

Description

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Technical Field

The present invention relates to methods and compositions for improved biological control of insect pests. More particularly, the present invention relates to the use and manipulation of a baculovirus gene and its gene product which is effective in controlling the growth and development of insects. The present invention also relates to genetically modified baculoviruses and other insect control agents that adversely affect infected insect pests.

Background of the Invention

Interest in the biological control of insect pests has arisen as a result of disadvantages of conventional chemical pesticides. Chemical pesticides generally affect beneficial as well as nonbeneficial species. Insect pests tend to acquire resistance to such chemicals so that new insect pest populations can rapidly develop that are resistant to these pesticides. Furthermore, chemical residues pose environmental hazards and possible health concerns. Biological control presents an alternative means of pest control which can reduce dependence on chemical pesticides.

The primary strategies for biological control include the deployment of naturally-occurring organisms which are pathogenic to insects (entomopathogens) and the development of crops that are more resistant to insect pests. Approaches include the identification and characterization of insect genes or gene products which may serve as suitable targets for insect control agents, the identification and exploitation of previously unused microorganisms (including the modification of naturally-occurring nonpathogenic microorganisms to render them pathogenic to insects), the modification and refinement of currently used entomopathogens, and the development of genetically engineered crops which display greater resistance to insect pests.

Viruses that cause natural epizootic diseases within insect populations are among the entomopathogens which have been developed as biological pesticides. Baculoviruses are a large group of viruses which infect only arthropods (Miller, L.K. (1981) in *Genetic Engineering in the Plant Sciences*, N. Panopoulos, (ed.), Praeger Publ., New York, pp. 203-224; Carstens, (1980) *Trends in Biochemical Science* 52:107-110; Harrap and Payne (1979) in *Advances in Virus Research*, Vol. 25, Lawler et al. (eds.), Academic Press, New York, pp. 273-355). Many baculoviruses infect insects which are pests of commercially important agricultural and forestry crops. Such baculoviruses are potentially valuable as biological control agents. Four different baculoviruses have been registered for use as insecticides by the U.S. Environmental Protection Agency. Among the advantages of baculoviruses as biological pesticides is their host specificity. Not only do baculoviruses as a group infect only arthropods, but also individual baculovirus strains usually only infect one or a few species of insects. Thus, they pose no risk to man or the environment, and can be used without adversely affecting beneficial insect species.

Baculovirus subgroups include nuclear polyhedrosis viruses (NPV), granulosis viruses (GV), and nonoccluded baculoviruses. In the occluded forms of baculoviruses, the virions (enveloped nucleocapsids) are embedded in a crystalline protein matrix. This structure, referred to as an inclusion or occlusion body, is the form found extraorganismally in nature and is responsible for spreading the infection between organisms. The characteristic feature of the NPV viruses is that many virions are embedded in each occlusion body. The NPV occlusion bodies are relatively large (up to 5 micrometers). Occlusion bodies of the GV viruses are smaller and contain a single virion each. The crystalline protein matrix of the occlusion bodies of both forms is primarily composed of a single 25,000 to 33,000 dalton polypeptide which is known as polyhedrin or granulin. Baculoviruses of the nonoccluded subgroup do not produce a polyhedrin or granulin protein, and do not form occlusion bodies.

In nature, infection is initiated when an insect ingests food contaminated with baculovirus particles, typically in the form of occlusion bodies. The occlusion bodies dissociate under the alkaline conditions of the insect midgut, releasing individual virus particles which then invade epithelial cells lining the gut. Within a host cell, the baculovirus migrates to the nucleus where replication takes place. Initially, certain specific viral proteins are produced within the infected cell via the transcription and translation of so-called "early genes." Among other functions, these proteins are required to allow replication of the viral DNA, which begins 4 to 6 hours after the virus enters the cell. Extensive viral DNA replication proceeds up to about 12 hours post-infection (pi). From about 8 to 10 hours pi, the infected cell produces large amounts of "late viral

gene products." These include components of the nucleocapsid which surrounds the viral DNA during the formation of progeny virus particles. Production of the progeny virus particles begins around 12 hours pi. Initially, progeny virus migrate to the cell membrane where they acquire an envelope as they bud out from the surface of the cell. This nonoccluded virus can then infect other cells within the insect. Polyhedrin synthesis begins from 12 to 18 hours after infection and increases to very high levels by 24 hours pi. At that time, there is a decrease in the number of budded virus particles, and progeny virus are then embedded in occlusion bodies. Occlusion body formation continues until the cell dies or lyses. Some baculoviruses infect virtually every tissue in the host insect so that at the end of the infection process, the entire insect is liquified, releasing extremely large numbers of occlusion bodies which can then spread the infection to other insects. (Reviewed in The Biology of Baculoviruses, Vol. I and II, Granados and Federici (eds.), CRC Press, Boca Raton, Florida, 1986.)

One significant disadvantage to using baculoviruses as pesticides is the length of time between virus ingestion and insect death. During this time, the pest insect continues to feed and damage crops. Because a grower is unlikely to apply the pesticide until after an infestation is apparent, it is critical that the time of feeding be minimized.

What is needed is a biological pesticide which reduces feeding by the insect before death. A biological pesticide is preferred because it creates less of an environmental hazard than a chemical pesticide. More rapid insect death is also desirable. The exploitation of (a) gene(s) that encodes (a) protein(s) which controls insect development and its incorporation into various organisms will allow improved biological control of insect pests.

Summary of the Invention

In the present invention a baculovirus gene and its gene product which affect the growth, development or behavior of insects are identified. The present invention provides methods for the use of this gene and gene product, as well as methods for the inactivation of this gene or its gene product in insect control strategies. In a preferred embodiment, the egt gene encodes ecdysteroid UDP-glucosyl transferase (EGT) of AcMNPV. Expression of the baculovirus egt gene causes the production of EGT which inactivates insect molting hormones, thus preventing the insect larva from molting or pupating. Inactivation of the baculovirus egt gene allows molting and pupation of the infected larva to proceed.

The present invention encompasses a broad range of insect control agents utilizing egt genes and their gene products. The insect control agents of the present invention either cause the inappropriate synthesis of the EGT protein or prevent the normal functioning or expression of the EGT protein in the insect pest so that normal development of the insect pest is disrupted.

Preferably, the organism containing the egt gene is a virus specific for the insect pest, and wherein the egt gene of the virus has been inactivated, thereby allowing the continued development of the insect host infected with the virus. Development of the insect host induces behavioral changes such as reduced feeding and, when infected by the viral pesticide, there is reduced growth and more rapid death. The present invention also includes a method for producing the improved viral pesticide. In addition, the present invention includes a method of controlling insect pests comprising exposing the insect pest to the improved viral pesticide.

The genetically altered viruses contemplated by the present invention are more efficient pesticides than prior art viruses. Baculoviruses, such as Autographa californica nuclear polyhedrosis virus (AcMNPV) and Orygia pseudotsugata nuclear polyhedrosis virus (OpMNPV), naturally express an egt gene whose expression prolongs the period of time an infected larva spends feeding before succumbing to the viral infection. The present invention includes inactivating the egt gene in the genome of viruses such as, but not limited to, baculoviruses. The egt gene can be inactivated by substituting in its place or inserting within it another gene such as the nonviral marker gene for β -galactosidase. It should be understood that any DNA sequence could be used to disrupt the egt gene as long as it disrupts expression of the egt coding sequence. Alternatively, all or part of the egt gene can be removed from the genome by deleting an appropriate DNA coding segment or the regulatory part of the genome that controls egt gene expression can be altered or removed. Baculoviruses with deletions inactivating the egt gene may also be produced by serial virus passage in insect cell culture. The resulting deleted viruses have the advantage that they contain no foreign DNA and differ from wild-type viruses only in that they lack a functional egt gene. Such modified baculoviruses function better as pest control agents than the viruses presently in use. As simple deletion mutants, they should also be acceptable to regulatory agencies (e.g., U.S. EPA) as genetically engineered pesticides because they do not contain heterologous DNA. Baculoviruses that do not express a functional egt gene can be modified by the insertion of genes other than egt which interfere with insect development,

thus improving the efficacy of such viruses as insect control agents.

The present invention also includes a method of controlling insect pests by infecting insect larvae with a mutant virus lacking an intact egt gene or incapable of expressing a functional EGT product. The mutant virus-infected larvae attempt to molt and pupate, consequently feeding for a shorter period of time than larvae infected with wild-type or other prior art viruses. The mutant virus-infected insect larvae also die

sooner than wild-type or other prior art virus-infected larvae. It is an object of the present invention to provide a recombinant virus that is a more effective pesticide than wild-type or prior art viruses. The present invention is exemplified by the recombinant AcMNPV designated vEGTZ, in which a portion of the egt gene has been deleted and replaced by lacZ, a bacterial gene encoding β -galactosidase. Any DNA sequence which inactivates the egt gene may be substituted, as is understood by those skilled in the art. The present invention is further exemplified by the recombinant baculovirus designated vEGTDEL, in which a portion of the egt gene has been deleted. Another embodiment is a baculovirus pesticide in which the egt gene has been removed. Also included are baculoviruses in which a naturally occurring mutation, including a deletion, has resulted in the inactivation of the egt gene.

Accordingly, it is an object of the present invention to provide an egt gene and its gene product that are useful in the control of insect pests. Also within the scope of the invention are substantially purified ecdysteroid UDP-glucosyl transferase and antibody specific for it. egt genes can be identified by sequence homology to the nucleotide sequence of an egt gene in Table 1 or after determination of the EGT enzyme activity and subsequent DNA analysis. Any DNA sequence encoding an ecdysteroid UDP-glucosyl transferase is an egt gene as defined herein. An egt protein may be purified using art-known techniques and the assay procedure described in Example 4. A substantially pure egt protein preparation is one which comprises at least about 70% (w/w) ecdysteroid UDP-glucosyl transferase. A recombinant egt protein is one which has been made in any organism other than that in which the gene encoding said protein naturally occurs. The recombinant protein is expressed as a result of genetic engineering or recombinant DNA technology.

It is another object of the present invention to provide a genetically engineered virus that is an effective pesticide and is also environmentally acceptable.

It is yet another object of the present invention to provide a recombinant pesticide that lacks a functional egt gene and expresses a second gene which interferes with insect development, where the second gene is not naturally present in the organism. This second gene is one which encodes a gene product affecting ecdysis. Such a gene product can be an insect hormone which affects insect development or an enzyme which inactivates an insect hormone which regulates ecdysis. Specific examples include prothoracicotropic hormone, eclosion hormone, and juvenile hormone esterase. When genes encoding these proteins are to be incorporated into an insect virus to produce an insect control agent, that virus must be one which does not contain an egt gene or one in which an egt gene has been inactivated.

It is yet another object of the present invention to provide a genetically modified organism for use as an insect control agent that expresses a genetically inserted egt gene, where no egt gene was naturally expressed. Such genetically modified organisms expressing EGT will adversely affect insect pests, resulting in less damage to plants.

It is a further object of this invention to provide insecticidal compositions suitable for agricultural application. Such compositions comprise an agriculturally suitable carrier as is understood in the art and an insect virus, such as a baculovirus, which has been genetically modified so that an egt of said virus is inactivated. Such an Egt⁻ baculovirus may be further genetically modified to express a heterologous gene encoding an insect hormone affecting ecdysis or an enzyme which inactivates an insect hormone affecting ecdysis. Heterologous genes whose gene products affect ecdysis include, but are not limited to, prothoracicotropic hormone, eclosion hormone and juvenile hormone esterase. Preferably, insecticidal compositions comprising genetically modified baculoviruses are formulated for spray application.

Also contemplated as insecticidal compositions are those comprising an agriculturally suitable carrier and genetically modified insect parasite. An insect parasite is an organism which lives or replicates in close association with an insect larva, and has adverse effects on that larvae. An insect parasite can be a bacterium, a fungus, a virus or another insect. Such a genetically modified insect parasite comprising an egt gene will be improved as an insect control agent by the inactivation of that egt gene.

Any of the above-noted insecticidal compositions may further comprise ingredients to stimulate insect feeding. The insecticidal compositions of the present invention can be ingested by insect pests after plant application, and those insect pests susceptible to the insect control agent in the insecticidal composition will exhibit reduced feeding and will die.

It is another object of the present invention to provide a modified biological pesticide that inhibits with the expression of egt gene or with the function of its gene product.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the AcMNPV genome showing the location of the egt gene. The AcMNPV genome is presented in map units and as EcoRI and HindIII restriction maps.

Fig. 2 is a schematic summary of the results from sequencing the egt gene and analyzing the sequence. Panel A depicts a restriction endonuclease map of the region. The results of the computer-assisted analysis of the sequence for open reading frames are shown in Panel B. The vertical lines indicate stop codons. The sequence is "translated" in all three potential open reading frames for each DNA strands (1, 2, 3, 1', 2', 3'). The open reading frame (2) corresponding to egt is labeled EGT.

Fig. 3 is a schematic representation of the structures of the egt gene regions of AcMNPV (A) and the recombinant viruses vEGTZ (B) and vEGTDEL (C). The hatched box represents the lacZ gene.

Fig. 4 is a schematic representation of agarose gel electrophoresis and Southern blot analysis for identification of an egt gene in the baculovirus OpMNPV.

Fig. 5 is a schematic restriction map of the OpMNPV genome.

Fig. 6 is a graph of the weight gain in control uninfected larvae or after infection of larvae at 4th instar with wt AcMNPV or vEGTZ.

Fig. 7 is a graph of larval mortality after infection of larvae at 4th instar with wt AcMNPV or vEGTZ.

Fig. 8 is a graph of the weight gain after infection of larvae at 5th instar with wt AcMNPV or vEGTZ.

Fig. 9 is a graph of larval mortality after infection of larvae at 5th instar with wt AcMNPV or vEGTZ.

Fig. 10 is a graph of larval mortality after infection of larvae at first instar with wt AcMNPV or vEGTZ at a concentration of 4.8×10^5 polyhedral inclusion bodies (PIB)/ml.

Fig. 11 is a graph of larval mortality after infection of larvae at first instar with wt AcMNPV or vEGTZ at a concentration of 2.4×10^6 PIB/ml.

Detailed Description of the Disclosed Embodiments

Lepidopteran insects undergo a well characterized sequence of events during development from egg to adult insect (see Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vols. 7 and 8, Kerkut and Gilbert (eds.), Pergamon Press, Oxford, 1984 for detailed reviews). After hatching, the insect larva enters a period of extensive feeding during which time it will molt several times to allow continued growth. Stages between successive molts are known as instars. At the end of the larval growth period, the larva pupates and finally emerges as an adult insect. The processes of molting and pupation (collectively termed ecdysis) are regulated by the concerted actions of several different groups of hormones. The initial stimulus is the release of prothoracicotropic hormone (PTTH) by certain brain cells. This stimulates the prothoracic glands to produce and secrete ecdysteroids, often referred to as insect molting hormones. In the presence of juvenile hormone, a larval molt will ensue, while in the absence of juvenile hormone, the larvae will pupate. Ecdysis hormone is also important in mediating several behavioral changes associated with ecdysis.

The baculovirus AcMNPV, which is used as a model system for much baculovirus research, interferes with the process of insect development discussed above in previously unsuspected manner. Insect larvae infected with AcMNPV are no longer able to molt or pupate because AcMNPV directs the synthesis of an enzyme, known as ecdysteroid UDP-glucosyl transferase (EGT), which specifically inactivates the insect ecdysteroids.

The gene encoding EGT was identified by the present inventors; it extends from 8.4 to 9.6 map units on the AcMNPV genome (Figs. 1 and 2). As shown in Panel C of Fig. 1, viral DNA fragments encompassing the egt gene have been cloned into the plasmids pUC19, Bluescript M13+ and Bluescript M13-. Figure 2 shows the restriction map of the egt region of the genome and the computer-assisted open reading frame analysis of the egt region. Only reading Frame 2 contains a relatively long open reading frame, which has been identified as the coding region for the egt gene. The nucleotide sequence of the egt gene and the deduced amino acid sequence of 506 amino acids are shown in Table 1. The coding sequence of egt extends from about nucleotide 149 to about nucleotide 1670.

In a preferred embodiment of the present invention, the egt gene of the baculovirus AcMNPV is inactivated by replacing a portion of the egt gene with a bacterial sequence encoding β -galactosidase. This recombinant baculovirus is designated vEGTZ. In a second preferred embodiment, part of the egt gene of AcMNPV is deleted without replacement, as shown in Fig. 3. A comparison of the proteins synthesized during wt AcMNPV and vEGTZ infection revealed that the EGT protein is a 60 kDa protein which is secreted from infected cells. An alternate mechanism for the inactivation of the insect virus egt gene is the insertion of a gene encoding an insect hormone affecting ecdysis or an enzyme which inactivates an insect

hormone affecting ecdysis, which gene is expressible in an insect cell infected with said insect virus.

A search of the Genbank database revealed 21 to 22% amino acid sequence homology between *egt* and several mammalian UDP-glucuronosyl transferases. Homology was also found to a plant UDP-glucosyl transferase. The alignment of the *egt* amino acid sequence with the amino acid sequences of certain of these enzymes is displayed in Table 2.

Table 1

**Nucleotide Sequence and Deduced
Amino Acid Sequence of AcMNPV *egt*.**

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1  GTCGACGCGC TTCTGCGTAT AATTGCACAC TAACATGTTG CCCTTTGAAC
51 TTGACCTCGA TTGTGTTAAT TTTTGGCTAT AAAAAGGTCA CCCTTTAAAA
101 TTTGTTACAT AATCAAATTA CCAGTACAGT TATTCGGTTT GAAGCAAAAT
    egt:  M
151 GACTATTCTC TGCTGGCTTG CACTGCTGTC TACGCTTACT GCTGTAAATG
    T I L C W L A L L S T L T A V N A
201 CGGCCAATAT ATTGGCCGTG TTTCCTACGC CAGCTTACAG CCACCATATA
    A N I L A V F P T P A Y S H H I
251 GTGTACAAAG TGTATATTGA AGCCCTTGCC GAAAAATGTC ACAACGTTAC
    V Y K V Y I E A L A E K C H N V T
301 GGTGTCGAAG CCCAACTGT TTGCGTATTC AACTAAACT TATTGCGGTA
    V V K P K L F A Y S T K T Y C G N
351 ATATCACGGA AATTAATGCC GACATGTCTG TTGAGCAATA CAAAAAACTA
    I T E I N A D M S V E Q Y K K L
401 GTGGCGAATT CGGCAATGTT TAGAAAGCGC GGAGTGGTGT CCGATACAGA
    V A N S A M F R K R G V V S D T D
451 CACGGTAACC GCCGCTAACT ACCTAGGCTT GATTGAAATG TTCAAAGACC
    T V T A A N Y L G L I E M F K D Q
501 AGTTTGACAA TATCAACGTG CGCAATCTCA TTGCCAACAA CCAGACGTTT
    F D N I N V R N L I A N N Q T F
551 GATTTAGTCG TCGTGGAAGC GTTTGCCGAT TATGCGTTGG TGTTTGGTCA
    D L V V V E A F A D Y A L V F G H
601 CTTGTACGAT CCGGCGCCCG TAATTCAAAT CGCGCCTGGC TACGGTTTGG
    L Y D P A P V I Q I A P G Y G L A
651 CGGAAAACTT TGACACGGTC GGCGCCGTGG CGCGGCACCC CGTCCACCAT
    E N F D T V G A V A R H P V H H
701 CCTAACATTT GCGCAGCAA TTTCGACGAC ACGGAGGCAA ACGTGATGAC
    P N I W R S N F D D T E A N V M T
751 GGAAATGCGT TTGTATAAAG AATTTAAAT TTTGGCCAAC ATGTCCAACG
    E M R L Y K E F K I L A N M S N A
801 CGTTGCTCAA ACAACAGTTT GGACCCAACA CACGACAAT TGAAAAACTA
    L L K Q Q F G P N T P T I E K L
851 CGCAACAAGG TGCAATTGCT TTTGCTAAAC CTGCATCCCA TATTTGACAA
    R N K V Q L L L L N L H P I F D N

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Table 1 (continued)

5	901	CAACCGACCC	GTGCCGCCCA	GCGTGCAGTA	TCTTGGCGGA	GGAATCCATC
		N R P	V P P S	V Q Y	L G G	G I H L
	951	TTGTAAAGAG	CGCGCCGTTG	ACCAAATTAA	GTCCGGTCAT	CAACGCGCAA
		V K S	A P L	T K L S	P V I	N A Q
10	1001	ATGAACAAGT	CAAAAAGCGG	AACGATTTAC	GTAAGTTTGT	GGTCGAGCAT
		M N K S	K S G	T I Y	V S F G	S S I
	1051	TGACACCAAA	TCGTTTGCAA	ACGAGTTTCT	TTACATGTTA	ATCAATACGT
		D T K	S F A N	E F L	Y M L	I N T F
15	1101	TCAAAACGTT	GGATAATTAC	ACCATATTAT	GGAAAATTGA	CGACGAAGTA
		K T L	D N Y	T I L W	K I D	D E V
	1151	GTAAAAAACA	TAACGTTGCC	CGCCAACGTA	ATCACGCAAA	ATTGGTTTAA
		V K N I	T L P	A N V	I T Q N	W F N
20	1201	TCAACGCGCC	GTGCTGCGTC	ATAAAAAAAT	GGCGGCGTTT	ATTACGCAAG
		Q R A	V L R H	K K M	A A F	I T Q G
	1251	GCGGACTACA	ATCGAGCGAC	GAGGCCTTGG	AAGCCGGGAT	ACCCATGGTG
		G L Q	S S D	E A L E	A G I	P M V
25	1301	TGTCTGCCCA	TGATGGGCGA	CCAGTTTAC	CATGCGCACA	AATTACAGCA
		C L P M	M G D	Q F Y	H A H K	L Q Q
	1351	ACTCGGCGTA	GCCCCGCGCT	TGGACACTGT	TACCGTTTCC	AGCGATCAAC
30		L G V	A R A L	D T V	T V S	S D Q L
	1401	TACTAGTGGC	GATAAACGAC	GTGTTGTTTA	ACGCGCCTAC	CTACAAAAAA
		L V A	I N D	V L F N	A P T	Y K K
35	1451	CACATGGCCG	AGTTATATGC	GCTCATCAAT	CATGATAAAG	CAACGTTTCC
		H M A E	L Y A	L I N	H D K A	T F P
	1501	GCCTCTAGAT	AAAGCCATCA	AATTCACAGA	ACGCGTAATT	CGATATAGAC
		P L D	K A I K	F T E	R V I	R Y R H
40	1551	ATGACATCAG	TCGTCAATTG	TATTCATTAA	AAACAACAGC	TGCCAATGTA
		D I S	R Q L	Y S L K	T T A	A N V
	1601	CCGTATTCAA	ATTACTACAT	GTATAAATCT	GTGTTTCTA	TTGTAATGAA*
		P Y S N	Y Y M	Y K S	V F S I	V M N
45	1651	TCACTTAACA	CACTTTAAAT	TACGTCAATA	AATGTTATTC	ACCATTATTT
		H L T	H F *			
	1701	ACCTGGTTTT	TTTGAGAGGG	GCTTTGTGCG	ACTGCGCACT	TCCAGCCTTT
50	1751	ATAAACGCTC	ACCAACCAAA	GCAGGTCATT	ATTGTGCCAG	GACGTTCAAA

Table 2

EGT	MTILCWLALLS-----TLTAVNAANILAVFPTPAYSHHIVYKVYIEALAEKCHNVTV
HUMUDPGAT	Msm....ALL.....fss.s.gkvL-V.PT-.fSH.m..K..ld.L.qr.HeVTV
MUSUDPGAT	M.....ALL.....f.sVk.gkvL-V.P-.fSH.m..Kl.ld.L.qr.HeVTV
RATUDPGAT	M.....ALf.....f.s.h.gkvL-V.P-.fSH.m..Ki.ld.L.qr.HeVTV
EGT	VKP-KLFAYSTKTYCGNITEI-NADMSVE-----QYKKLVANSAMFRKRGVSDTDT
HUMUDPGAT	l.s...isf..ns.....Ev....lt.....-...KqLV...A....kd.....s
MUSUDPGAT	lrP...y....K...G...E...t.vS.d.....K.v...t.-...Rd.....
RATUDPGAT	lKP...F....K...d...EI.st.iS.d.....K.L...t.-...Rd.....
EGT	VTAANYLGLIEMFKDQF-DNINVRNLIANNQ--TFDLVVVEAFADYALVFGHLYDPA
HUMUDPGAT	f.dilr....D.vs.kkLm.k.Q...FDvVl.dal..fg.liaeL...p
MUSUDPGAT	l.....f.d.F....D.vs.keLmtk.Q...FDvll.dpiA..g.liaeL.q.p
RATUDPGAT	i.....f...y.....D.vs.kqLmtk.Q...FDvl..dpiA..g.liaeL.h.p
EGT	PVI--QIAPGYGLAENFDTVGAVAR-HPVHHPNIW-RSNFDDTEANVMTEMRLYKEF
HUMUDPGAT	.V...rfsPGYai..h.g.l...p...PV..sel..q..F.e...Nmi-.v-LY.EF
MUSUDPGAT	.l...rfsPGY.i..s.g.....p...PV..s.l..q..F.e...Nmi-.M-LY.dF
RATUDPGAT	.l....fsPG..L..sig.....p...PV..s.l..k..F.D...Nmi-.M-LY.dF
EGT	--KIL-ANMSNALLKQQFGPNTPTIEKLNRNKVQLLLNLHPIDNNRPVPPSVQYLG
HUMUDPGAT	..qIf..k..d.f..e.lG..T-Tl.....K.di.Li.....Fq..hPl.PnVefv-
MUSUDPGAT	..gmf..k..dsf..e.lG..T-Tl.....q.em.Li..n..le..hP..PnVdYv-
RATUDPGAT	L..k..dtf..e.lG..T-Tvd....sKvei.Li.....l...hP..PnVdYi-
EGT	GGIHLVKSAPLTKLSPVINAQMNSKSGTIYVSFGSSIDTKSFANEFLYMLINTFKT
HUMUDPGAT	GGLH...a.PL.K....f-.Q.s..ng..vf-SlGS.v-.n.seE...vi.sal..
MUSUDPGAT	GGLH...a.PL.K....f-.Q.s..g..vf-SlGS.v-.n.teE...i..al..
RATUDPGAT	GGLH...a.PL.K....f-.Q.s..g..vf-SlGS.v-.n.teE...L..al..
ZMAYUDPGT	285 qp..G..YVSFGT....rp...El...L.ds...
EGT	LDNYTILWKIDDEVVKNITLPANVITONWFNQRAVLRHKKMAAFITOGGLQSSDEAL
HUMUDPGAT	i.-..vLWrfDgn....l.L.t.l--.kwi.Q..lL.H.K..AFiThGG.ng...Ai
MUSUDPGAT	i.-..vLWKfDg....l...t.v--.kwI.Q..lL.H.K..AFhThGG.ng...Eai
RATUDPGAT	i.-..vLWKfDg....l...t.v--.kwI.Q..lL.H.K..AFvThGG.ng...Eai
ZMAYUDPGT	L.....W.l....l...a.g..l...W..Q.AVLRH..vgAFvThaG..S..EgL
EGT	EAGIPMVCPLPMGDQFYHAHKLQQLGVARALDVTVTSSDQLLVAINDVLFNAPTYKK
HUMUDPGAT	.p.IPMV.vPl.aDQ..n...mk..G.A.sLD..TmSS.dLL.Alk.Vi-N.P.YK.
MUSUDPGAT	..GIPMi.iPl.GeQ..n...m...G.A.ALn..TmS..dvL.AleeVi-..P.YKK
RATUDPGAT	..GIPMi.iPl.GDQ..n...m...G.A.sLn..TmS..dfL.AleeVi-d.P.YKK
ZMAYUDPGT	.sGvPM.C.P..GDQ..nAr.v.hvG.G.Afe.-amtS..v..Aveell.....rr
EGT	HMAELYALINHDKATFPPLDKAIAKFTERVIRYRHDISRQLYSLKTTAANVPYSNYM
HUMUDPGAT	n...L-s.IhHDqp...PLDrA-.F....v-.RH..akhL....-A.dl....f.
MUSUDPGAT	n...L-s.IhHDqp...PLDrA-.F....v-.RH..akhL.pL---g.Nl....f.
RATUDPGAT	nv..L-s.IhHDqp...PLDrA-.F....I-.RH..akhL.pL---g.NlP---Y.
ZMAYUDPGT	..AEL.ALv.e.g.....K.frF.E.V.R.
EGT	YKSVFSIVMNLTHF*
HUMUDPGAT	Y.S-l.v....La.....-.....*
MUSUDPGAT	Y.S-l.vi...Ls.....*
RATUDPGAT	Y.S-l.vi...LT.F.....*

Table 2 illustrates the alignment of the *egt* amino acid sequence with a selection of UDP-glucuronosyl transferases and a UDP-glucosyl transferase from other species. the predicted amino acid sequence of *egt* is compared to human (HUMUDPGAT) (Jackson et al. (1987) Biochem. J. 242:581; mouse (MUSUDPGAT) (Kimura and Owens (1987) Eur. J. Biochem. 168:515; and rat (RATUDPGAT) (MacKenzie (1987) J. Biol. Chem. 262:9744); UDP-glucuronosyl transferases and maize UDP-glucosyl transferase (ZMAYUDPGT) (Ralston et al. (1988) Genetics 119:185), using the FASTP algorithm (Lipman and Pearson (1985) Science 227:1435), as implemented by International Biotechnologies Inc. Upper case letters denote exact matches;

lower case letters denote substitutions which occur frequently among related proteins, (Dayhoff, (1978) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Vol. 5, Supplement 3, Silver Spring, MD); dots represent substitutions which occur infrequently; hyphens indicate gaps in a sequence; and a caret marks where an amino acid has been deleted from the sequence. The amino acids between the
 5 arrows are deleted from the egt gene in vEGTZ and vEGTDEL. The homology of the AcMNPV gene to known UDP-glucose and UDP-glucuronosyl transferases supports the identification of this AcMNPV sequence as the egt coding sequence.

In mammals, the UDP-glucuronosyl transferases catalyze the transfer of glucuronic acid to a wide variety of both exogenous and endogenous lipophilic substrates (reviewed in Glucuronidation of Drugs and
 10 Other Compounds, Dutton (ed.), CRC Press, Boca Raton, Florida, 1986). This conjugation reaction is important in the detoxification and safe elimination of certain drugs and carcinogens. In addition, the normal metabolism and disposal of various endogenous compounds such as bilirubin and steroid hormones, proceed via conjugation with glucuronic acid. Available evidence on insect systems indicate that sugar conjugation reactions of this type involve glucose rather than glucuronic acid transfer (reviewed in Smith
 15 (1977) in Drug Metabolism - From Microbe to Man, Parke and Smith (eds.), Taylor and Francis Ltd., London, pp. 219-232). As in mammals, a wide variety of exogenous and endogenous compounds are subject to conjugation in insects.

The inventors have shown that the AcMNPV EGT protein is a UDP-glucosyl transferase which specifically conjugates glucose with ecdysteroids such as ecdysone, 20-hydroxyecdysone and makisterone
 20 A (see Table 3). Neither lysates nor extracellular medium of uninfected cells or VEGTZ-infected cells modify ecdysone. Most of the ecdysteroid glucosyl transferase activity expressed by AcMNPV-infected cells is secreted into the extracellular medium; only a relatively small level of activity is observed in AcMNPV-infected cell lysates.

Using the AcMNPV egt gene as a probe, an egt gene has been identified in another baculovirus, Orgyia
 25 pseudotsugata nuclear polyhedrosis virus (OpMNPV), as shown in Fig. 4. It will be recognized by those skilled in the art with the benefit of this disclosure that the egt gene of any baculovirus, insect virus or insect can be located, characterized and isolated in a similar manner to that exemplified herein. egt genes with at least 70% nucleotide sequence homology to the sequence in Table 1 are considered equivalent to the sequence in Table 1, provided those homologous genes encode an enzyme which is an ecdysteroid
 30 UDP-glucosyl transferase.

Functional equivalents of the egt gene are those which also catalyze the inactivation of ecdysteroids such as ecdysone by transferring a glucose moiety from UDP-glucose to the ecdysteroid(s). Those functional equivalents of egt may be identified using the assay methods described herein.

With the location, identification and isolation of an egt gene, the skilled artisan can inactivate that gene
 35 using the teachings of present disclosure and art-known techniques to produce a more effective insect control agent.

By comparing the properties of vEGTZ with the properties of wild-type (wt) AcMNPV, the present inventors have shown that egt expression prevents the insect from molting or pupating. Insects infected with wt AcMNPV do not molt or pupate, but insects infected with vEGTZ molt and attempt to pupate (see Table
 40 4, in Example II).

By inhibiting molting and pupation, wt AcMNPV infection can actually prolong larval feeding time. Larvae infected at the beginning of fifth instar (the last larval instar) with wt virus continue feeding until death 5 or 6 days after infection. However, uninfected larvae stop feeding 2 to 3 days after entering fifth instar in preparation for pupation (see Fig. 8). Similar effects are observed when larvae at earlier instars are
 45 examined. Uninfected larvae cease feeding for approximately 24 hours during the molt, while larva infected by wt AcMNPV do not molt and consequently do not stop feeding (see Fig. 6).

Recombinant baculoviruses which lack a functional egt gene do not prolong larval feeding time. Thus, larvae infected with vEGTZ at early fifth instar cease feeding two days after infection in preparation for pupation (see Fig. 8). However, they do not pupate and instead succumb to the viral infection even more
 50 rapidly than larvae infected with wt virus, as shown in Fig. 9. Similarly, larvae infected with vEGTZ early in fourth instar cease feeding two days after infection to molt, and then die more rapidly than wt-infected larvae (see Figs. 6 and 7). The more rapid killing by a baculovirus lacking a functional egt gene is most dramatically seen when newly hatched first instar larvae are infected with wt AcMNPV and with vEGTZ as shown in Figs. 10 and 11. Larvae infected with vEGTZ succumb to the viral infection 3 to 4 days sooner
 55 than larvae infected with wt AcMNPV. Therefore, recombinant baculoviruses lacking a functional egt gene are considerably more effective as insect control agents than wild-type baculoviruses. It will be apparent to those skilled in the art with the benefit of this disclosure that the egt gene can be rendered nonfunctional in any baculovirus or insect virus by any means known to the art.

The effects described above and in the following examples will be more dramatic in the field. vEGTZ-infected larvae exhibit difficulty in molting, and do so successfully only under carefully controlled laboratory conditions. When temperature and light are not rigorously controlled, many insects fail to complete the molt. These insects do not recommence feeding and die shortly thereafter.

Although the length of time progeny virus can accumulate in larvae infected with baculoviruses lacking a functional egt gene is somewhat truncated and the infected insect displays reduced growth, there is substantial production of progeny virus. The amount of virus obtained per larva following vEGTZ infection of late instar larvae is about half that obtained with wt virus. This is sufficient to allow transmission of the virus in the field and cost-effective preparation of large quantities of virus particles.

In another embodiment of the present invention, an insect virus lacking a functional egt gene is modified by genetic engineering techniques so that its effectiveness as a biological control agent is further enhanced by incorporating a second gene whose product affects insect development.

The gene encoding PTTH (a peptide hormone) can be inserted into the viral genome with the egt gene deleted and PTTH can be expressed at levels sufficiently high to affect ecdysis. Insect larvae infected with such a virus experience extreme disruption in the hormonal control of development. These insects become sick rapidly resulting in severely compromised growth and development, reduced feeding, and earlier death.

Eclosion hormone is also a small peptide hormone whose gene can be inserted into the viral genome with a nonfunctional egt gene, using techniques known to the art. Because eclosion hormone governs many behavioral changes associated with ecdysis, insects infected by an Egt⁻ virus producing sufficiently high levels of this hormone will display behavioral and/or developmental abnormalities, such as reduced feeding.

A prime regulator of juvenile hormone titers in the insect is the enzyme juvenile hormone esterase, which inactivates juvenile hormone. A recombinant virus lacking a functional egt gene and expressing sufficiently high levels of juvenile hormone esterase can adversely affect insect behavior and/or development.

It is important to note that, while all of the above genes could be added to wild-type virus genome, they would not be expected to significantly affect insect behavior in the wild-type virus because expression of the egt gene by wild-type virus inactivates the ecdysteroid molting hormones and ecdysis is prevented, regardless of the production of other hormones. Thus, successful strategies involving the generation of viruses designed to interfere with insect ecdysis depend upon prior inactivation of the egt gene, as described in this invention.

It will be understood by those skilled in the art that mutant organisms lacking an intact egt gene or incapable of expressing a functional egt product and those which are further genetically modified so as to express another hormone modifying enzyme or a peptide hormone are included as insect control agents of the present invention.

The egt gene product has been shown to interfere with insect development in a powerful and specific manner. Ecdysteroids play a critical role in the development of essentially all insect species (reviewed in Comprehensive Insect Physiology Biochemistry and Pharmacology, Kerkut and Gilbert (eds.), Pergamon Press, Oxford, 1984). Thus, egt itself has considerable potential for use in broad-based insect control strategies. For example, in a fourth preferred embodiment of the present invention, crop species can be genetically engineered to constitutively produce the EGT protein by any art-known technique. Insects feeding upon such crops will be unable to complete development to adult insects, thereby providing effective long-term crop protection. Similarly, bacteria normally present in or on the plant or insect can be genetically engineered by techniques known to the art to produce the EGT enzyme. Such bacteria will likewise function as effective biological control agents.

Similarly, nonphytopathogenic, plant-colonizing bacteria may be genetically modified to express the EGT gene. Plants colonized by those genetically modified bacteria will be protected from insect pests susceptible to the action of the expressed protein(s). Nonphytopathogenic, plant-colonizing bacteria are described, for example, in U.S. Patent No. 4,798,723, and genetic modifications of certain plant-colonizing bacteria are described in U.S. Patent No. 4,771,131 and EPO Publication No. 0185005. Other plant-colonizing bacteria are known to the art. Any means known to the art may be used to introduce expressible genes such as those listed above to produce insect control agents.

Ingestion of such genetically modified plant material or of plant material colonized by such genetically modified plant-colonizing bacteria by a susceptible insect will result in disruption of normal development in that insect. It is known in the art that certain insects, e.g., aphids, have particularly permeable gut tissue. The skilled artisan understands the molecular biological steps necessary to construct such a plant-expressible gene and the steps necessary to incorporate such a gene into the genome of the plant.

It is likely that insects themselves express an egt gene at specific stages during their life cycle and that this constitutes an important component of the mechanisms regulating development. Thus, it is possible

that an insect egt gene might be a suitable target for novel insect control strategies. The disclosure of the present invention provides the means for designing such strategies.

An additional embodiment of the present invention, ecdysteroid analogs which bind to the EGT enzyme, but which cannot be cleaved and released from the enzyme can be designed. Such "suicide substrates" will competitively bind to and inhibit or block the action of the EGT enzyme, consequently interfering with insect development. Alternatively, recombinant organisms can be genetically engineered employing teachings provided herein and as understood in the art so that expression of the insect egt gene is prevented through the production of antisense RNA, for example.

As used herein, an insect control agent is a composition or the active ingredient of a composition which has an adverse affect on insect pests. Feeding by insects is reduced in response to the insect control agent, normal insect ecdysis is disrupted and death of the insect ensues. An insect control agent of this invention can be an insect virus genetically engineered to inactivate a gene encoding an ecdysteroid modifying enzyme or one which is further engineered to express a heterologous gene encoding a protein which affects insect development, or it may be a plant, insect parasite or a nonphytopathogenic bacterium, any of which has been genetically engineered to express a heterologous gene encoding a protein which affects ecdysis.

Insecticidal compositions suitable for applications to plants to control insect pests comprise an agriculturally suitable carrier and an insect control agent. Application of an insecticidal composition of this invention can protect plants from insect pests by reducing feeding by and killing of susceptible insects.

The skilled artisan knows how to choose an insect control agent, e.g., an insect virus, which is suitable for the control of a particular insect pest.

It will be understood by those skilled in the art that the insect pests can be exposed to the insect control agent of the present invention by conventional methods including ingestion, inhalation or direct contact of the insect control agent.

Also contemplated as insect control agents are plants and entomopathogenic microorganisms which have been genetically altered to express at least one protein which affects insect development. Examples of such a protein include, but are limited to, prothoracicotropic hormone, eclosion hormone, juvenile hormone esterase and ecdipone glucosyl transferase.

Insect parasites, including bacteria viruses, fungi and other insects, can also be genetically modified to express egt. Parasitism of appropriate insects by such insect parasites will result in disruption of normal development in addition to symptomology normally associated with the unmodified parasite. The disruption of development in infected insects exacerbates the insect disease state. Feeding by and infection of the insect pest will reduce feeding and hasten death.

DNA sequences encoding an EGT protein which affect insect development disclosed herein, expressed under the regulatory control of a promoter appropriate to the organism, may be used to genetically modify an organism to produce an insect control agent. Target organisms for genetic modification include insect parasites, plants and nonphytopathogenic plant-colonizing bacteria.

The egt gene, gene product, antibodies directed against the latter, and all other reagents derived from or connected with the above are all within the scope of the present invention. The EGT protein can be substantially purified using the assay described herein and techniques known in the art.

A primary use of the genetically engineered baculoviruses of the present invention will be as components of agricultural compositions for applying to plants to effect the biological control of insect pests of plants. Many variations of preparing such agriculturally suitable compositions for insect control are known in the art.

The concentration of the insect control agent that will be required to produce insecticidally effective agricultural compositions for plant protection will depend on the type of organism and mutation used and the formulation of the composition. The insectically effective concentration of the insect control agent within the composition can readily be determined experimentally, as will be understood by the skilled artisan. For example, the insectically effective concentration of a virus can be readily determined using variations of techniques described in any of Examples VI - XI.

Agricultural compositions must be suitable for agricultural use and dispersal in fields. Generally, components of the composition must be non-phytotoxic and not detrimental to the integrity of the occluded virus. Foliar applications must not damage or injure plant leaves. In addition to appropriate solid or, more preferably, liquid carriers, agricultural compositions may include sticking and adhesive agents, emulsifying and wetting agents, but no components which deter insect feeding or any viral functions. It may also be desirable to add components which protect the insect control agent from UV inactivation. Agricultural compositions for insect pest control may also include agents which stimulate insect feeding.

Reviews describing methods of application of biological insect control agents and agricultural application are available. See, for example, Couch and Ignoffo (1981) in Microbial Control of Pests and Plant Disease 1970-1980, Burges (ed.), chapter 34, pp. 621-634; Corke and Rishbeth, *ibid*, chapter 39, pp. 717-732; Brockwell (1980) in Methods for Evaluating Nitrogen Fixation, Bergersen (ed.) pp. 417-488; Burton (1982) in Biological Nitrogen Fixation Technology for Tropical Agriculture, Graham and Harris (eds.) pp. 105-114; and Roughley (1982) *ibid*, pp. 115-127; The Biology of Baculoviruses, Vol. II, *supra*.

This invention is illustrated by the following examples, which are not to be construed in any way as imposing limitations on the scope thereof. It is understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Example I

The position of the egt gene on the AcMNPV genome is illustrated in Fig. 1. A scale in map units is presented above the map of the AcMNPV genome. To determine the nucleotide sequence of this gene and flanking regions, and to allow for subsequent manipulation of the gene, it is first necessary to clone several DNA fragments encompassing this region into plasmid vectors (see T. Maniatis et al. (1982) in Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, for standard cloning procedures). Panel A shows a linear map of the AcMNPV genome after cleavage with restriction endonucleases EcoRI and HindIII. Panel B is an enlargement of the genome from 7.6 to 11.1 map units showing the location of the egt gene. The original AcMNPV strain used is the L1 strain (Lee and Miller (1978) *J. Virol.* 27:754). The cloned DNA fragments and the names of the resultant plasmids are shown in Fig. 1, Panel C. Fragment 1, which extends from the PstI site at 7.6 mu to a BamHI site at 11.1 mu, is cloned into the plasmid vector pUC19; fragments 2 and 3 (from PstI (7.6 mu) to EcoRI (8.65 mu) and from EcoRI (8.65 mu) to Sall (10.5 mu), respectively) are both cloned into the vectors Bluescript M13+ and Bluescript M13-(Stratagene, San Diego, California). Fragment 5 (BstEII (8.35 mu) to BstEII (8.7 mu) is cloned into Bluescript M13+.

A large number of subclones of BCPsE and BCES plasmids are then generated (Henikoff (1984) *Gene* 28:351). These subclones have progressively larger deletions of the viral insert so that they contain differing amounts of viral DNA, ranging from less than 50 base pairs to the entire viral fragment. Many of these subclones and the plasmid BCB, are then sequenced (Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463) so that the entire egt gene is sequenced in both directions. The nucleotide sequences obtained are then analyzed by computer for the presence of open reading frames which can encode proteins using programs of Pustell and Kafatos (1984) *Nucl. Acids Res.* 12:643-655 and Devereaux et al. (1984) *Nucl. Acids Res.* 12:387-396. This analysis indicates that the egt gene encodes a protein of 506 amino acids. The nucleotide sequence of the egt gene and the predicted amino acid sequence of the egt gene product, are presented in Table 1.

Example II

To construct recombinant viruses incapable of expressing a functional egt gene, further manipulation of the plasmid clones described in Example I is required. The plasmid pUCBCPsB is cleaved with restriction endonucleases EcoRI and XbaI (see Fig. 3 for sites within the egt gene) and the small fragment is discarded. The Escherichia coli lacZ gene, excised from pSKS104 (Casadaban et al. (1983) *Methods Enzymol.* 100:293-303) with EcoRI and AhaIII, is then inserted between the EcoRI and XbaI sites after the XbaI overhanging ends are filled in using T4 DNA polymerase. The resultant plasmid is designated pEGTZ. In this plasmid, the inserted lacZ gene is in frame with the preceding egt coding sequences. Alternatively, the plasmid pEGTDEL is constructed by simply ligating the EcoRI and XbaI sites together (after both sites have been blunt-ended) without inserting any sequences between them.

All viruses are derived originally from AcMNPV L-1 (Lee and Miller (1978) *supra*), and are plaque-purified and propagated in the Spodoptera frugiperda IPLB-SF-21 cell line (SF cells) (Vaughn et al. (1977) *In Vitro* 13:213-217) using methods described previously (Lee and Miller (1978); Miller et al. (1986) Genetic Engineering, Principles and Methods, Vol. 8 (eds. J. Setlow and A. Hollaender), Plenum Press, N.Y., pp. 277-298, 1986).

The plasmid pEGTZ is then cotransfected with wt AcMNPV DNA into SF cells as described in Miller et al. (1986) *supra*. This procedure allows for homologous recombination to take place between sequences in the viral and plasmid DNAs, resulting in replacement of the viral egt gene with the egt-lacZ gene fusion

from the plasmid. Because the remaining egt coding sequence is in frame with the lacZ sequences, such a recombinant virus will produce a fusion protein comprising the first 84 amino acids of egt joined to β -galactosidase. The recombinant virus, termed vEGTZ, can be identified because β -galactosidase expression gives rise to blue viral plaques in the presence of a chromogenic indicator such as 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). A diagram of the egt gene of vEGTZ is presented in Fig. 3, Panel B.

Recombinant virus vEGTDEL is obtained by cotransfecting the plasmid pEGTDEL and DNA from the virus vEGTZ into SF cells. Homologous recombination results in the replacement of the egt-lacZ fusion gene in vEGTZ with the deleted egt gene from pEGTDEL. The recombinant virus vEGTDEL is identified by its failure to form blue plaques in the presence of X-gal. The structure of the egt gene of vEGTDEL is shown in Fig. 3, Panel C.

Example III

The product of the egt gene is identified by comparing proteins synthesized by wt AcMNPV (which makes EGT) to those produced by vEGTZ or vEGTDEL (which cannot make EGT). SF cells are infected either with wt AcMNPV or with vEGTZ at a multiplicity of infection (MOI) of 20 as described in O'Reilly and Miller (1990) J. Virol. 64:1321-1328. Uninfected cells are also analyzed. After 6 hours of infection, the cells are incubated in the presence of the radioactively labeled [³⁵S]-methionine for 1 hour to radioactively label all proteins made during that period. The cells are then lysed and the proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli et al. (1970) Nature 227:680-685). Any proteins secreted from the cells are also harvested and analyzed. After SDS-PAGE, the radiolabeled proteins are detected by autoradiography. A 60 kDa protein is secreted from wt AcMNPV-infected cells but not from vEGTZ-infected cells or uninfected cells. This 60 kDa protein cannot be detected in lysates of wt-AcMNPV infected cells, showing that it is secreted from the cell. These data demonstrate that the product of the egt gene is a 60 kDa secreted protein, properties in good agreement with the nucleotide and amino acid sequence data.

Example IV

The enzymatic activity of the EGT protein is identified by comparing SF cells that are infected with wt AcMNPV or vEGTZ. SF cells infected with wt AcMNPV or vEGTZ are described in Example III. Twelve hours pi the cells and extracellular media are collected and processed separately. Uninfected cells are treated in parallel. Cell lysates or extracellular media are incubated in the presence of 1 mM UDP-glucose and 0.25 μ Ci [³H]ecdysone as described in O'Reilly and Miller (1989) Science 245:1110-1112. Ecdysteroid UDP-glucosyl transferase activity in the cell lysates or media will catalyze the transfer of glucose from the UDP-glucose to ecdysone to form an ecdysone-glucose conjugate. Ecdysone and the ecdysone-glucose conjugate are separated from one another by silica gel thin layer chromatography (Bansal and Gessner (1988) Anal. Biochem. 109:321) and visualized by autoradiography. Ecdysone-glucose conjugates (G) are only formed when wt AcMNPV-infected cell lysate or extracellular medium is assayed. No conjugates are observed when uninfected or vEGTZ-infected cell lysates or media are used, showing that the activity is due to egt expression. Most of the activity is located in the extracellular medium, in agreement with the data discussed in Example III. Proof that glucose is conjugated to ecdysone is obtained by assaying wt-infected lysates as described above except that the UDP-glucose is replaced with the radiolabeled UDP-[U-¹⁴C]-glucose. Reactions carried out with unlabeled UDP-glucose or labeled UDP-[U-¹⁴C]glucose, with [³H]-ecdysone present in both reactions, and scintillation counting of the conjugate shows that both ³H from the ecdysone and ¹⁴C from the glucose can be detected. These data prove that the egt gene product is a UDP-glucosyl transferase which catalyses the transfer of glucose from UDP-glucose to ecdysone.

Experiments are then performed to more thoroughly investigate the substrate specificity of EGT. In these experiments various substrates (1mM) are incubated in the presence of extracellular medium from wt-AcMNPV infected cells containing significant egt activity. As controls to ensure that any conjugation observed is due to EGT, each substrate is also incubated with media from uninfected and vEGTZ-infected cells, which cannot produce EGT. 0.05 μ Ci UDP-[U-¹⁴C] glucose are added to each reaction. Any compounds which function as substrates for EGT will be conjugated with glucose; they can be detected because the glucose is radioactively labeled.

TABLE 3

Substrate specificity of EGT		
Substrate	Mock (pmol Glucose transferred)	wild type (pmol Glucose transferred)
p-Aminobenzoic acid	--	--
Bilirubin	--	--
Chloramphenicol	--	--
Diethylstilbestrol	--	--
Ecdysone	--	155.7
β -Estradiol	--	--
20-Hydroxyecdysone	--	87.8
Hydroxyquinoline	--	--
Makisterone A	--	89.6
(-) Menthol	--	--
Methylumbelliferol	--	--
α -Naphthol	--	--
p-Nitrophenol	--	--
Phenolphthalein	--	--
Testosterone	--	--
α -Tetralol	--	--
Substrates are incubated in the presence of medium derived from appropriately infected cells and 0.05 μ Ci UDP-[U- 14 C]glucose. Amounts of glucose transferred are calculated after scintillation counting of the appropriate regions of the chromatography plates.		

One further control is the addition of UDP-[U- 14 C]glucuronic acid to a separate set of reactions with medium from wt-infected cells to demonstrate that glucuronic acid is not transferred by this reaction. The data obtained are presented in Table 3. The only substrates identified are ecdysone, 20-hydroxyecdysone and makisterone A, all of which are ecdysteroids. No conjugation is observed using medium from mock- or vEGTZ-infected cells, confirming that the activity observed is due to expression of *egt*. No conjugation is observed when UDP-[U- 14 C]glucuronic acid is used, demonstrating the EGT does not transfer glucuronic acid.

Example V

To demonstrate that other baculoviruses also contain genes with substantial homology to the AcMNPV *egt* gene, the DNA of OpMNPV is isolated and digested separately with the restriction endonucleases *EcoRI*, *BamHI*, and *HindIII*. These enzymes cleave the viral DNA into several fragments of different sizes whose position in the OpMNPV genome are already known (Leisy et al. (1984) J. Virol. 52:699). Southern hybridizations are performed as described in T. Maniatis et al. (1982) *supra*. An internal fragment of the AcMNPV *egt* gene is excised from the plasmid BCES with the enzymes *EcoRI* and *XbaI* (see Fig. 1). This fragment is radioactively labeled with 32 P and used as a probe to identify any related sequences in the OpMNPV genome. Under appropriate conditions, as understood in the art, a DNA fragment will bind to another fragment of DNA which contains sequences similar or identical to it. Thus, the AcMNPV *egt* probe should bind to any OpMNPV DNA fragments on the nylon membrane which contain related DNA sequences. The position of the bound probe can be visualized by exposing the membrane to X-ray film. The *egt* probe is first hybridized with the nylon membrane under conditions of low stringency (1 M sodium chloride, 0.3 M sodium citrate, 5% dextran sulfate, 5x Denhardt's solution, and 0.25% SDS at 37°C). This allows the probe to hybridize to relatively distantly related sequences. The stringency of hybridization is then increased in increments, by increasing the temperature of hybridization or by adding formamide to the hybridization solution, until specifically hybridizing bands are observed. The hybridization conditions for Fig. 4 are 1 M sodium chloride, 0.3 M sodium citrate, 5% dextran sulfate, 5x Denhardt's solution, and 0.25% SDS at 68°C, for 15 h. The membrane is then washed twice for 15 min each in 0.3 M sodium chloride, 0.1 M sodium citrate, 0.1% SDS at 68°C. Fig. 4 shows that the AcMNPV *egt* probe binds to specific fragments of OpMNPV DNA, namely *EcoRI* fragment B, *BamHI* fragment A and *HindIII* fragments N and S. Note that the OpMNPV *egt* gene is at the same relative position in the genome as the AcMNPV gene (Fig. 5). Similar

protocols can be applied for the identification of egt-homologous genes in other entomopathogens. It is understood that for highly divergent sequences, it will be necessary to confirm EGT activity using the assay method described in Example IV. Then a molecular genetic analysis of the genome will be required to isolate the gene encoding the EGT enzyme using methodology known to the art.

Alternatively, the EGT gene may also be found in other organisms using the specific assay for ecdysteroid UDP-glucosyl transferase activity described in Example IV. This assay is used to identify the enzyme during purification by conventional biochemical techniques. Once purified, a partial amino acid sequence of the EGT protein is determined. This information is used to generate an oligonucleotide probe which is then used to locate the EGT gene in the genome.

Example VI

Hemolymph titers of ecdysteroids fluctuate in a cyclic manner to regulate both larval-larval and larval-pupal molts and since glucose conjugation is suspected to inactivate ecdysteroids (Warren et al. (1986) J. Liq. Chromatogr. 9:1759; Thompson et al. (1987) Arch. Insect Biochem. Physiol. 4:1; Thompson et al. (1988) Arch. Insect Biochem. Physiol. 7:157), it is probable that egt expression during AcMNPV infection disrupts the normal developmental process of the infected insect. To demonstrate such a disruption, newly-ecdysed fourth instar S. frugiperda larvae are infected by injection with wt AcMNPV or vEGTZ and monitored daily for any perturbations in their development. One cohort of larvae is injected with tissue culture fluid as a negative control. The results of this experiment are presented in Table 4 below. All larvae injected with tissue culture fluid (mock-infected) molt to fifth instar as expected. Only one of sixteen larvae infected with wt virus makes this transition. In contrast, all larvae infected with the mutant vEGTZ undergo a fourth-to-fifth instar molt. Thus, egt expression by wt AcMNPV clearly and specifically inhibits host molting. Both infected groups of larvae subsequently succumb to viral infection, showing that disruption of egt does not prevent vEGTZ from killing its insect host.

TABLE 4

Inhibition of molting by AcMNPV infection.		
Virus	Molting	Mortality
Mock	16	0
Wild Type	1	16
vEGTZ	16	16

Fourth instar *S. frugiperda* larvae are injected with 1×10^5 pfu wt AcMNPV or vEGTZ in 5 μ l. Mock-infected larvae are injected with 5 μ l tissue culture fluid. Each cohort includes 16 larvae which are maintained on artificial diet (R. L. Burton (1969) ARS publication, pp. 33-134), at 28°C with a 14:10 hour light:dark cycle. Larvae are monitored daily for ecdysis, and mortality is recorded at day 7.

Similar results are obtained with larvae injected at early fifth instar. Using newly ecdysed fifth instar larvae, no wt-infected larvae show any signs of pupation, while the majority of vEGTZ-infected larvae display several behavioral modifications (feeding cessation, wandering, and spinning) characteristic of an impending larval-pupal molt. However, all virus-infected larvae died before pupation. These data show that AcMNPV infection prevents the insect larvae from molting or pupating. Further, the data show that this disruption of the insects' development is due to the expression of egt.

Example VII

In vivo bioassays of wild-type (wt) AcMNPV and vEGTZ reveal that egt gene expression prolongs the time the infected larvae spend feeding and that disruption of egt improves the characteristics of the virus as a pesticide. In these studies, S. frugiperda larvae are injected either with wt AcMNPV or vEGTZ early in the 4th instar. For comparison, control larvae are injected with tissue culture medium containing no virus. The larvae are checked daily for weight gain, signs of molting or pupation, and mortality. The average daily weight gains of the different groups of larvae, along with the percent mortality, are plotted in Figs. 6 and 7 respectively. Control larvae show moderate growth for the first two days. During the second day, they all molt to 5th instar. They then grow dramatically for two more days before they stop feeding in preparation for pupation. Only 1 out of 16 wt AcMNPV-infected larvae molt, and instead the larvae show continuous growth for three days following infection. At this stage, they begin to get sick, but no larvae die until day 5. All wt AcMNPV-infected larvae are dead by day 6. In contrast, all vEGTZ-infected larvae undergo a 4th to 5th instar molt and during this period, and they cease to feed. This accounts for the absence of growth from day 1 to day 2. After molting, they resume feeding but begin to show signs of sickness by day 3. They begin to die 4 days after infection and all are dead by day 5. Larvae infected with an AcMNPV derivative lacking a functional egt display reduced feeding and die more quickly post infection than those infected with wild-type AcMNPV.

These phenomena are even more clearly observed following infection of 5th instar larvae (Figs. 8 and 9). As expected, control larvae show considerable growth for two days before they stop feeding in preparation for pupation. This feeding cessation is accompanied by a dramatic weight loss. Wt AcMNPV-infected larvae show no signs of such a feeding cessation; they continue to feed and gain weight for two more days before they begin to get sick. Mortality is not complete until seven days after infection.

In summary, it can be seen that vEGTZ-infected larvae, like the controls, feed only for the first two days after infection. After this point, there is a dramatic weight loss as they prepare for pupation. However, none of these larvae pupate; they show signs of illness by day three and are all dead six days after infection. Again, vEGTZ infection brings about reduced feeding and earlier death.

Example VIII

The effects of vEGTZ and wt AcMNPV infection on newly-hatched first instar S. frugiperda larvae are compared. Neonate S. frugiperda are fed a diet containing various concentrations of vEGTZ or wt AcMNPV polyhedral inclusion bodies (PIBs), and monitored daily for mortality. The results obtained for two separate doses are presented in the Figs. 10 and 11. It can be seen that, at both doses, vEGTZ-infected larvae show considerable mortality substantially sooner than larvae infected with the wt virus. In general, larvae infected with the recombinant virus are killed between three and four days earlier than wt-infected larvae. This result provides further support that baculoviruses comprising inactivated egt genes function better as biological pesticides than wt baculoviruses with intact egt genes.

Example IX

To construct recombinant viruses which express prothoracicotropic hormone (PTTH), the PTTH gene is cloned into the transplacement plasmid pEVmodXIV, which plasmid is described in U.S. Patent Application Serial No. 07/353,847, filed May 17, 1989, which is incorporated by reference herein. This plasmid includes sequences upstream and downstream of the AcMNPV polyhedrin gene, which mediate homologous recombination of the expressible PTTH gene into the Egt⁻ virus. A multiple cloning site is positioned at the usual site of polyhedrin translation initiation, downstream from the LSXIV-modified polyhedrin promoter (Rankin et al., Gene, 70:39 (1988)). The Bombyx mori PTTH gene is excised from the plasmid pBc22k-C19 (Kawakami et al., Science, 247:1333, (1990)) by digestion with the restriction enzyme HindIII. The HindIII cohesive ends are filled in with T4 DNA polymerase, and the plasmid then cleaved with EcoRI. pEVmodXIV

is cleaved with KpnI, the KpnI overhanging end is removed with T4 DNA polymerase, and the plasmid then cleaved with EcoRI. The fragment containing the PTTH gene is cloned into the transplacement plasmid via these sites. In the resultant plasmid, pEVPTTH, the PTTH gene is located immediately downstream from the LSXIV-modified polyhedrin promoter.

6 Recombinant viruses expressing PTTH are obtained by cotransfecting pEVPTTH into SF cells with vEGTDEL or wt AcMNPV DNA. Recombination between the flanking polyhedrin sequences in the viral DNA and those flanking the PTTH gene in pEVPTTH results in the replacement of the polyhedrin gene with PTTH. Viruses in which this recombination event has taken place are identified by their occlusion-negative phenotype (Miller et al., in Genetic Engineering, Principles and Methods, Vol. 8, J. Setlow and A. Hollaender, eds., Plenum Press N.Y., 1986, pp. 277-298). The respective recombinant viruses are designated vEGT⁻PTTH and vWTPPTTH.

15 The method for producing recombinant viruses expressing juvenile hormone esterase is similar. First, the Heliothis virescens juvenile hormone esterase gene (Hanzlik et al. (1989) J. Biol. Chem. 264:12419) is excised from the plasmid pJHE16B (Hammock et al. (1990) Nature 344:458) by digestion with EcoRI and KpnI. The transplacement plasmid pEVmodXIV is also cleaved with EcoRI and KpnI and the juvenile hormone esterase gene-containing fragment is inserted between these sites. pEVJHE thus includes polyhedrin gene sequences flanking the juvenile hormone esterase gene. The recombinant viruses vEGT⁻JHE and vWTJHE are obtained by cotransfection of pEVJHE with vEGTZ or wt AcMNPV DNA, respectively, and screening for occlusion-negative plaques.

20 To construct recombinant viruses expressing eclosion hormone, the Manduca sexta eclosion hormone gene is excised from the plasmid pF5-3 (horodyski et al. (1989) Proc. Natl. Acad. Sci. USA, 86:8123) by digestion with EcoRI and HpaI. pEVmodXIV is cleaved with KpnI, the KpnI overhanging end removed with T4 DNA polymerase, and then cleaved with EcoRI. Insertion of the eclosion hormone gene-containing fragment into the transplacement plasmid yields the recombinant pVEEH, in which the eclosion hormone gene is positioned downstream from the LSXIV-modified polyhedrin promoter. Cotransfection of this plasmid with vEGTZ or vDA26Z DNA allows the isolation of the recombinant viruses vEGT⁻EH and vDA26ZEH, respectively. vDA26Z is a recombinant virus with the E. coli lacZ gene inserted in the DA26 gene (O'Reilly et al., J. Gen Virol., In Press). The function of the DA26 gene is not known, and vDA26Z is phenotypically wild-type with respect to ecdysis. Because of the presence of the lacZ gene, vDA26Z derivatives give rise to blue plaques in the presence of X-gal.

Example X

35 To assess the *in vivo* effects of these recombinant viruses, S. frugiperda larvae are injected with 2×10^5 pfu of vEGTDEL, vEGT⁻PTTH, vWTPPTTH, or both vEGT⁻PTTH and vEGT⁻JHE (1×10^5 pfu each) late in 3rd instar or early in 4th instar. The daily percent mortality is presented in Table 5. For all EGT⁻ viruses, the infected insects proceed through 4th instar and display head capsule slippage, which indicates an impending molt, by day 3. After infection by vEGT⁻PTTH alone or by infection with vEGT⁻PTTH and vEGT⁻JHE together, expression of PTTH causes the infected insects to become sick rapidly, and significant mortality is observed by day 4. By contrast, insects infected with vEGTDEL do not show considerable mortality until day 5. As expected, insects infected with vWTPPTTH never show signs of molting into 5th instar. No head capsule slippage is observed and the insects do not show considerable mortality until day 6. Expression of the PTTH in the absence of EGT thus advances the time of death following infection by 1 day compared to vEGTDEL. These data demonstrate the improvement of the pesticial properties of baculoviruses by expressing genes affecting insect ecdysis, and show that egt must be inactivated for such strategies to be effective.

TABLE 5

Virus	% Mortality					
	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
vEGTDEL	4.5	4.5	68.2	90.9	90.9	95.5
vWTPPTTH	0	0	13.0	39.1	78.3	95.7
vEGT ⁻ PTTH	4.2	62.5	87.5	100.0		
vEGT ⁻ PTTH and vEGT ⁻ JHE	8.7	60.9	91.3	95.7	100.0	

Example XI

Generation of occlusion-positive viruses which express heterologous proteins affecting insect development can be accomplished by the following scheme. First, an EGT⁻ occlusion-negative virus expressing β -galactosidase is constructed as follows. The *E. coli lacZ* gene, encoding the enzyme β -galactosidase, is cloned into the transplacement vector pSynVI⁻ described in U.S. Patent Application Serial No. 07/353,847, filed May 17, 1989. pSynVI⁻ includes sequences upstream and downstream from the AcMNPV polyhedrin gene, with a multiple cloning site located immediately downstream from a synthetic modified polyhedrin promoter. The *lacZ* gene is excised from the plasmid pSKS105 (Casadaban et al. (1983) *supra*) by cleavage with *Pst*I. The *Pst*I overhanging end is removed with mung bean nuclease, and the plasmid cleaved with *Sst*II. pSynVI⁻ is cleaved with *Eco*RV and *Sst*II and the *lacZ* gene cloned into these sites. The resultant plasmid is termed pSynVI⁻ gal, and contains the *lacZ* gene immediately downstream of the synthetic modified polyhedrin promoter. pSynVI⁻ gal is cotransfected into SF cells with vEGTDEL DNA, and occlusion-negative plaques expressing β -galactosidase are isolated, giving the virus vEGT-SynVI⁻ gal.

To construct an occlusion-positive recombinant virus which expresses PTTH, the PTTH gene is cloned into the transplacement vector pSpXIVVI+X3.

To construct pSpXIVVI+X3, an intermediate plasmid pSpXIVVI+ is first constructed. pSpLSXIVVI+CAT (described in United States Patent Application Serial No. 07/353,847, filed May 17, 1989) is cut with *Bgl*II and the ends filled in with DNA polymerase. Plasmid pSynVI+ wtp (described United States Patent Application Serial No. 07/353,847, filed May 17, 1989) is cut with *Eco*RV and *Sac*I and the small *Eco*RV-*Sac*I fragment is purified. The fragments of the two plasmids are ligated and pSpXIVVI+ is selected. The pSpXIVVI+ plasmid is the same as pSpLSXIVVI+CAT except that the multicloning site from the *Bgl*II site to the *Sac*I site is the same as that of pSynVI+ wtp. To construct multiple cloning site #3 (described in United States Patent Application Serial No. 07/353,847, filed May 17, 1989) a polylinker is inserted between the *Eco*RI-*Sac*I sites of pSpXIVVI+. The sequence of the multicloning site in pSPXIVVI+X3 from the fused *Bgl*II site to the *Sac*I site is:

```

AGATCATC   GAATTCTCGAG   CTGCAGATCT   GTCGACCCGGG   AATAAA
GAGCTC
EcoRV/BglI  EcoRI-XhoI   PstI-BglI   SalI-SmaI   poly A
SacI

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This plasmid includes an intact polyhedrin gene under the control of a wild-type polyhedrin promoter. A synthetic modified polyhedrin promoter is located upstream of, and in the orientation opposite to the polyhedrin gene. A multiple cloning site is positioned to allow insertion of genes to be expressed under the control of the synthetic modified polyhedrin promoter. The PTTH gene is excised from the plasmid pBc22k-C19 (Kawakami et al. (1990) *supra*) by digestion with *Hind*III. The *Hind*III overhanging end is filled in the T4 DNA polymerase and the plasmid cleaved with *Eco*RI. pSpXIVVI+X3 is cleaved with *Eco*RI and *Sma*I and the PTTH gene cloned into these sites, yielding the plasmid pSpPTTH. In this plasmid, the PTTH gene is downstream from the synthetic modified polyhedrin promoter, which is located adjacent to, and in the opposite orientation to the wild-type polyhedrin promoter and coding sequences. Both the PTTH and polyhedrin genes are flanked by sequences upstream and downstream of the polyhedrin gene in AcMNPV.

pSpPTTH is cotransfected into SF cells with vEGT-SynVI⁻ gal DNA. Recombination between the polyhedrin upstream and downstream sequences in the viral DNA and those flanking the PTTH and polyhedrin genes in pSpPTTH results in the replacement of the *lacZ* gene of vEGT-SynVI⁻ gal with the PTTH and polyhedrin genes from pSpPTTH. The recombinant virus vEGT-SpPTTH is identified by an occlusion-positive, β -galactosidase-negative phenotype.

To construct an occlusion-positive virus expressing juvenile hormone esterase, the juvenile hormone esterase gene is excised from the plasmid pJHE16B (Hammock et al. (1990) *supra*) by digestion with *Kpn*I, removal of the overhanging end with T4 DNA polymerase, and digestion with *Eco*RI. The juvenile hormone esterase gene is then cloned into the plasmid pSpXIVVI+X3 which has been cleaved with *Eco*RI and *Sma*I. The resultant plasmid, pSpJHE, is cotransfected with vEGT-SynVI⁻ gal DNA to generate the recombinant virus vEGT-SpJHE. This occlusion-positive virus expresses juvenile hormone esterase under the control of the synthetic modified polyhedrin promoter.

Similarly, the eclosion hormone gene is excised from the plasmid pF5-3 (Horodyski et al. (1990) *supra*) by digestion with *Eco*RI and *Hpa*I and cloned into pSpXIVVI+X3 which has been cleaved with *Eco*RI and

Smal. The resultant plasmid, pSpEH, is cotransfected with vEGT-SynVI-gal DNA to generate the recombinant virus vEGT-SpEH.

The occlusion-positive, EGT⁻ viruses which are further genetically modified to express a protein affecting ecdysis (an insect peptide hormone or an enzyme which inactivates insect hormones) will reduce feeding and cause more rapid death of infected insect larvae than will wt baculoviruses or baculoviruses genetically altered only to inactivate the egt gene.

Because the insect control agents of Example XI are occluded, these viruses can be incorporated into insecticidally effective, agriculturally acceptable compositions which can be applied to infected crops. Ingestion of such occluded viral particles result in the propagation of those viruses in the field, and spread of insect control agent. Infection will cause insect death and hence, protection of the crops from insect pests.

It should be understood that the foregoing relates only to preferred specific embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

Claims

1. An insect control agent comprising an insect parasite in which a naturally occurring gene encoding an ecdysteroid-modifying enzyme is inactivated.
2. The insect control agent of claim 1 wherein said gene is an egt gene.
3. The insect control agent of claim 2 wherein said insect parasite is an insect virus.
4. The insect control agent of claim 3 wherein said insect virus is a baculovirus.
5. The insect control agent of claim 4 wherein said baculovirus is a nuclear polyhedrosis virus.
6. The insect control agent of claim 5 wherein said nuclear polyhedrosis virus is selected from the group consisting of Autographa californica nuclear polyhedrosis virus and Orgyia pseudotsugata nuclear polyhedrosis virus.
7. The insect control agent of claim 6 wherein said insect virus comprising an inactivated gene encoding ecdysteroid UDP-glucosyl transferase is one of vEGTZ and vEGTDEL.
8. The insect control agent of claim 3 wherein said insect virus has been further modified by the insertion of at least one heterologous gene, wherein said heterologous gene(s) are expressible in insect cells infected with said virus, and wherein said gene(s) encode(s) a protein affecting ecdysis.
9. The insect control agent of claim 8 wherein said heterologous gene is selected from the group of genes encoding proteins consisting of prothoracicotrophic hormone, eclosion hormone and juvenile hormone esterase.
10. The insect control agent of claim 9 which is selected from the group consisting of vEGT-SpPTTH, vEGT-SpEH and vEGT-SpJHE.
11. A method of producing an improved insect control agent comprising the steps of:
 - (a) isolating a gene which encodes a protein which affects ecdysis; and
 - (b) inserting said gene into an organism which does not naturally express said gene.
12. The method of claim 11 wherein said gene encodes a protein selected from the group of proteins affecting ecdysis consisting of ecdysteroid UDP-glucosyl transferase, prothoracicotrophic hormone, eclosion, hormone and juvenile hormone esterase.
13. The method of claim 11 wherein said organism is selected from the group consisting of insect parasites, nonphytopathogenic, plant-colonizing bacteria, entomopathogenic bacteria, entomopathogenic fungi and plants.

Smal. The resultant plasmid, pSpEH, is cotransfected with vEGT-SynVI-gal DNA to generate the recombinant virus vEGT-SpEH.

The occlusion-positive, EGT⁻ viruses which are further genetically modified to express a protein affecting ecdysis (an insect peptide hormone or an enzyme which inactivates insect hormones) will reduce feeding and cause more rapid death of infected insect larvae than will wt baculoviruses or baculoviruses genetically altered only to inactivate the egt gene.

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It should be understood that the foregoing relates only to preferred specific embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

Claims

1. An insect control agent comprising an insect parasite in which a naturally occurring gene encoding an ecdysteroid-modifying enzyme is inactivated.
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3. The insect control agent of claim 2 wherein said insect parasite is an insect virus.
4. The insect control agent of claim 3 wherein said insect virus is a baculovirus.
5. The insect control agent of claim 4 wherein said baculovirus is a nuclear polyhedrosis virus.
6. The insect control agent of claim 5 wherein said nuclear polyhedrosis virus is selected from the group consisting of Autographa californica nuclear polyhedrosis virus and Orgyia pseudotsugata nuclear polyhedrosis virus.
7. The insect control agent of claim 6 wherein said insect virus comprising an inactivated gene encoding ecdysteroid UDP-glucosyl transferase is one of vEGTZ and vEGTDEL.
8. The insect control agent of claim 3 wherein said insect virus has been further modified by the insertion of at least one heterologous gene, wherein said heterologous gene(s) are expressible in insect cells infected with said virus, and wherein said gene(s) encode(s) a protein affecting ecdysis.
9. The insect control agent of claim 8 wherein said heterologous gene is selected from the group of genes encoding proteins consisting of prothoracicotrophic hormone, eclosion hormone and juvenile hormone esterase.
10. The insect control agent of claim 9 which is selected from the group consisting of vEGT-SpPTTH, vEGT-SpEH and vEGT-SpJHE.
11. A method of producing an improved insect control agent comprising the steps of:
 - (a) isolating a gene which encodes a protein which affects ecdysis; and
 - (b) inserting said gene into an organism which does not naturally express said gene.
12. The method of claim 11 wherein said gene encodes a protein selected from the group of proteins affecting ecdysis consisting of ecdysteroid UDP-glucosyl transferase, prothoracicotrophic hormone, eclosion, hormone and juvenile hormone esterase.
13. The method of claim 11 wherein said organism is selected from the group consisting of insect parasites, nonphytopathogenic, plant-colonizing bacteria, entomopathogenic bacteria, entomopathogenic fungi and plants.



14. A method for producing an improved insect control agent comprising the step of inactivating a gene encoding an ecdysteroid-modifying enzyme in an organism, said gene being a natural part of the genome of said organism.
- 5 15. The method of claim 14 wherein said gene encodes ecdysteroid UDP-glucosyl transferase.
16. The method of claim 15 wherein said organism is an insect control agent of any of claims 3-6.
17. The method of claim 16, wherein an Autographa californica nuclear polyhedrosis virus derivative with a deletion inactivating the gene encoding ecdysteroid UDP-glucosyl transferase is isolated after serial passage of said virus in insect cells.
- 10 18. The method of claim 16 wherein said nuclear polyhedrosis virus is one of vEGTZ and vEGTDEL.
- 15 19. The method of claim 14 further comprising the step of genetically altering said organism by the insertion of a heterologous gene expressible in an insect cell and which gene encodes a protein affecting ecdysis.
- 20 20. The method of claim 19 wherein said second gene is selected from the group consisting of genes encoding prothoracicotrophic hormone, eclosion hormone and juvenile hormone esterase.
21. A method for controlling an insect comprising the step of exposing said insect to an insect control agent, which insect control agent has been genetically engineered to express a gene encoding an ecdysteroid-modifying enzyme, wherein said enzyme adversely affects said insect.
- 25 22. A method for insect control comprising the step of exposing the insect to an insect control agent of any of claims 1-10.
23. A method for insect control comprising the step of exposing an insect to an insect control agent containing a gene encoding an ecdysteroid-modifying enzyme, wherein said gene has been inactivated by the insertion of a heterologous gene expressible in an insect cell infected with said virus, which heterologous gene encodes a protein affecting ecdysis.
- 30 24. The method of claim 23 wherein said second gene is selected from the group consisting of genes encoding prothoracicotrophic hormone, eclosion hormone and juvenile hormone esterase, which genes are expressible in insect cells infected with said virus.
- 35 25. An insecticidal composition comprising an insect control agent of any of claims 1-10 and an agriculturally suitable carrier, said insect control agent having been genetically modified so as to inactivate a naturally occurring gene encoding ecdysteroid UDP-glucosyl transferase.
- 40 26. A recombinant DNA molecule comprising a nucleotide sequence of a gene encoding ecdysteroid UDP-glucosyl transferase.
- 45 27. The recombinant DNA molecule of claim 26 wherein said sequence is the sequence of Table 1, from about nucleotide 149 to about nucleotide 1670, or a functional equivalent thereof.
28. An insect virus prepared by the method of any of claims 11-12, 14-17 and 19-20.
- 50 29. A method of preparing vEGTZ comprising the steps of:
 - a) digesting pUCBCPsB with restriction endonucleases EcoRI and XbaI and purifying a resulting large fragment;
 - b) excising the lacZ gene from pSKS104 with restriction endonucleases EcoRI and AhaII;
 - c) Ligating said lacZ gene with said large EcoRI-XbaI fragment of pUCBCPsB after making the XbaI end blunt with T4 DNA polymerase to form pEGTZ;
 - d) cotransfecting pEGTZ and AcMNPV into SF cells;
 - e) identifying viral recombinants by β -galactosidase expression; and
 - f) purifying said recombinant virus, which is vEGTZ.
- 55



30. A method of preparing vEGTDEL comprising the steps of:

- a) digesting pUCBCPsB with restriction endonucleases EcoRI and XbaI and purifying a resulting large fragment;
- b) making blunt both ends of said large DNA fragment of step (a);
- c) ligating said large DNA fragment of step (b);
- d) cotransfecting pEGTDEL and vEGTZ into SF cells to allow for homologous recombination;
- e) identifying recombinant vEGTDEL by failure to express β -galactosidase; and
- f) purifying said recombinant vEGTDEL.

31. A substantially pure ecdysteroid UDP-glucosyl transferase having an amino acid sequence of Table 1, or an amino acid sequence with at least about 70% homology thereto and having the biological activity of said ecdysteroid UDP-glucosyl transferase.

Patentansprüche

1. Insektenbekämpfungsmittel, umfassend einen Insektenparasit, in welchem ein natürlich vorkommendes Gen, das für ein Ecdysteroid-modifizierendes Enzym codiert, inaktiviert ist.
2. Insektenbekämpfungsmittel nach Anspruch 1, bei welchem das genannte Gen ein egt-Gen ist.
3. Insektenbekämpfungsmittel nach Anspruch 2, bei welchem der Insektenparasit ein Insektenvirus ist.
4. Insektenbekämpfungsmittel nach Anspruch 3, bei welchem das Insektenvirus ein Baculovirus ist.
5. Insektenbekämpfungsmittel nach Anspruch 4, bei welchem das Baculovirus ein nucleares Polyhedrosis-Virus ist.
6. Insektenbekämpfungsmittel nach Anspruch 5, bei welchem das nucleare Polyhedrosis-Virus ausgewählt ist aus der Gruppe bestehend aus dem nuclearen Polyhedrosis-Virus Autographa californica und dem nuclearen Polyhedrosis-Virus Orgyia pseudotsugata.
7. Insektenbekämpfungsmittel nach Anspruch 6, bei welchem das genannte Insektenvirus, das ein inaktiviertes, für Ecdysteroid UDP-Glucosyltransferase codierendes Gen umfaßt, vEGTZ oder vEGTDEL ist.
8. Insektenbekämpfungsmittel nach Anspruch 3, bei welchem das Insektenvirus weiter modifiziert ist durch Einsetzen von zumindest einem heterologen Gen, wobei das (die) heterologe(n) Gen(e) in Insektenzellen exprimierbar ist (sind), die mit diesem Virus infiziert sind, und wobei diese(s) Gen(e) für ein Protein codiert (codieren), das die Ecdysis beeinflusst.
9. Insektenbekämpfungsmittel nach Anspruch 8, bei welchem das heterologe Gen ausgewählt ist aus der Gruppe von Genen, die für Proteine aus der Gruppe prothoracicotropes Hormon, Eclosionshormon und juvenile Hormonesterase codieren.
10. Insektenbekämpfungsmittel nach Anspruch 9, das ausgewählt ist aus der Gruppe bestehend aus vEGT-SpPTTH, vEGT-SpEH und vEGT-SpJHE.
11. Verfahren zur Herstellung eines verbesserten Insektenbekämpfungsmittels, das folgende Stufen umfaßt:
 - (a) Isolierung eines Gens, das für ein Protein codiert, das die Ecdysis beeinflusst; und
 - (b) Einsetzen dieses Gens in einen Organismus, der dieses Gen natürlicherweise nicht exprimiert.
12. Verfahren nach Anspruch 11, bei welchem dieses Gen für ein Protein codiert, das ausgewählt ist aus der Gruppe der Proteine, die die Ecdysis beeinflussen, bestehend aus Ecdysteroid UDP-Glucosyltransferase, prothoracicotropes Hormon, Eclosionshormon und juvenile Hormonesterase.
13. Verfahren nach Anspruch 11, bei welchem dieser Organismus ausgewählt ist aus der Gruppe bestehend aus Insektenparasiten, nicht-phytopathogenen, pflanzenkolonisierenden Bakterien, entomopathogenen Bakterien, entomopathogenen Pilzen und Pflanzen.



14. Verfahren zur Herstellung eines verbesserten Insektenbekämpfungsmittels, das folgende Stufen umfaßt: Inaktivierung eines Gens, das für ein Ecdysteroid-modifizierendes Enzym codiert, in einem Organismus, wobei dieses Gen ein natürlicher Teil des Genoms dieses Organismus ist.
- 5 15. Verfahren nach Anspruch 14, bei welchem dieses Gen für Ecdysteroid UDP-Glucosyltransferase codiert.
16. Verfahren nach Anspruch 15, bei welchem dieser Organismus ein Insektenbekämpfungsmittel nach einem der Ansprüche 3 bis 6 ist.
- 10 17. Verfahren nach Anspruch 16, bei welchem ein Derivat des nuclearen Polyhedrosis-Virus Autographa californica mit einer Deletion, die das für Ecdysteroid UDP-Glucosyltransferase codierende Gen inaktiviert, nach einem Seriedurchgang dieses Virus in Insektenzellen isoliert wird.
- 15 18. Verfahren nach Anspruch 16, bei welchem das nucleare Polyhedrosis-Virus vEGTZ oder vEGTDEL ist.
19. Verfahren nach Anspruch 14, das außerdem umfaßt die Stufe der genetischen Veränderung dieses Organismus durch Einsetzen eines in einer Insektenzelle exprimierbaren heterologen Gens, das für ein Protein codiert, das die Ecdysis beeinflusst.
- 20 20. Verfahren nach Anspruch 19, bei welchem dieses zweite Gen ausgewählt ist aus der Gruppe bestehend aus Genen, die für prothoracicotropes Hormon, Eclosionshormon und juvenile Hormonesterase codiert.
- 25 21. Verfahren zur Bekämpfung eines Insekts, umfassend eine Stufe, bei welcher das Insekt einem Insektenbekämpfungsmittel ausgesetzt wird, wobei das Insektenbekämpfungsmittel genetisch verändert wurde, um ein Gen zu exprimieren, das für ein Ecdysteroid-modifizierendes Enzym codiert, und dieses Enzym auf das genannte Insekt nachteilig wirkt.
- 30 22. Verfahren zur Insektenbekämpfung, umfassend die Stufe, bei welcher das Insekt einem Insektenbekämpfungsmittel nach einem der Ansprüche 1 bis 10 ausgesetzt wird.
23. Verfahren zur Insektenbekämpfung, umfassend den Schritt, bei welchem ein Insekt einem Insektenbekämpfungsmittel ausgesetzt wird, das ein Gen enthält, das für ein Ecdysteroid-modifizierendes Enzym codiert, wobei dieses Gen durch Einsetzen eines heterologen Gens inaktiviert wurde, welches in einer mit dem genannten Virus infizierten Insektenzelle exprimierbar ist, und wobei das heterologe Gen für ein die Ecdysis beeinflussendes Protein codiert.
- 35 24. Verfahren nach Anspruch 23, bei welchem dieses zweite Gen ausgewählt ist aus der Gruppe bestehend aus Genen, die für prothoracicotropes Hormon, Eclosionshormon und juvenile Hormonesterase codieren, wobei diese Gene in Insektenzellen exprimierbar sind, die mit dem genannten Virus infiziert sind.
- 40 25. Insektizide Zusammensetzung, umfassend ein Insektenbekämpfungsmittel nach einem der Ansprüche 1 bis 10 und einen in der Landwirtschaft verwendbaren Träger, wobei das Insektenbekämpfungsmittel genetisch so modifiziert wurde, daß es ein natürlich auftretendes Gen, das für Ecdysteroid UDP-Glucosyltransferase codiert, inaktiviert.
- 45 26. Rekombinantes DNA-Molekül, umfassend eine Nucleotidsequenz eines Gens, das für Ecdysteroid UDP-Glucosyltransferase codiert.
- 50 27. Rekombinantes DNA-Molekül nach Anspruch 26, bei welchem die genannte Sequenz eine Sequenz von Tabelle 1 von etwa Nucleotid 149 bis etwa Nucleotid 1670 oder ein funktionelles Äquivalent derselben ist.
- 55 28. Insektenvirus, hergestellt nach dem Verfahren nach einem der Ansprüche 11, 12, 14 bis 17, 19 und 20.
29. Verfahren zur Herstellung von vEGTZ, das folgende Stufen umfaßt:



- a) Abbau von pUCBCPsB mit den Restriktionsendonucleasen EcoRI und XbaI und Reinigung eines resultierenden großen Fragmentes;
- b) Excision des lacZ-Gens aus pSRS104 mit den Restriktionsendonucleasen EcoRI und AhaII;
- c) Ligation dieses lacZ-Gens mit diesem großen EcoRI-XbaI-Fragment von pUCBCPsB nach Abstumpfen des XbaI-Endes mit T4 DNA Polymerase zur Bildung von pEGTZ;
- d) Cotransfektion von pEGTZ und AcMNPV in SF-Zellen;
- e) Identifizierung der viralen Rekombinanten durch β -Galactosidase-Expression; und
- f) Reinigung dieses rekombinanten Virus, der vEGTZ ist.

30. Verfahren zur Herstellung von vEGTDEL, das folgende Stufen umfaßt:

- a) Abbau von pUCBCPsB mit den Restriktionsendonucleasen EcoRI und XbaI und Reinigen eines resultierenden großen Fragmentes;
- b) Abstumpfen beider Enden dieses großen DNA-Fragmentes von Stufe a);
- c) Ligation dieses großen DNA-Fragmentes von Stufe b);
- d) Cotransfektion von pEGTDEL und vEGTZ in SF-Zellen, um homologe Rekombination zu ermöglichen;
- e) Identifizierung des rekombinanten vEGTDEL durch Nichtstattfinden der Expression von β -Galactosidase; und
- f) Reinigung dieses rekombinanten vEGTDEL.

31. Praktisch reine Ecdysteroid UDP-Glucosyltransferase, die eine Aminosäuresequenz von Tabelle 1 oder eine mindestens zu 70 % dazu homologe Aminosäuresequenz aufweist und die biologische Aktivität von Ecdysteroid UDP-Glucosyltransferase besitzt.

Revendications

1. Agent insecticide comprenant un parasite d'insecte dans lequel on inactive un gène naturel codant pour une enzyme modificatrice d'ecdystéroïde.
2. Agent insecticide de la revendication 1 dans lequel ledit gène est un gène egt.
3. Agent insecticide de la revendication 2 dans lequel ledit parasite d'insecte est un virus d'insecte.
4. Agent insecticide de la revendication 3 dans lequel ledit virus d'insecte est un baculovirus.
5. Agent insecticide de la revendication 4 dans lequel ledit baculovirus est un virus de polyhedrose nucléaire.
6. Agent insecticide de la revendication 5 dans lequel ledit virus de polyhedrose nucléaire est choisi dans le groupe constitué par le virus de la polyhedrose nucléaire Autographa californica et le virus de la polyhedrose nucléaire Orygia pseudotsugata.
7. Agent insecticide de la revendication 6 dans lequel ledit virus d'insecte comprenant un gène inactivé codant pour l'ecdystéroïde UDP-glucosyl transférase est vEGTZ ou vEGTDEL.
8. Agent insecticide de la revendication 3 dans lequel ledit virus d'insecte a encore été modifié par l'insertion d'au moins un gène hétérologue, où le(s)dit(s) gène(s) hétérologue(s) est(sont) expressible(s) dans des cellules d'insectes infectées par ledit virus, et où le(s)dit(s) gène(s) code(nt) pour une protéine affectant la mue.
9. Agent insecticide de la revendication 8 dans lequel ledit gène hétérologue est choisi dans le groupe de gènes codant pour des protéines constituées de l'hormone prothoracicotropique, l'hormone de l'éclosion et l'estérase de l'hormone juvénile.
10. Agent insecticide de la revendication 9 qui est choisi dans le groupe constitué par vEGT SpPTTH, vEGT SpEH et vEGT SpJHE.
11. Procédé de préparation d'un agent insecticide amélioré comprenant les étapes de:



- (a) isolement d'un gène qui code pour une protéine qui affecte la mue; et
 (b) insertion dudit gène dans un organisme qui n'exprime pas naturellement ledit gène.

12. Procédé de la revendication 11 dans lequel ledit gène code pour une protéine choisie dans le groupe de protéines affectant la mue constitué par l'ecdystéroïde UDP-glucosyl transférase, l'hormone prothoracicotrophique, l'hormone de l'éclosion et l'estérase de l'hormone juvénile.
13. Procédé de la revendication 11 dans lequel ledit organisme est choisi dans le groupe constitué par les parasites d'insectes, les bactéries non-phytopathogènes colonisant les plantes, les bactéries entomopathogènes, les champignons et plantes entomopathogènes.
14. Procédé de préparation d'un agent insecticide amélioré comprenant l'étape d'inactivation d'un gène codant pour une enzyme modificatrice d'ecdystéroïde dans un organisme, ledit gène étant une partie naturelle du génome dudit organisme.
15. Procédé de la revendication 14 dans lequel ledit gène code pour l'ecdystéroïde UDP-glucosyl transférase.
16. Procédé de la revendication 15 dans lequel ledit organisme est un agent insecticide de l'une quelconque des revendications 3-6.
17. Procédé de la revendication 16 dans lequel on isole un dérivé de virus de polyhedrose nucléaire Autographa californica avec une délétion inactivant le gène codant pour l'ecdystéroïde UDP-glucosyl transférase après passages en série dudit virus dans des cellules d'insectes.
18. Procédé de la revendication 16 dans lequel ledit virus de polyhedrose nucléaire est vEGTZ ou vEGTDEL.
19. Procédé de la revendication 14 comprenant en outre l'étape consistant à modifier génétiquement ledit organisme par insertion d'un gène hétérologue expressible dans une cellule d'insecte et qui code pour une protéine affectant la mue.
20. Procédé de la revendication 19 dans lequel ledit second gène est choisi dans le groupe constitué par les gènes codant pour l'hormone prothoracicotrophique, l'hormone de l'éclosion et l'estérase de l'hormone juvénile.
21. Procédé pour lutter contre un insecte comprenant l'étape d'exposition dudit insecte à un agent insecticide, lequel agent insecticide a été traité par génie génétique pour exprimer un gène codant pour une enzyme modificatrice d'ecdystéroïde, ladite enzyme affectant négativement ledit insecte.
22. Procédé pour lutter contre un insecte comprenant l'étape consistant à exposer l'insecte à un agent insecticide de l'une quelconque des revendications 1-10.
23. Procédé pour lutter contre un insecte comprenant l'étape d'exposition d'un insecte à un agent insecticide contenant un gène codant pour une enzyme modificatrice d'ecdystéroïde, ledit gène ayant été inactivé par l'insertion d'un gène hétérologue expressible dans une cellule d'insecte infectée avec ledit virus, lequel gène hétérologue code pour une protéine affectant la mue.
24. Procédé de la revendication 23 dans lequel le second gène est choisi dans le groupe constitué par les gènes codant pour l'hormone prothoracicotrophique, l'hormone de l'éclosion et l'estérase de l'hormone juvénile, ces gènes étant expressibles dans des cellules d'insectes infectées par ledit virus.
25. Composition insecticide comprenant un agent insecticide de l'une quelconque des revendications 1-10 et un support approprié en agriculture, ledit agent insecticide ayant été génétiquement modifié de manière à inactiver un gène naturel codant pour l'ecdystéroïde UDP-glucosyl transférase.
26. Molécule d'ADN recombinant comprenant une séquence nucléotidique d'un gène codant pour l'ecdystéroïde UDP-glucosyl transférase.



27. Molécule d'ADN recombinante de la revendication 26 dans laquelle ladite séquence est la séquence du tableau 1, des environs du nucléotide 149 aux environs du nucléotide 1670, ou un de ses équivalents fonctionnels.

5 28. Virus d'insecte préparé par le procédé de l'une quelconque des revendications 11-12, 14-17 et 19-20.

29. Procédé de préparation de vEGTZ comprenant les étapes consistant à:

a) digérer pUCBCPsB avec les endonucléases de restriction EcoRI et XbaI et purifier un grand fragment résultant;

10 b) exciser le gène lacZ de pSRS104 avec les endonucléases de restriction EcoRI et AhaII;

c) ligaturer ledit gène lacZ avec ledit grand fragment EcoRI-XbaI de pUCBCPsB après avoir rendu franche l'extrémité XbaI avec l'ADN polymérase T4 pour former pEGTZ;

d) cotransfecter pEGTZ et AcMNPV dans des cellules SF;

e) identifier les recombinants viraux par expression de la bêta-galactosidase; et

15 f) purifier ledit virus recombinant, qui est vEGTZ.

30. Procédé de préparation de vEGTDEL comprenant les étapes consistant à:

a) digérer pUCBCPsB avec les endonucléases de restriction EcoRI et XbaI et purifier un grand fragment résultant;

20 b) rendre franches les deux extrémités dudit grand fragment d'ADN de l'étape (a);

c) ligaturer ledit grand fragment d'ADN de l'étape (b);

d) cotransfecter pEGTDEL et vEGTZ dans des cellules SF pour permettre une recombinaison homologue;

e) identifier vEGTDEL recombinant par inaptitude à exprimer la bêta-galactosidase; et

25 f) purifier ledit vEGTDEL recombinant.

31. Ecdystéroïde UDP-glucosyl transférase sensiblement pure ayant une séquence d'acides aminés du tableau 1, ou une séquence d'acides aminés ayant une homologie d'au moins environ 70% avec une de ces séquences et ayant l'activité biologique de ladite ecdystéroïde UDP-glucosyl transférase.

