The sequence of the toxin coding region shown in Figure 10 differs from the native cDNA sequence in 27 of 61 codons; these changes were introduced so that codon usage in the synthetic LghIT2 coding region reflects overall codon usage in the AcMNPV genome (Ayres et al (1994)).

Assembly of the DNA fragment containing the LqhIT2 coding region was carried out in several steps. First, four oligonucleotides were synthesized which collectively represent both strands of the LqhIT2 coding region and a small amount of 3' flanking linker DNA (Figure 10). Prior to use, the 5' termini of oligonucleotides LqhIT2F2 (which comprises the 3' portion of the (+) strand) and LqhIT2R3 (which comprises the 3' portion of the (-) strand) were phosphorylated using the procedure described in Example 3. 40 pmol of each oligonucleotide were annealed in 10 μl of 50 mM NaCl by heating briefly to 95°C and then slowly cooling the mixture to 60°C. The mixture was adjusted to a total volume of 40 μl and the two halves of the LqhIT2 coding region (LqhIT2F1: LqhIT2R3 and LqhIT2F2:LqhIT2R4) were ligated. Due to the presence of incomplete synthesis products in each of the oligonucleotides, the initial ligation produced a heterogeneous mixture of fragments ranging from 200 - 1000 bp in length. The desired product was isolated from this mixture by PCR, using 0.5 μl of the ligation reaction as a source of template and oligonucleotides LqhIT2 PCRF (phosphorylated at its 5' terminus)

and LqhIT2 PCRR as primers (Figure 10). Amplification was carried out for 25 cycles of 1 min at 94°C, 1.5 min at 55°C and 3 min at 72°C, as described in Example 3. Following synthesis, the DNA was digested with BamH I, which cuts in the linker segment adjacent to the termination codon, and the desired 190 bp 5'-blunt/3'-BamH I fragment was purified by gel electrophoresis and cloned into MEVS vectors pMEV1/ADK, pMEV2/ADK, pMEV5/ADK and pMEV6/ADK as outlined in Example 3 and Figure 5. The sequence of the LqhIT2 coding region in each vector was confirmed by DNA sequencing.

B. ω-ACTX-HV1

The ω-atracotoxins are a family of insecticidal peptide toxins isolated from Australian funnel web spiders. One of the best studied members of this group is ω-atracotoxin-HV1 (ω-ACTX-HV1), a 37-residue peptide toxin isolated from the venom of the Blue Mountains funnel web spider *Hadronyche versuta* (International patent application AU93/00039). ω-ACTX-HV1 inhibits insect voltage-gated calcium channels and is lethal to *Helicoverpa armigera* larvae, but it is harmless to newborn mice.

Based on the amino acid sequence of ω-ACTX-HV1, two oligonucleotides were designed that represent the two strands of the ω-ACTX-HV1 coding region and a small amount of 3' flanking linker DNA. Codon usage within the ω-ACTX-HV1 coding region is designed to reflect overall codon usage in the AcMNPV genome (Ayres et al (1994)). The sequences of these oligonucleotides are shown in Figure 11. Due to the length of oligonucleotides ACTXHV1F and ACTXHV1R, there was a strong likelihood that each was significantly contaminated with incomplete synthesis products. Accordingly, a PCR strategy similar to that described above for LqhIT2 was used to synthesize an ω-ACTX-HV1 coding region fragment suitable for cloning into MEVS vectors. In this case, 0.1 pmol of each oligonucleotide were combined and used as the template for PCR amplification of the desired full length ω-ACTX-HV1 product. The primers for the reaction were ACTXPCRF (phosphorylated at its 5' terminus as described above) and ACTXPCRR. The PCR reaction was performed as described above, except that amplification was carried out for 15 cycles of 1 min at 94°C, 1.5 min at 55°C and 3 min at 72°C. Following amplification, the DNA was cleaved with BamH I, which cuts in the linker segment adjacent to the termination codon, and the desired 118 bp 5'-blunt/3'-BamH I fragment was purified by gel electrophoresis and cloned into MEVS vectors

pMEV1/ADK, pMEV2/ADK, pMEV5/ADK and pMEV6/ADK. The sequence of the ω-ACTX-HV1 coding region in each vector was confirmed by DNA sequencing.

Example 5: Construction of Recombinant PxNPVs

The following experiments were performed to construct recombinant PxNPVs encoding insecticidal toxins.

Viral DNA was prepared from occlusion bodies (O'Reilly *et al.* 1994). The purified DNA was digested with Bsu 36I and Sse 8387I using an approximately 5 units of enzyme/μg DNA. Size exclusion chromatography or sucrose gradient purification was used to separate the viral genomic DNA from the excised fragment. The resulting linearized viral DNA was ethanol precipitated and resuspended in 10 mM Tris-HCl pH 8.0/ 1 mM EDTA at a concentration of 0.2 - 1 μg/μl.

0.5 μg of the linearized DNA was then ligated with 15 ng of a purified Bsu 36I/Sse 8387I digested fragment from an appropriate MEVs vector in a reaction volume of 5 μl overnight at 15°C. The mixture was then used to transfect Sf-9 cells as described in Example 1. The culture was refed with fresh medium 24 hours after transfection. After an additional 72 hours, the culture supernatant was harvested, diluted, and used to re-infect Sf-9 cells for plaque isolation. Random plaques were picked and used to infect wells of a 48-well plate containing 6 x 10^4 Sf-9 cells/well in 0.5 mls TNM-FH complete media. Recombinants were identified using primers specific for the inserted gene. Virus from plaques giving positive wells were purified through two additional rounds of plaque purification.

The following table provides information on the various recombinant PxNPVs which have been prepared.

Virus	MEVs used
PxEGTDEL/35K ADK-AaIT	pMEV4/ ADK-AaIT
PxEGTDEL/hsp70Bam ADK-AaIT	pMEV5/ ADK-AaIT
PxEGTDEL/ DA26 tox34	pMEV1/ tox34
PxEGTDEL/hsp70Bam tox34	pMEV5/ tox34
PxEGTDEL/hsp70Xba tox 34	pMEV6/ tox34
PxEGTDEL/DA26 ADK-tox34S	pMEV1/ ADK-tox34S
PxEGTDEL/DA26 ADK-tox34L	pMEV1/ ADK-tox34L
PxEGTDEL/6.9K ADK-tox34S	pMEV2/ ADK-tox34S

Virus	MEVs used
PxEGTDEL/6.9K ADK-tox34L	pMEV2/ ADK-tox34L
Ac(V8)EGTDEL/DA26 ADK-AaIT	pMEV4/ ADK-AaIT
PxEGTDEL/DA26 ADK-LqhIT2	pMEV1/ ADK-LqhIT2
PxEGTDEL/6.9K ADK-LqhIT2	pMEV2/ ADK-LqhIT2
PxEGTDEL/hsp70 Bam ADK-LqhIT2	pMEV5/ ADK-LqhIT2
PxEGTDEL/hsp70 Xba ADK-LqhIT2	pMEV6/ ADK-LqhIT2
PxEGTDEL/DA26 ADK-ω-ACTX-HV1	pMEV1/ ADK-ω-ACTX-HV1
PxEGTDEL/6.9K ADK-ω-ACTX-HV1	pMEV2/ ADK-ω-ACTX-HV1
PxEGTDEL/hsp70 Bam ADK-ω-ACTX-HV1	pMEV5/ ADK-ω-ACTX-HV1
PxEGTDEL/hsp70 Xba ADK-ω-ACTX-HV1	pMEV6/ ADK-ω-ACTX-HV1

Example 6: Efficacy of Recombinant PxNPVs Expressing Insecticidal Proteins

The following experiments were performed to test the insecticidal potency of recombinant PxNPVs according to the present invention.

A standard diet overlay assay was performed on second instar *Plutella xylostella* larvae (2-3 days old, 0.15-0.3 mg) to determine the dosage required to achieve 50% mortality in the test insect population (i.e., the LC_{50}). Viral stocks were serially diluted in 0.01% sodium dodecyl sulfate and 50 μl aliquots of the viral suspensions were used to surface contaminate 15 mm round arenas containing linseed oil-augmented Stoneville diet (Southland Products Incorporated, Lake Village, AK). Individual larvae were placed in each arena and allowed to feed on the contaminated diet for the duration of the test. Dead larvae were scored twice daily over the initial three days of the test then at least daily until the onset of pupation in six to seven days. Data from replicates were pooled and analyzed by probit analysis (Finney, 1952). The median time for lethality to occur (LT_{50}) was determined by probit analysis of the time to death at a single dose.

The results are shown in the following tables. For purposes of comparison, all occlusion body (OB) concentrations are expressed as OBs/16cm^2 of diet.

rPxNPV	LC_{50} (OBs/16cm^2)	LT_{50} (days) @ 4.5 x 10^4
PxEGTDEL/35K ADK-AaIT	9.34×10^2	2.69
wt PxNPV	5.97×10^4	4.39

Probit values were generated from three replicates with approximately 32 larvae/dose treatment.

rPxNPV	LC_{50} (OBs/16cm^2)	LT_{50} (days) @ 4.5 x 10^3	LT_{50} (days) @ 4.5 x 10^4
PxEGTDEL/ 35K ADK AaIT	7 x 10^2	3.7	3.0
PxEGTDEL/hsp70Bam ADK-AaIT	6 x 10^2	3.7	2.7
Ac(V8)EGTDEL/DA26 ADK-AaIT	6.3 x 10^5	ND*	4.0

* ND not determined
Probit values were generated from four replicates with approximately 32 larvae/dose treatment.

These results indicate that (i) the addition of a toxin gene to the PxNPV genome not only increases the speed of kill of the virus but also dramatically lowers the effective LC_{50} on *Plutella xylostella* larvae; and (ii) recombinant PxNPVs have a dramatically lowered LC_{50} with a much faster speed of kill when compared to a closely related recombinant AcNPV. Since *Plutella xylostella* is an important vegetable pest, this finding is of critical importance in considering the choice of a recombinant baculovirus for use as a biopesticide in the vegetable market.

The following tables present data from assays of recombinant PxNPVs expressing a second insect-selective toxin, tox34.

rPxNPV	LT_{50} (days) @ 4.5 x 10^3	LT_{50} (days) @ 4.5 x 10^4
PxEGTDEL/DA26 tox34	4.5	3.4
PxEGTDEL/hsp70Bam tox34	2.6	1.9
PxEGTDEL/hsp70Xba tox34	2.0	1.8
PxEGTDEL/ DA26 ADK-tox34S	3.7	2.7
PxEGTDEL/DA26 ADK-tox34L	3.1	2.4
PxEGTDEL/6.9K ADK-tox34S	2.9	1.6
PxEGTDEL/6.9K ADK-tox34L	3.2	1.8
PxEGTDEL/ hsp70Bam ADK-AaIT	3.0	2.2

Probit values were generated from four replicates with approximately 32 larvae/dose treatment.

rPxNPV	Mean LC_{50} (OBs/16cm^2)	LT_{50} (days) @ 4.5 x 10^3
PxEGTDEL/ hsp70 Xba tox34	0.8 x 10^2	2.4

PxEGTDEL/hsp70 Bam ADK-AaIT	2.9×10^2	3.3
wt PxNPV	165×10^2	$4.6 @ 4.5 \times 10^4$

The data indicate that the increased efficacy of recombinant PxNPVs is not limited to AaIT-expressing constructs. The tox34 expressing PxNPVs exhibit as low an LT_{50} and LC_{50} as the AaIT-expressing recombinant. In some cases (i.e., PxEGTDEL/hsp70Xba tox34) expression of tox34 by the recombinant PxNPV results in an LT_{50} significantly lower than the AaIT-expressing recombinants.

All patents, patent applications, articles, publications, and test methods mentioned above are hereby incorporated by reference in their entirety.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the invention.

Claims:

1. An isolated recombinant *Plutella xylostella* baculovirus (PxNPV) having /a genetic alteration relative to wild-type PxNPV, wherein said alteration is selected from the group consisting of (a) introduction or deletion of a restriction site; (b) modification, deletion, or duplication of a viral-encoded gene; (c) introduction of a gene encoding a heterologous protein; and (d) any combination of any of the foregoing.

2. A recombinant baculovirus as defined in claim 1 having incorporated within its genome a nucleic acid sequence encoding an insect-modifying substance operably linked to a promoter capable of activating transcription in an insect cell.

3. A recombinant baculovirus as defined in claim 2, wherein said insect-modifying substance is an insecticidal toxin.

4. A recombinant baculovirus as defined in claim 3, wherein said toxin is selected from the group consisting of AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

5. A recombinant baculovirus as defined in claim 4, wherein said toxin is selected from the group consisting of AaIT, TxP-1, LqhIT2, and ω-atracotoxin.

6. A recombinant baculovirus as defined in claim 4, further comprising a sequence encoding a heterologous signal peptide, wherein said signal peptide-encoding sequence is fused in-frame to the sequence encoding said toxin.

7. A recombinant baculovirus as defined in claim 6, wherein said signal peptide is selected from the group consisting of the pBMHPC-12 signal sequence from *Bombyx mori*; the adipokinetic hormone signal sequence from *Manduca sexta*; the apolipophorin signal sequence from *Manduca sexta*; the chorion signal sequence from *Bombyx mori*; the cuticle signal sequence from *Drosophila melanogaster*; the esterase-6

* C L M S *

signal sequence from *Drosophila melanogaster*; and the sex-specific signal sequence from *Bombyx* mori.

8. A recombinant baculovirus as defined in claim 2, wherein said promoter is an hsp70 promoter.

9. A recombinant baculovirus as defined in claim 8, wherein said promoter is selected from the group consisting of hsp70 Bam and hsp70 Xba.

10. A recombinant baculovirus as defined in claim 3, wherein said promoter is selected from the group consisting of DA26, 35K, 6.9K, polyhedrin, hsp70, and actin promoters.

11. A recombinant baculovirus as defined in claim 3, wherein said genome has been further modified to inactivate the viral egt gene.

12. A recombinant baculovirus as defined in claim 3 further comprising a nucleic acid sequence encoding juvenile hormone esterase (JHE) operably linked to a promoter capable of activating transcription in an insect cell.

13. A recombinant baculovirus as defined in claim 5, wherein said promoter is an hsp70 promoter.

14. A recombinant *Plutella xylostella* baculovirus having incorporated into its genome a sequence encoding TxP-1 operably linked to a *Drosophila* hsp70 promoter.

15. A recombinant *Plutella xylostella* baculovirus having incorporated into its genome a sequence encoding AaIT operably linked to a *Drosophila* hsp70 promoter.

16. A recombinant baculovirus as defined in claim 1, wherein said genetic alteration forms a cloning site not present in wild-type PxNPV.

* C L M S *

17. A recombinant baculovirus as defined in claim 16, wherein a nucleic acid sequence encoding an insect-modifying substance operably linked to a promoter capable of activating transcription in an insect cell is incorporated at said cloning site.

18. A recombinant baculovirus as defined in claim 17, wherein said insect-modifying substance is an insecticidal toxin.

19. A recombinant baculovirus as defined in claim 18, wherein said toxin is selected from the group consisting of AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

20. A recombinant baculovirus as defined in claim 19, wherein said promoter is selected from the group consisting of DA26, 35K, 6.9K, polyhedrin, hsp70, and actin promoters.

21. A recombinant baculovirus as defined in claim 20, wherein said genome has been further modified to inactivate the viral egt gene.

22. A recombinant baculovirus as defined in claim 21 further comprising a nucleic acid sequence encoding juvenile hormone esterase (JHE) operably linked to a promoter capable of activating transcription in an insect cell.

23. A recombinant *Plutella xylostella* baculovirus selected from the group consisting of ATCC VR-2607, ATCC VR-2608, and ATCC VR-2609.

24. A direct ligation vector comprising genomic DNA isolated from a recombinant baculovirus as defined in claim 1.

* C L M S *

25. A direct ligation vector as defined in claim 24, wherein said DNA further comprises DNA encoding an insect-modifying substance operably linked to a promoter capable of activating transcription in an insect cell.

26. A direct ligation vector as defined in claim 25, wherein said insect-modifying substance comprises a toxin selected from the group consisting of AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

27. A direct ligation vector as defined in claim 26, further comprising a sequence encoding a heterologous signal peptide, wherein said signal peptide-encoding sequence is fused in-frame to the sequence encoding said toxin.

28. A direct ligation vector as defined in claim 27, wherein said signal peptide is selected from the group consisting of the pBMHPC-12 signal sequence from *Bombyx mori*; the adipokinetic hormone signal sequence from *Manduca sexta*; the apolipophorin signal sequence from *Manduca sexta*; the chorion signal sequence from *Bombyx mori*; the cuticle signal sequence from *Drosophila melanogaster*; the esterase-6 signal sequence from *Drosophila melanogaster*; and the sex-specific signal sequence from *Bombyx* mori.

29. A direct ligation vector as defined in claim 25, wherein said genome has been further modified to inactivate the viral egt gene.

30. A direct ligation vector as defined in claim 25, further comprising a nucleic acid sequence encoding juvenile hormone esterase (JHE) operably linked to a promoter capable of activating transcription in an insect cell.

31. A direct ligation vector comprising genomic DNA isolated from a recombinant baculovirus as defined in claim 16.

* C L M S *

32. A direct ligation vector comprising genomic DNA isolated from a recombinant baculovirus as defined in claim 19.

33. A direct ligation vector comprising genomic DNA isolated from a recombinant baculovirus as defined in claim 23.

34. An insecticidal composition comprising a recombinant *Plutella xylostella* baculovirus (PxNPV) having a genetic alteration relative to wild-type PxNPV and an agriculturally acceptable carrier, wherein said alteration is selected from the group consisting of (a) introduction or deletion of a restriction site; (b) modification, deletion, or duplication of a viral-encoded gene; (c) introduction of a gene encoding a heterologous protein; and (d) any combination of any of the foregoing.

35. An insecticidal composition as defined in claim 34, wherein said recombinant baculovirus has incorporated within its genome a nucleic acid sequence encoding an insect-modifying substance operably linked to a promoter capable of activating transcription in an insect cell.

36. An insecticidal composition as defined in claim 35, wherein said insect-modifying substance is an insecticidal toxin selected from the group consisting of AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

37. An insecticidal composition as defined in claim 36, wherein said recombinant baculovirus further comprises a sequence encoding a heterologous signal peptide, wherein said signal peptide-encoding sequence is fused in-frame to the sequence encoding said toxin.

38. An insecticidal composition as defined in claim 37, wherein the signal peptide is selected from the group consisting of the pBMHPC-12 signal sequence from

Bombyx mori; the adipokinetic hormone signal sequence from *Manduca sexta*; the apolipophorin signal sequence from *Manduca sexta*; the chorion signal sequence from *Bombyx mori*; the cuticle signal sequence from *Drosophila melanogaster*; the esterase-6 signal sequence from *Drosophila melanogaster*; and the sex-specific signal sequence from *Bombyx* mori.

39. An insecticidal composition as defined in claim 36, wherein said genome has been further modified to inactivate the viral egt gene.

40. An insecticidal composition as defined in claim 36, wherein said genetic alteration forms a cloning site not present in wild-type PxNPV.

41. An insecticidal composition as defined in claim 40, wherein said baculovirus further comprises a nucleic acid sequence encoding an insect-modifying substance operably linked to a promoter capable of activating transcription in an insect cell incorporated at said cloning site.

42. An insecticidal composition as defined in claim 41, wherein said insect-modifying substance is an insecticidal toxin.

43. An insecticidal composition as defined in claim 42, wherein said toxin is selected from the group consisting of AaIT, AaHIT1, AaHIT2, LqhIT2, LqqIT1, LqqIT2, BjIT1, BjIT2, LqhP35, LqhαIT, SmpIT2, SmpCT2, SmpCT3, SmpMT, DK9.2, DK11, DK12, μ-agatoxin, King Kong toxin, Pt6, NPS-326, NPS-331, NPS-373, Tx4(6-1), TxP-1, ω-atracotoxins, α-conotoxins, μ-conotoxins, chlorotoxin and ω-conotoxins.

44. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 2 and an agriculturally acceptable carrier.

45. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 4 and an agriculturally acceptable carrier.

* C L M S *

1 46. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 7 and an agriculturally acceptable carrier.

1 47. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 14 and an agriculturally acceptable carrier.

1 48. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 15 and an agriculturally acceptable carrier.

1 49. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 20 and an agriculturally acceptable carrier.

1 50. An insecticidal formulation comprising a recombinant *Plutella xylostella* baculovirus as defined in claim 23 and an agriculturally acceptable carrier.

1 51. A method for killing insects, which comprises contacting said pests with an insecticidally effective amount of an insecticidal formulation as defined in claim 44.

1 52. A method for killing insects, which comprises contacting said pests with an insecticidally effective amount of an insecticidal formulation as defined in claim 45.

1 53. A method for killing insects, which comprises administering an insecticidally effective amount of an insecticidal formulation as defined in claim 46.

1 54. A method for killing insects, which comprises administering an insecticidally effective amount of an insecticidal formulation as defined in claim 47.

1 55. A method for killing insects, which comprises administering an insecticidally effective amount of an insecticidal formulation as defined in claim 48.

1 56. A method for killing insects, which comprises administering an insecticidally effective amount of an insecticidal formulation as defined in claim 49.

1 57. A method for killing insects, which comprises administering an insecticidally effective amount of an insecticidal formulation as defined in claim 50.

