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(54) Title: NOVEL <i>BACILLUS THURINGIENSIS</i> TOXINS ACTIVE AGAINST CORN ROOTWORM LARVAE		
(57) Abstract Disclosed and claimed are toxins and genes from <i>Bacillus thuringiensis</i> strains designated PS80JJ1, PS158D5, PS167P, PS169E, PS177F1, PS177G, PS204G4, PS204G6 which can be used to control corn rootworm. Mutants which retain the activity of the parent strain can be used to control the pest. Further, isolated spores or purified toxins from these isolates can be used to control corn rootworm. Genes encoding δ-endotoxins can be removed from these strains using standard well-known techniques, and transferred to other hosts. Expression of the δ-endotoxin in such hosts results in control of corn rootworm larvae.		

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DESCRIPTIONNOVEL *BACILLUS THURINGIENSIS* TOXINS ACTIVE
AGAINST CORN ROOTWORM LARVAE

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Cross-Reference to a Related Application

This application is a continuation-in-part of co-pending application Serial No. 07/999,186, filed on December 31, 1992.

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Background of the Invention

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The soil microbe *Bacillus thuringiensis* (*B.t.*) is a Gram-positive, spore-forming bacterium characterized by parasporal crystalline protein inclusions. These inclusions often appear microscopically as distinctively shaped crystals. The proteins can be highly toxic to pests and specific in their toxic activity. Certain *B.t.* toxin genes have been isolated and sequenced, and recombinant DNA-based *B.t.* products have been produced and approved for use. In addition, with the use of genetic engineering techniques, new approaches for delivering these *B.t.* endotoxins to agricultural environments are under development, including the use of plants genetically engineered with endotoxin genes for insect resistance and the use of stabilized intact microbial cells as *B.t.* endotoxin delivery vehicles (Gaertner, F.H., L. Kim [1988] *TIBTECH* 6:S4-S7). Thus, isolated *B.t.* endotoxin genes are becoming commercially valuable.

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Until the last ten years, commercial use of *B.t.* pesticides has been largely restricted to a narrow range of lepidopteran (caterpillar) pests. Preparations of the spores and crystals of *B. thuringiensis* subsp. *kurstaki* have been used for many years as commercial insecticides for lepidopteran pests. For example, *B. thuringiensis* var. *kurstaki* HD-1 produces a crystalline δ -endotoxin which is toxic to the larvae of a number of lepidopteran insects.

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In recent years, however, investigators have discovered *B.t.* pesticides with specificities for a much broader range of pests. For example, other species of *B.t.*, namely *israelensis* and *tenebrionis* (a.k.a. *B.t.* M-7, a.k.a. *B.t. san diego*), have been used commercially to control insects of the orders Diptera and Coleoptera, respectively (Gaertner, F.H. [1989] "Cellular Delivery Systems for Insecticidal Proteins: Living and Non-Living Microorganisms," in *Controlled Delivery of Crop Protection Agents*, R.M. Wilkins, ed., Taylor and Francis, New York and London, 1990, pp. 245-255). See also Couch, T.L. (1980) "Mosquito Pathogenicity of *Bacillus thuringiensis* var. *israelensis*," *Developments in Industrial Microbiology* 22:61-76; Beegle, C.C., (1978) "Use of Entomogenous Bacteria in Agroecosystems," *Developments in Industrial Microbiology* 20:97-104. Krieg, A., A.M. Huger, G.A. Langenbruch, W. Schnetter (1983) *Z. ang. Ent.* 96:500-508, describe *Bacillus thuringiensis* var. *tenebrionis*, which is reportedly active against two beetles in the order Coleoptera. These are the Colorado potato beetle, *Leptinotarsa decemlineata*, and *Agelastica alni*.

Recently, new subspecies of *B.t.* have been identified, and genes responsible for active δ -endotoxin proteins have been isolated (Höfte, H., H.R. Whiteley [1989] *Microbiological Reviews* 52(2):242-255). Höfte and Whiteley classified *B.t.* crystal protein genes into 4 major classes. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Diptera-specific). The discovery of strains specifically toxic to other pests has been reported. (Feitelson, J.S., J. Payne, L. Kim [1992] *Bio/Technology* 10:271-275).

The cloning and expression of a *B.t.* crystal protein gene in *Escherichia coli* has been described in the published literature (Schnepf, H.E., H.R. Whiteley [1981] *Proc. Natl. Acad. Sci. USA* 78:2893-2897). U.S. Patent 4,448,885 and U.S. Patent 4,467,036 both disclose the expression of *B.t.* crystal protein in *E. coli*. U.S. Patents 4,797,276 and 4,853,331 disclose *B. thuringiensis* strain *tenebrionis* (a.k.a. M-7, a.k.a. *B.t. san diego*) which can be used to control coleopteran pests in various environments. U.S. Patent No. 4,918,006 discloses *B.t.* toxins having activity against Dipterans. U.S. Patent No. 4,849,217 discloses *B.t.* isolates which have activity against the alfalfa weevil. U.S. Patent No. 5,208,077 discloses coleopteran-active *Bacillus thuringiensis* isolates. U.S. Patent No. 5,151,363 and U.S. Patent No. 4,948,734 disclose certain isolates of *B.t.* which have activity against nematodes. As a result of extensive research and investment of resources, other patents have issued for new *B.t.* isolates and new uses of *B.t.* isolates. However, the discovery of new *B.t.* isolates and new uses of known *B.t.* isolates remains an empirical, unpredictable art.

Approximately 9.3 million acres of U.S. corn are infested with corn rootworm species complex each year. The corn rootworm species complex includes the northern corn rootworm, *Diabrotica barberi*, the southern corn rootworm, *D. undecimpunctata howardi*, and the western corn rootworm, *D. virgifera virgifera*. The soil-dwelling larvae of these *Diabrotica* species feed on the root of the corn plant, causing lodging. Lodging eventually reduces corn yield and often results in death of the plant. By feeding on cornsilks, the adult beetles reduce pollination and, therefore, detrimentally effect the yield of corn per plant. In addition, adults and larvae of the genus *Diabrotica* attack cucurbit crops (cucumbers, melons, squash, etc.) and many vegetable and field crops in commercial production as well as those being grown in home gardens.

Control of corn rootworm has been partially addressed by cultivation methods, such as crop rotation and the application of high nitrogen levels to stimulate the growth of an adventitious root system. However, chemical insecticides are relied upon most heavily to guarantee the desired level of control. Insecticides are either banded onto or incorporated into the soil. The major problem associated with the use of chemical insecticides is the development of resistance among the treated insect populations.

Brief Summary of the Invention

The subject invention concerns novel materials and methods for controlling corn rootworm. The materials and methods of the subject invention result from the unexpected discovery that certain *B.t.* isolates, as well as toxins from these isolates, have activity against this pest.

More specifically, the methods of the subject invention use *B.t.* microbes, or variants thereof, and/or their toxins, to control corn rootworms. Specific *B.t.* microbes useful according to the invention are *B.t.* PS80JJ1, *B.t.* PS158D5, *B.t.* PS167P, *B.t.* PS169E, *B.t.* PS177F1, *B.t.* PS177G, *B.t.* PS204G4, and *B.t.* PS204G6. Further, the subject invention also includes the use of variants of the exemplified *B.t.* isolates which have substantially the same corn rootworm-active properties as the specifically exemplified *B.t.* isolates. Such variants would include, for example, mutants. Procedures for making mutants are well known in the microbiological art. Ultraviolet light and nitrosoguanidine are used extensively toward this end.

The subject invention also includes the use of genes from the *B.t.* isolates of the invention which genes encode the corn rootworm-active toxins.

Still further, the invention includes the treatment of substantially intact *B.t.* cells, and recombinant cells containing the genes of the invention, treated to prolong the corn rootworm activity when the substantially intact cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or a combination of chemical and physical means, so long as the chosen means do not deleteriously affect the properties of the pesticide, nor diminish the cell's capability of protecting the pesticide. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes active upon ingestion by a target insect.

Finally, the subject invention concerns plants cells transformed with genes of the subject invention which encode corn rootworm-active toxins.

Brief Description of the Sequences

SEQ ID NO. 1 – is the N-terminal amino acid sequence for a toxin obtainable from PS204G6.

SEQ ID NO. 2 – is an oligonucleotide probe used for cloning a gene from PS204G6.

SEQ ID NO. 3 – is a forward primer used for PCR amplification of the 80JJ1 and 167P genes.

SEQ ID NO. 4 – is a reverse primer used for PCR amplification of the 80JJ1 and 167P genes.

SEQ ID NO. 5 – is the nucleotide sequence of gene 80JJ1.

SEQ ID NO. 6 – is the amino acid sequence of protein 80JJ1.

Detailed Disclosure of the Invention

Certain *Bacillus thuringiensis* stains useful according to the subject invention are disclosed in U.S. Patent 5,151,363. The disclosure of the cultures and their taxonomic characteristics are incorporated herein by reference to said patent. The *B.t.* isolates of the subject invention

5 have been deposited in the permanent collection of the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, USA. The culture repository numbers of the *B.t.* strains are as follows:

	<u>Culture</u>	<u>Repository No.</u>	<u>Deposit Date</u>
10	<i>B.t.</i> strain PS80JJ1	NRRL B-18679	July 17, 1990
	<i>B.t.</i> strain PS158D5	NRRL B-18680	July 17, 1990
	<i>B.t.</i> strain PS167P	NRRL B-18681	July 17, 1990
	<i>B.t.</i> strain PS169E	NRRL B-18682	July 17, 1990
	<i>B.t.</i> strain PS177F1	NRRL B-18683	July 17, 1990
15	<i>B.t.</i> strain PS177G	NRRL B-18684	July 17, 1990
	<i>B.t.</i> strain PS204G4	NRRL B-18685	July 17, 1990
	<i>B.t.</i> strain PS204G6	NRRL B-18686	July 17, 1990
	<i>E. coli</i> NM522 (pMYC2365)	NRRL-	
	<i>E. coli</i> NM522 (pMYC2379)	NRRL B-21155	Nov. 3, 1993

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Certain of these culture deposits are now available to the public by virtue of the issuance of U.S. Patent No. 5,151,363.

Other cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

30 Genes and toxins. The genes and toxins useful according to the subject invention include not only the full length sequences disclosed but also fragments of these sequences, variants, mutants, and fusion proteins which retain the characteristic pesticidal activity of the toxins specifically exemplified herein. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences which encode the same toxins or which encode equivalent toxins having

35 pesticidal activity. As used herein, the term "equivalent toxins" refers to toxins having the same or essentially the same biological activity against the target pests as the claimed toxins.

It should be apparent to a person skilled in this art that genes encoding active toxins can be identified and obtained through several means. The specific genes exemplified herein may be obtained from the isolates deposited at a culture depository as described above. These genes, or portions or variants thereof, may also be constructed synthetically, for example, by use of a gene synthesizer. Variations of genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal31* or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these toxins.

Equivalent toxins and/or genes encoding these equivalent toxins can be derived from *B.t.* isolates and/or DNA libraries using the teachings provided herein. There are a number of methods for obtaining the pesticidal toxins of the instant invention. For example, antibodies to the pesticidal toxins disclosed and claimed herein can be used to identify and isolate other toxins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the toxins which are most constant and most distinct from other *B.t.* toxins. These antibodies can then be used to specifically identify equivalent toxins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or western blotting. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in this art. The genes which encode these toxins can then be obtained from the microorganism.

Fragments and equivalents which retain the pesticidal activity of the exemplified toxins would be within the scope of the subject invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding the same, or essentially the same, toxins. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino acid substitutions, deletions, additions, or insertions which do not materially affect pesticidal activity. Fragments retaining pesticidal activity are also included in this definition.

A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are detectable nucleotide sequences. These sequences may be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO93/16094. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample have substantial homology. Preferably, hybridization is conducted under stringent conditions by techniques well-

known in the art, as described, for example, in Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY., pp. 169-170. Detection of the probe provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying toxin-encoding genes of the subject invention. The nucleotide segments which are used as probes according to the invention can be synthesized using DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Certain toxins of the subject invention have been specifically exemplified herein. Since these toxins are merely exemplary of the toxins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent toxins (and nucleotide sequences coding for equivalent toxins) having the same or similar pesticidal activity of the exemplified toxin. Equivalent toxins will have amino acid homology with an exemplified toxin. This amino acid homology will typically be greater than 75%, preferably be greater than 90%, and most preferably be greater than 95%. The amino acid homology will be highest in critical regions of the toxin which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 1 provides a listing of examples of amino acids belonging to each class.

Table 1.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin.

The toxins of the subject invention can also be characterized in terms of the shape and location of toxin inclusions, which are described above.

5 Recombinant Hosts. The toxin-encoding genes harbored by the isolates of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable microbial hosts, e.g., *Pseudomonas*, the microbes can be applied to the situs of the pest, where they will proliferate and be ingested. The result is a control of the pest.
10 Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest.

Where the *B.t.* toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes
15 be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably,
20 provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*,
25 *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Cluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*,
30 *Agrobacterium tumefaciens*, *Rhodopseudomonas spheroides*, *Xanthomonas campestris*, *Rhizobium melioli*, *Alcaligenes entrophus*, and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C. diffluens*, *C. laurentii*, *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odor*, *Cluyveromyces veronae*, and *Aureobasidium pollulans*. Of particular interest are the pigmented
35 microorganisms.

A wide variety of ways are available for introducing a *B.t.* gene encoding a toxin into a microorganism host under conditions which allow for stable maintenance and expression of the

gene. These methods are well known to those skilled in the art and are described, for example, in United States Patent No. 5,135,867, which is incorporated herein by reference.

5 Treatment of cells. As mentioned above, *B.t.* or recombinant cells expressing a *B.t.* toxin can be treated to prolong the toxin activity and stabilize the cell. The pesticide microcapsule that is formed comprises the *B.t.* toxin within a cellular structure that has been stabilized and will protect the toxin when the microcapsule is applied to the environment of the target pest. Suitable host cells may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, 10 organisms which produce substances toxic to higher organisms could be used, where the toxic substances are unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi.

15 The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

20 Treatment of the microbial cell, e.g., a microbe containing the *B.t.* toxin gene, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability of protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Lugol iodine, Bouin's fixative, various acids and Helly's fixative (See: Humason, Gretchen L., 25 *Animal Tissue Techniques*, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host environment. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like. Methods for treatment of microbial cells are disclosed in United States Patent Nos. 4,695,455 and 4,695,462, which are incorporated herein by reference. 30

35 The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of cell treatment should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of treatment should retain at least a substantial portion of the bio-availability or bioactivity of the toxin.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the *B.t.* gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; survival in aqueous environments; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

Growth of cells. The cellular host containing the *B.t.* insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B.t.* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The *B.t.* cells of the invention can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle the bacteria can be harvested by first separating the *B.t.* spores and crystals from the fermentation broth by means well known in the art. The recovered *B.t.* spores and crystals can be formulated into a wettable powder, liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers, and other components to facilitate handling and application for particular target pests. These formulations and application procedures are all well known in the art.

Formulations. Formulated bait granules containing an attractant and spores and crystals of the *B.t.* isolates, or recombinant microbes comprising the genes obtainable from the *B.t.* isolates disclosed herein, can be applied to the soil. Formulated product can also be applied as a seed-coating or root treatment or total plant treatment at later stages of the crop cycle. Plant and soil treatments of *B.t.* cells may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal-additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

As would be appreciated by a person skilled in the art, the pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide

while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the pest, e.g., soil and foliage, by spraying, dusting, sprinkling, or the like.

Mutants. Mutants of the isolates of the invention can be made by procedures well known in the art. For example, an asporogenous mutant can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A smaller percentage of the asporogenous mutants will remain intact and not lyse for extended fermentation periods; these strains are designated lysis minus (—). Lysis minus strains can be identified by screening asporogenous mutants in shake flask media and selecting those mutants that are still intact and contain toxin crystals at the end of the fermentation. Lysis minus strains are suitable for a cell treatment process that will yield a protected, encapsulated toxin protein.

To prepare a phage resistant variant of said asporogenous mutant, an aliquot of the phage lysate is spread onto nutrient agar and allowed to dry. An aliquot of the phage sensitive bacterial strain is then plated directly over the dried lysate and allowed to dry. The plates are incubated at 30°C. The plates are incubated for 2 days and, at that time, numerous colonies could be seen growing on the agar. Some of these colonies are picked and subcultured onto nutrient agar plates. These apparent resistant cultures are tested for resistance by cross streaking with the phage lysate. A line of the phage lysate is streaked on the plate and allowed to dry. The presumptive resistant cultures are then streaked across the phage line. Resistant bacterial cultures show no lysis anywhere in the streak across the phage line after overnight incubation at 30°C. The resistance to phage is then reconfirmed by plating a lawn of the resistant culture onto a nutrient agar plate. The sensitive strain is also plated in the same manner to serve as the positive control. After drying, a drop of the phage lysate is placed in the center of the plate and allowed to dry. Resistant cultures showed no lysis in the area where the phage lysate has been placed after incubation at 30°C for 24 hours.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Culturing of *B.t.* Isolates of the Invention

A subculture of the *B.t.* isolates, or mutants thereof, can be used to inoculate the following medium, a peptone, glucose, salts medium.

	Bacto Peptone	7.5 g/l
	Glucose	1.0 g/l
	KH ₂ PO ₄	3.4 g/l
	K ₂ HPO ₄	4.35 g/l
5	Salt Solution	5.0 ml/l
	CaCl ₂ Solution	5.0 ml/l
	pH 7.2	
	Salts Solution (100 ml)	
10	MgSO ₄ ·7H ₂ O	2.46 g
	MnSO ₄ ·H ₂ O	0.04 g
	ZnSO ₄ ·7H ₂ O	0.28 g
	FeSO ₄ ·7H ₂ O	0.40 g
15	CaCl ₂ Solution (100 ml)	
	CaCl ₂ ·2H ₂ O	3.66 g

The salts solution and CaCl₂ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30° C on a rotary shaker at 200 rpm for 64 hr.

The above procedure can be readily scaled up to large fermentors by procedures well known in the art.

The *B.t.* spores and/or crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

Example 2 – Purification of Protein and Amino Acid Sequencing

The *Bacillus thuringiensis* (*B.t.*) isolates were cultured as described in Example 1 or can be cultured using other standard media and fermentation techniques well-known in the art. Delta-endotoxins were isolated and purified by harvesting toxin protein inclusions by standard sedimentation centrifugation. Recovered parasporal inclusion bodies of some of the isolates were partially purified by sodium bromide (26-40%) isopycnic gradient centrifugation (Pfannenstiel, M.A., E.J. Ross, V.C. Kramer, K.W. Nickerson [1984] *FEMS Microbiol. Lett.* 21:39). Thereafter the individual toxin proteins were resolved by solubilizing the crystalline protein complex in alkali buffer and fractionating the individual proteins by DEAE-sepharose CL-6B (Sigma Chem. Co., St. Louis, MO) chromatography by step-wise increments of increasing concentrations of an NaCl-

containing buffer (Reichenberg, D., in *Ion Exchangers in Organic and Biochemistry* [C. Calmon and T.R.E. Kressman, eds.], Interscience, New York, 1957).

Fractions containing a protein toxic to corn rootworm were bound to PVDF membrane (Millipore, Bedford, MA) by western blotting techniques (Towbin, H., T. Staehelin, K. Gordon [1979] *Proc. Natl. Acad. Sci. USA* 76:4350) and the N-terminal amino acids determined by the standard Edman reaction with an automated gas-phase sequenator (Hunkapiller, M.W., R.M. Hewick, W.L. Dreyer, L.E. Hood [1983] *Meth. Enzymol.* 91:399).

The sequence obtained from the PS204G6 20-25 kDa polypeptide was:

G N F N X E K D Y D (SEQ ID NO. 1)

where X represents an amino acid residue with an undetermined identity. From this sequence data oligonucleotide probes were designed by utilizing a codon frequency table assembled from available sequence data of other *B.t.* toxin genes. The probes can be synthesized on an Applied Biosystems, Inc. DNA synthesis machine.

Example 3 – Molecular Cloning and Expression of Gene Encoding a Toxin from *Bacillus thuringiensis* Strain PS204G6

Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density, at 600 nm, of 1.0. Cells were pelleted by centrifugation and resuspended in protoplast buffer (20 mg/ml lysozyme in 0.3M sucrose, 25mM Tris-Cl (pH 8.0), 25mM EDTA). After incubation at 37°C for 1 hour, protoplasts were lysed by two cycles of freezing and thawing. Nine volumes of a solution of 0.1 M NaCl, 0.1% SDS, 0.1 M Tris-Cl were added to complete lysis. The cleared lysate was extracted twice with phenol:chloroform (1:1). Nucleic acids were precipitated with two volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in TE buffer and RNase was added to a final concentration of 50 µg/ml. After incubation at 37°C for 1 hour, the solution was extracted once each with phenol:chloroform (1:1) and TE-saturated chloroform. DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3M NaOAc and two volumes of ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in TE buffer.

An oligonucleotide probe with the following sequence was synthesized based on the PS204G6 20-25 kDa toxin peptide sequence:

5' AGACGTGGATCCGAAATTTTAATTTT

GAAAA(AG)GA(CT)TA(CT)GA 3'

(SEQ ID NO. 2)

This oligonucleotide contains a 5' *Bam*HI cloning site and is mixed at three positions as shown. This probe was radiolabeled with ³²P and used in standard hybridizations of Southern blots of

PS204G6 total cellular DNA. Hybridizing bands included an approximately 2.4 kbp *Hind*III fragment. This DNA fragment contains all or a fragment of this PS204G6 toxin gene.

A gene library was constructed from PS204G6 DNA partially digested with *Sau*3A. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 9.3 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, purified on an Elutip-D ion exchange column (Schleicher and Schuell, Keene, NH), and recovered by ethanol precipitation. The *Sau*3A inserts were ligated into *Bam*HI-digested LambdaGem-11 (Promega, Madison, WI). Recombinant phage were packaged and plated on *E. coli* KW251 cells. Plaques were screened by hybridization with the radiolabeled probe described above. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for isolation of DNA by standard procedures (Maniatis *et al*, *supra*).

For subcloning the gene encoding the PS204G6 toxin, preparative amounts of phage DNA were digested with *Eco*RI+*Sal*I and electrophoresed on an agarose gel. The approximately 5.5 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as described above. The purified DNA insert was ligated into *Eco*RI+*Sal*I-digested pHTBlueII (an *E. coli*/*B.thuringiensis* shuttle vector comprised of pBluescript S/K (Stratagene, La Jolla, CA) and the replication origin from a resident *B.t.* plasmid [D. Lereclus *et al.* (1989) *FEMS Microbiology Letters* 60:211-218]). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). β -galactosidase transformants were screened by restriction digestion of alkaline lysate plasmid minipreps. The desired plasmid construct, pMYC2365, contains a toxin gene that is novel compared to other δ -endotoxin genes.

pMYC2365 was introduced into the acrycristalliferous (*Cry*⁻) *B.t.* host, *Cry*B (A, Aronson, Purdue University, West Lafayette, IN), by electroporation. Expression of an approximately 75-85 kDa toxin was demonstrated by SDS-PAGE analysis. The polypeptide profile of the cloned toxin was similar to that of purified native PS204G6 crystals. In addition to the 75-85 kDa polypeptide, both native and cloned toxins exhibited the approximately 20-25 kDa polypeptide.

Example 4 – Cloning and Expression of a Novel Toxin Gene from *Bacillus thuringiensis* strain PS80JJ1

Total cellular DNA was prepared from *B.t.* cells as described in Example 3. An approximately 700-800 bp DNA fragment from a novel PS80JJ1 130 kDa toxin gene was obtained by polymerase chain reaction (PCR) amplification using PS80JJ1 cellular DNA and the following primers:

“Forward”: 5' GGACCAGGATTTACAGG(TA)GG(AG)(AG)A 3'

(SEQ ID NO. 3)

“Reverse”: 5' TAACGTGTAT(AT)CG(CG)TTTTAATTT(TA)GA(CT)TC 3'
(SEQ ID NO. 4).

5 The DNA fragment was cloned into pBluescript S/K (Stratagene, LaJolla, CA) and partially sequenced by dideoxynucleotide DNA sequencing methodology (Sanger *et al.* [1977] *Proc. Natl. Acad. Sci. USA* 74:5463-5467) using Sequenase (US Biochemicals, Cleveland, OH). DNA sequences unique to at least one PS80JJ1 toxin gene were identified by computer comparison with other known δ -endotoxin genes.

10 The 700-800 bp DNA fragment was radiolabelled with ^{32}P and used in standard hybridizations of Southern blots of PS80JJ1 total cellular DNA. Hybridizing bands included an approximately 1.8 kbp *EcoRI* fragment and an approximately 9.5 kbp *HindIII* fragment. These hybridizing DNA bands contain toxin genes or restriction fragments of toxin genes from PS80JJ1.

15 A gene library was constructed from PS80JJ1 DNA partially digested with *NdeII*. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 9.3 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, purified on an Elutip-D ion exchange column (Schleicher and Schuell, Keene, NH), and recovered by ethanol precipitation. The *NdeII* inserts were ligated into *BamHI*-digested LambdaGem-11 (Promega, Madison, WI). Recombinant phage were packaged and plated on *E. coli* KW251 cells. Plaques were screened by hybridization with the probe described above. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for isolation of DNA by standard procedures
20 (Maniatis *et al.*, *supra*).

For subcloning the gene encoding the PS80JJ1 130 kDa toxin, preparative amounts of phage DNA were digested with *XhoI* and electrophoresed on an agarose gel. The approximately 12 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as described above. The purified DNA insert was
25 ligated into *XhoI*-digested pHTBlueII (an *E. coli/B. thuringiensis* shuttle vector comprised of pBluescript S/K [Stratagene, La Jolla, CA] and the replication origin from a resident *B.t.* plasmid [D. Lereclus *et al.* [1989] *FEMS Microbiology Letters* 60:211-218]). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). β -galactosidase- transformants were screened by restriction digestion of alkaline lysate plasmid minipreps as above. The desired
30 plasmid construct, pMYC2379, contains a toxin gene that is novel compared to other toxin genes containing insecticidal proteins.

Sequence analysis of the toxin gene revealed that it encodes a protein of approximately 130,000 daltons, deduced from the DNA sequence. The nucleotide and deduced amino acid sequences are shown in SEQ ID NOS. 5 and 6, respectively.

35 pMYC2379 was introduced into the acrySTALLIFEROUS (Cry^-) *B.t.* host, CryB (A. Aronson, Purdue University, West Lafayette, IN), by electroporation. Expression of the 130kDa toxin was demonstrated by SDS-PAGE analysis.

The PS80JJ1 toxin gene encoded by pMYC2379 was sequenced using the ABI373 automated sequencing system and associated software.

Example 5 – Restriction Fragment Length Polymorphism Analysis of δ -endotoxin Genes From *Bacillus thuringiensis* strain PS167P

Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells as described in Example 3.

An approximately 700-800 bp DNA fragment from novel PS167P 130 kDa toxin genes was obtained by polymerase chain reaction (PCR) amplification using PS167P cellular DNA and the primers shown in SEQ ID NO. 3 and SEQ ID NO. 4. This DNA fragment was cloned into pBluescript S/K (Stratagene, LaJolla, CA) and partially sequenced by dideoxynucleotide DNA sequencing methodology (Sanger *et al.*, *supra*) using Sequenase (US Biochemicals, Cleveland, OH). DNA sequences unique to at least two PS167P toxin genes were identified by computer comparison with other known δ -endotoxin genes.

The 700-800 bp DNA fragment was radiolabeled with ^{32}P and used in standard hybridizations of Southern blots of PS167P total cellular DNA. Hybridizing bands included approximately 1.8 kbp and 2.3 kbp *EcoRI* fragments and approximately 5.5 kbp and 8.0 kbp *HindIII* fragments. These DNA fragments contain toxin genes or restriction fragments of toxin genes unique to PS167P.

Example 6 – Insertion of Toxin Genes Into Plants

One aspect of the subject invention is the transformation of plants with genes encoding the insecticidal toxin. The transformed plants are resistant to attack by the target pest.

Genes encoding pesticidal toxins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the *B.t.* toxin can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and

the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasterdam, Chapter 5; Fraley *et al.*, *Crit. Rev. Plant Sci.* 4:1-46; and An *et al.* (1985) *EMBO J.* 4:277-287.

Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, or electroporation as well as other possible methods. If agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.* [1978] *Mol. Gen. Genet.* 163:181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the

normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In a preferred embodiment of the subject invention, plants will be transformed with genes wherein the codon usage has been optimized for plants. Also, advantageously, plants encoding a truncated toxin will be used. The truncated toxin typically will encode about 55% to about 80% of the full length toxin. Methods for creating synthetic *B.t.* genes for use in plants are known in the art.

Example 7 – Cloning of *B.t.* Genes Into Insect Viruses

A number of viruses are known to infect insects. These viruses include, for example, baculoviruses and entomopoxviruses. In one embodiment of the subject invention, genes encoding the insecticidal toxins, as described herein, can be placed within the genome of the insect virus, thus enhancing the pathogenicity of the virus. Methods for constructing insect viruses which comprise *B.t.* toxin genes are well known and readily practiced by those skilled in the art. These procedures are described, for example, in Merryweather *et al.* (Merryweather, A.T., U. Weyer, M.P.G. Harris, M. Hirst, T. Booth, R.D. Possee (1990) *J. Gen. Virol.* 71:1535-1544) and Martens *et al.* (Martens, J.W.M., G. Honee, D. Zuidema, J.W.M. van Lent, B. Visser, J.M. Vlak (1990) *Appl. Environmental Microbiol.* 56(9):2764-2770).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: MYCOGEN CORPORATION
- (ii) TITLE OF INVENTION: Novel Bacillus thuringiensis Toxins Active Against Corn Rootworm Larvae
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: SALIWANCHIK, DAVID R.
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 - (C) CITY: Gainesville
 - (D) STATE: Florida
 - (E) COUNTRY: US
 - (F) ZIP: 32606
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/999,186
 - (B) FILING DATE: 31-DEC-1992
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: SALIWANCHIK, DAVID R.
 - (B) REGISTRATION NUMBER: 31,794
 - (C) REFERENCE/DOCKET NUMBER: MA82
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 904-375-8100
 - (B) TELEFAX: 904-372-5800

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: protein
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Asn Phe Asn Xaa Glu Lys Asp Tyr Asp
 5 10

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 41 bases
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (synthetic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGACGTGGAT CCGGAAATTT TAATTTTGAA AARGAYTAYG A

41

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 bases
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGACCAGGAT TTACAGGWWG RRA

23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAACGTGTAT WCGSTTTTAA TTTWGAYTC

29

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3561 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGATTGTA ATTTACAATC ACAACAAAAT ATTCCTTATA ATGTATTAGC AATACCAGTA	60
TCTAATGTTA ATGCGTTGGT TGATACAGCT GGAGATTTAA AAAAAGCATG GGAAGAATTT	120
CAAAAACTG GTTCTTTTTT ATTAACAGCT TTACAACAAG GATTTTCTGC CTCACAAGGA	180
GGAGCATTC AATTATTTAAC ATTATTACAA TCAGGAATAT CATTAGCTGG TTCTTTTGTC	240
CCTGGAGGTA CTTTTGTAGC ACCCATTGTT AATATGGTTA TTGGTTGGTT ATGGCCACAT	300
AAAAACAAGA CAGCGGATAC AGAAAATTTA ATAAAATTAA TTGATGAAGA AATTCAAAAA	360
CAATTAAACA AAGCCTTATT AGACCAAGAT AGAAACAATT GGACCTCTTT TTTAGAAAGT	420
ATATTTGATA CTTCAGCTAC AGTAAGTAAT GCAATTATAG ATGCACAGTG GTCAGGTACT	480
GTAGATACTA CAAATAGACA ACAAAAACT CCAACAACAT CAGATTATCT AAATGTTGTT	540
GGAAAATTTG ATTCAGCGGA TTCTTCAATT ATAATAATG AAAATCAAAT AATGAATGGC	600
AACTTTGACG TAGCTGCAGC ACCCTATTTT GTTATAGGAG CAACATTACG TCTTTCATTA	660
TATCAATCTT ATATTAAATT TTGTAATAGT TGGATTGATG CAGTTGGATT TAGTACAAAT	720
GATGCTAATA CACAAAAGC TAATTTAGCT CGTACGAAAT TAACTATGCG TACTACAATT	780
AATGAATATA CACAAGAGT TATGAAAGTT TTTAAAGATT CCAAGAATAT GCCTACAATA	840
GGTACTAATA AATTTAGTGT TGATGCTTAT AATGTATATG TTAAAGGAAT GACATTAAAT	900
GTTTTAGATA TGGTAGCAAT ATGGTCTTCA TTATATCCAA ATGATTATAC TTCACAAACA	960
GCCATAGAAC AAACACGTGT CACTTTTTTCA AATATGGTTG GACAAGAAGA AGGTACAGAT	1020
GGAACCCTAA AAATTTACAA TACTTTTGAT TCTCTTAGTT ATCAACATAG CCTAATACCT	1080
AATAATAATG TTAATTTAAT TTCTTATTAT ACTGATGAAT TGCAAAATCT AGAATTAGCA	1140
GTATATACTC CTAAAGGTGG AAGTGGATAC GCTTATCCTT ATGGATTAT TTTAAATTAT	1200
GCAAACAGCA ACTACAAATA TGGTGATAAT GATCCAACAG GCAAACCATT AAATAAACAA	1260
GATGGACCTA TACAACAAAT AAATGCAGCA ACTCAAAACA GTAAATATCT AGATGGAGAA	1320
ACAATAAATG GAATAGGGGC ATCCTTACCT GGTATTGTGA CTACAGGATG TTCAGCAACA	1380
GAACAACCTT TTAGTTGTAC TTCTACTGCT AATAGCTATA AAGCAAGCTG TAATCCTTCA	1440

GATACTAATC	AAAAAATTAA	TGCTTTATAT	GCTTTTACAC	AAACTAATGT	AAAGGGAAGC	1500
ACGGGGAAAT	TAGGAGTACT	GGCAAGTCTT	GTTCCATATG	ATTTAAATCC	TAAAAATGTA	1560
TTTGGTGAAT	TAGATTGAGA	TACAAATAAT	GTTATCTTAA	AAGGAATTCC	TGCAGAAAAA	1620
GGGTATTTTC	CTAATAATGC	GCGACCTACT	GTTGTAAAAG	AATGGATTAA	TGGTGCAAGT	1680
GCTGTACCAT	TTTATTCAGG	AAATACTTTA	TTTATGACGG	CTACGAATTT	AACAGCTACT	1740
CAATATAAAA	TTAGAATACG	TTATGCAAAT	CCAAATTCAG	ATACTCAAAT	CGGTGTACTA	1800
ATTACGCAAA	ATGGTTCTCA	AATTTCCAAT	AGTAATCTAA	CACTTTATAG	TACTACTGAT	1860
TCAAGTATGA	GTAATAATTT	ACCACAAAAT	GTATATGTCA	CAGGGGAAAA	TGGAAATTAT	1920
ACACTTCTAG	ATTTATATAG	TACTACTAAT	GTTTTATCAA	CAGGAGATAT	TACATTAAAA	1980
CTTACAGGAG	GAAATCAAAA	AATATTTATT	GATCGAATAG	AATTTATTCC	TACTATGCCT	2040
GTACCTGCTC	CTACTAATAA	CACTAATAAC	AATAACGGCG	ATAACGGCAA	TAACAATCCC	2100
CCACACCACG	GTTGTGCAAT	AGCTGGTACA	CAACAACCTT	GTTCTGGACC	ACCTAAGTTT	2160
GAACAAGTAA	GTGATTTAGA	AAAAATTACA	ACGCAAGTAT	ATATGTTATT	CAAATCTTCT	2220
TCGTATGAAG	AATTAGCTCT	AAAAGTTTCT	AGCTATCAAA	TTAATCAAGT	GGCATTGAAA	2280
GTTATGGCAC	TATCTGATGA	AAAGTTTGT	GAAGAAAAAA	GATTGTTACG	AAAATTAGTC	2340
AATAAAGCAA	ACCAATTACT	AGAAGCACGT	AACCTACTAG	TAGGTGGAAA	TTTTGAAACA	2400
ACTCAAAATT	GGGTACTTGG	AACAAATGCT	TATATAAATT	ATGATTCGTT	TTTATTTAAT	2460
GGAAATTATT	TATCCTTACA	ACCAGCAAGT	GGATTTTTCA	CATCTTATGC	TTATCAAAAA	2520
ATAGATGAGT	CAACATTAAA	ACCATATACA	CGATATAAAG	TTTCTGGATT	CATTGGGCAA	2580
AGTAATCAAG	TAGAACTTAT	TATTTCTCGT	TATGGAAAAG	AAATTGATAA	AATATTAAAT	2640
GTTCCATATG	CAGGGCCTCT	TCCTATTACT	GCTGATGCAT	CGATAACTTG	TTGTGCACCA	2700
GAAATAGACC	AATGTGATGG	GGGGCAATCT	GATTCCTCATT	TCTTCAACTA	TAGCATCGAT	2760
GTAGGTGCAC	TTCACCCAGA	ATTAAACCCCT	GGCATTGAAA	TTGGTCTTAA	AATTGTGCAA	2820
TCAAATGGTT	ATATAACAAT	TAGTAATCTA	GAAATTATTG	AAGAACGTCC	ACTTACAGAA	2880
ATGGAAATTC	AAGCAGTCAA	TCGAAAAGAT	CACAAATGGA	AAAGAGAAAA	ACTTCTAGAA	2940
TGTGCAAGTG	TTAGTGAAC	TTTACAACCA	ATCATTAATC	AAATCGATTG	ATTGTTCAAA	3000
GATGCAAACT	GGTATAATGA	TATTCCTCCT	CATGTCACAT	ATCAAACCTCT	AAAAAATATT	3060
ATAGTACCCG	ATTTACCAAA	ATTAAAAACAT	TGGTTCATAG	ATCATCTCCC	AGGTGAATAT	3120
CATGAAATTG	AACAACAAAT	GAAAGAAGCT	CTAAAACATG	CATTTACACA	ATTAGACGAG	3180
AAAAATTTAA	TCCACAATGG	TCACCTTGCA	ACTAACTTAA	TAGATTGGCA	AGTAGAAGGT	3240
GATGCTCGAA	TGAAAGTATT	AGAAAATAAT	GCTTTGGCAT	TACAACTTTC	CAATTGGGAT	3300
TCTAGTGTTC	CACAATCTAT	TGATATATTA	GAATTTGATG	AAGATAAAGC	ATATAAACTT	3360
CGCGTATATG	CTCAAGGAAG	CGGAACAATC	CAATTTGGAA	ACTGTGAAGA	TGAAGCCATC	3420
CAATTTAATA	CAAACCTCATT	CGTATATAAA	GAAAAAATAA	TCTATTTTCA	TACCCCATCA	3480
ATTAACCTTAC	ACATACAATC	AGAAGGTTCT	GAATTCGTTG	TAAGTAGTAT	CGACCTCGTT	3540
GAATTATCAG	ACGACGAATA	A				3561

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1186 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asp Cys Asn Leu Gln Ser Gln Gln Asn Ile Pro Tyr Asn Val Leu
 1 5 10 15
 Ala Ile Pro Val Ser Asn Val Asn Ala Leu Val Asp Thr Ala Gly Asp
 20 25 30
 Leu Lys Lys Ala Trp Glu Glu Phe Gln Lys Thr Gly Ser Phe Ser Leu
 35 40 45
 Thr Ala Leu Gln Gln Gly Phe Ser Ala Ser Gln Gly Gly Ala Phe Asn
 50 55 60
 Tyr Leu Thr Leu Leu Gln Ser Gly Ile Ser Leu Ala Gly Ser Phe Val
 65 70 75 80
 Pro Gly Gly Thr Phe Val Ala Pro Ile Val Asn Met Val Ile Gly Trp
 85 90 95
 Leu Trp Pro His Lys Asn Lys Thr Ala Asp Thr Glu Asn Leu Ile Lys
 100 105
 Leu Ile Asp Glu Glu Ile Gln Lys Gln Leu Asn Lys Ala Leu Leu Asp
 115 120 125
 Gln Asp Arg Asn Asn Trp Thr Ser Phe Leu Glu Ser Ile Phe Asp Thr
 130 135 140
 Ser Ala Thr Val Ser Asn Ala Ile Ile Asp Ala Gln Trp Ser Gly Thr
 145 150 155 160
 Val Asp Thr Thr Asn Arg Gln Gln Lys Thr Pro Thr Thr Ser Asp Tyr
 165 170 175
 Leu Asn Val Val Gly Lys Phe Asp Ser Ala Asp Ser Ser Ile Ile Thr
 180 185 190
 Asn Glu Asn Gln Ile Met Asn Gly Asn Phe Asp Val Ala Ala Ala Pro
 195 200 205
 Tyr Phe Val Ile Gly Ala Thr Leu Arg Leu Ser Leu Tyr Gln Ser Tyr
 210 215 220
 Ile Lys Phe Cys Asn Ser Trp Ile Asp Ala Val Gly Phe Ser Thr Asn
 225 230 235 240
 Asp Ala Asn Thr Gln Lys Ala Asn Leu Ala Arg Thr Lys Leu Thr Met
 245 250 255
 Arg Thr Thr Ile Asn Glu Tyr Thr Gln Arg Val Met Lys Val Phe Lys
 260 265 270
 Asp Ser Lys Asn Met Pro Thr Ile Gly Thr Asn Lys Phe Ser Val Asp
 275 280 285
 Ala Tyr Asn Val Tyr Val Lys Gly Met Thr Leu Asn Val Leu Asp Met
 290 295 300
 Val Ala Ile Trp Ser Ser Leu Tyr Pro Asn Asp Tyr Thr Ser Gln Thr
 305 310 315 320
 Ala Ile Glu Gln Thr Arg Val Thr Phe Ser Asn Met Val Gly Gln Glu
 325 330 335
 Glu Gly Thr Asp Gly Thr Leu Lys Ile Tyr Asn Thr Phe Asp Ser Leu
 340 345 350
 Ser Tyr Gln His Ser Leu Ile Pro Asn Asn Asn Val Asn Leu Ile Ser
 355 360 365
 Tyr Tyr Thr Asp Glu Leu Gln Asn Leu Glu Leu Ala Val Tyr Thr Pro
 370 375 380
 Lys Gly Gly Ser Gly Tyr Ala Tyr Pro Tyr Gly Phe Ile Leu Asn Tyr
 385 390 395 400
 Ala Asn Ser Asn Tyr Lys Tyr Gly Asp Asn Asp Pro Thr Gly Lys Pro
 405 410 415
 Leu Asn Lys Gln Asp Gly Pro Ile Gln Gln Ile Asn Ala Ala Thr Gln
 420 425 430
 Asn Ser Lys Tyr Leu Asp Gly Glu Thr Ile Asn Gly Ile Gly Ala Ser
 435 440 445

Leu Pro Gly Tyr Cys Thr Thr₄₅₅ Gly Cys Ser Ala Thr₄₆₀ Glu Gln Pro Phe
 Ser Cys Thr Ser Thr Ala₄₇₀ Asn Ser Tyr Lys Ala₄₇₅ Ser Cys Asn Pro Ser₄₈₀
 Asp Thr Asn Gln Lys₄₈₅ Ile Asn Ala Leu Tyr₄₉₀ Ala Phe Thr Gln Thr₄₉₅ Asn
 Val Lys Gly Ser₅₀₀ Thr Gly Lys Leu Gly₅₀₅ Val Leu Ala Ser Leu₅₁₀ Val Pro
 Tyr Asp Leu₅₁₅ Asn Pro Lys Asn Val₅₂₀ Phe Gly Glu Leu Asp₅₂₅ Ser Asp Thr
 Asn Asn Val Ile Leu Lys Gly₅₃₅ Ile Pro Ala Glu Lys₅₄₀ Gly Tyr Phe Pro
 Asn Asn Ala Arg Pro Thr₅₅₀ Val Val Lys Glu Trp₅₅₅ Ile Asn Gly Ala Ser₅₆₀
 Ala Val Pro Phe Tyr₅₆₅ Ser Gly Asn Thr Leu₅₇₀ Phe Met Thr Ala Thr₅₇₅ Asn
 Leu Thr Ala Thr₅₈₀ Gln Tyr Lys Ile Arg₅₈₅ Ile Arg Tyr Ala Asn₅₉₀ Pro Asn
 Ser Asp Thr₅₉₅ Gln Ile Gly Val Leu₆₀₀ Ile Thr Gln Asn Gly₆₀₅ Ser Gln Ile
 Ser Asn Ser Asn Leu Thr Leu₆₁₅ Tyr Ser Thr Thr Asp₆₂₀ Ser Ser Met Ser
 Ser Asn Leu Pro Gln Asn₆₃₀ Val Tyr Val Thr Gly₆₃₅ Glu Asn Gly Asn Tyr₆₄₀
 Thr Leu Leu Asp Leu₆₄₅ Tyr Ser Thr Thr Asn₆₅₀ Val Leu Ser Thr Gly₆₅₅ Asp
 Ile Thr Leu Lys Leu Thr Gly Gly Asn₆₆₅ Gln Lys Ile Phe Ile₆₇₀ Asp Arg
 Ile Glu Phe Ile Pro Thr Met Pro₆₈₀ Val Pro Ala Pro Thr₆₈₅ Asn Asn Thr
 Asn Asn Asn Asn Gly Asp Asn₆₉₅ Gly Asn Asn Asn Pro₇₀₀ Pro His His Gly
 Cys Ala Ile Ala Gly Thr₇₁₀ Gln Gln Leu Cys Ser₇₁₅ Gly Pro Pro Lys Phe₇₂₀
 Glu Gln Val Ser Asp₇₂₅ Leu Glu Lys Ile Thr₇₃₀ Thr Gln Val Tyr Met₇₃₅ Leu
 Phe Lys Ser Ser₇₄₀ Ser Tyr Glu Glu Leu₇₄₅ Ala Leu Lys Val Ser₇₅₀ Ser Tyr
 Gln Ile Asn₇₅₅ Gln Val Ala Leu Lys₇₆₀ Val Met Ala Leu Ser₇₆₅ Asp Glu Lys
 Phe Cys₇₇₀ Glu Glu Lys Arg Leu₇₇₅ Leu Arg Lys Leu Val₇₈₀ Asn Lys Ala Asn
 Gln Leu Leu Glu Ala Arg₇₉₀ Asn Leu Leu Val Gly₇₉₅ Gly Asn Phe Glu Thr₈₀₀
 Thr Gln Asn Trp Val₈₀₅ Leu Gly Thr Asn Ala₈₁₀ Tyr Ile Asn Tyr Asp₈₁₅ Ser
 Phe Leu Phe Asn₈₂₀ Gly Asn Tyr Leu Ser₈₂₅ Leu Gln Pro Ala Ser₈₃₀ Gly Phe
 Phe Thr Ser₈₃₅ Tyr Ala Tyr Gln Lys₈₄₀ Ile Asp Glu Ser Thr₈₄₅ Leu Lys Pro
 Tyr Thr₈₅₀ Arg Tyr Lys Val Ser₈₅₅ Gly Phe Ile Gly Gln₈₆₀ Ser Asn Gln Val
 Glu Leu Ile Ile Ser Arg₈₇₀ Tyr Gly Lys Glu Ile₈₇₅ Asp Lys Ile Leu Asn₈₈₀
 Val Pro Tyr Ala Gly₈₈₅ Pro Leu Pro Ile Thr₈₉₀ Ala Asp Ala Ser Ile₈₉₅ Thr

[illegible]

Claims

1 1. A method for controlling corn rootworms comprising contacting said corn rootworms
2 with a corn rootworm-controlling amount of a *Bacillus thuringiensis* isolate, or a toxin of said
3 *Bacillus thuringiensis* isolate, wherein said isolate is selected from the group consisting of PS80JJ1,
4 having the identifying characteristics of NRRL B-18679; PS158D5, having the identifying
5 characteristics of NRRL B-18680; PS167P, having the identifying characteristics of NRRL B-
6 18681; PS169E, having the identifying characteristics of NRRL B-18682; PS177F1, having the
7 identifying characteristics of NRRL B-18683; PS177G, having the identifying characteristics of
8 NRRL B-18684; PS204G4, having the identifying characteristics of NRRL B-18685; and PS204G6,
9 having the identifying characteristics of NRRL B-18686; and mutants thereof which retain activity
10 against corn rootworms.

1 2. A method, according to claim 1, further comprising incorporating said *Bacillus*
2 *thuringiensis* isolate, or a spore or toxin from said isolate, into a bait granule and placing said
3 granule on or in the soil when planting corn or later in the crop cycle.

1 3. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is PS80JJ1,
2 having the identifying characteristics of NRRL B-18679.

1 4. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is
2 PS158D5, having the identifying characteristics of NRRL B-18680.

1 5. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is PS167P,
2 having the identifying characteristics of NRRL B-18681.

1 6. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is PS169E,
2 having the identifying characteristics of NRRL B-18682.

1 7. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is
2 PS177F1, having the identifying characteristics of NRRL B-18683.

1 8. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is PS177G,
2 having the identifying characteristics of NRRL B-18684.

1 9. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is
2 PS204G4, having the identifying characteristics of NRRL B-18685.

1 10. The method, according to claim 1, wherein said *Bacillus thuringiensis* isolate is
2 PS204G6, having the identifying characteristics of NRRL B-18686.

1 11. A composition of matter for controlling corn rootworms comprising a *Bacillus*
2 *thuringiensis* isolate selected from the group consisting of PS80JJ1, having the identifying
3 characteristics of NRRL B-18679; PS158D5, having the identifying characteristics of NRRL B-
4 18680; PS167P, having the identifying characteristics of NRRL B-18681; PS169E, having the
5 identifying characteristics of NRRL B-18682; PS177F1, having the identifying characteristics of
6 NRRL B-18683; PS177G, having the identifying characteristics of NRRL B-18684; PS204G4,
7 having the identifying characteristics of NRRL B-18685; and PS204G6, having the identifying
8 characteristics of NRRL B-18686; and mutants thereof which retain activity against corn
9 rootworms, in association with an agricultural carrier appropriate for use in controlling corn
10 rootworm.

1 12. A method for controlling corn rootworms comprising contacting said corn rootworms
2 with a corn rootworm-controlling effective amount of a pesticidal composition comprising
3 substantially intact treated cells having prolonged pesticidal activity when applied to the
4 environment of the corn rootworm, wherein said insecticide is produced by a *Bacillus thuringiensis*
5 gene from a *Bacillus thuringiensis* isolate selected from the group consisting of PS80JJ1, having
6 the identifying characteristics of NRRL B-18679; PS158D5, having the identifying characteristics
7 of NRRL B-18680; PS167P, having the identifying characteristics of NRRL B-18681; PS169E,
8 having the identifying characteristics of NRRL B-18682; PS177F1, having the identifying
9 characteristics of NRRL B-18683; PS177G, having the identifying characteristics of NRRL B-
10 18684; PS204G4, having the identifying characteristics of NRRL B-18685; and PS204G6, having
11 the identifying characteristics of NRRL B-18686; and mutants thereof which retain activity against
12 corn rootworms.

1 13. An isolated polynucleotide comprising DNA wherein said DNA encodes a toxin
2 which is active against corn rootworms and wherein said DNA or variant thereof is from a *Bacillus*
3 *thuringiensis* isolate selected from the group consisting of PS80JJ1, having the identifying
4 characteristics of NRRL B-18679; PS158D5, having the identifying characteristics of NRRL B-
5 18680; PS167P, having the identifying characteristics of NRRL B-18681; PS169E, having the
6 identifying characteristics of NRRL B-18682; PS177F1, having the identifying characteristics of
7 NRRL B-18683; PS177G, having the identifying characteristics of NRRL B-18684; PS204G4,
8 having the identifying characteristics of NRRL B-18685; and PS204G6, having the identifying

9 characteristics of NRRL B-18686; and mutants thereof which retain activity against corn
10 rootworms.

1 14. The polynucleotide, according to claim 13, from *Bacillus thuringiensis* isolate PS204G6
2 having a *Hind*III fragment of approximately 2.4 kbp which hybridizes with SEQ ID NO. 2.

1 15. The polynucleotide, according to claim 13, which encodes the amino acid sequence
2 of SEQ ID NO. 6.

1 16. The polynucleotide, according to claim 15, having the nucleotide sequence of SEQ
2 ID NO. 5.

1 17. The polynucleotide, according to claim 13, from *Bacillus thuringiensis* isolate PS167P
2 having a fragment selected from the group consisting of *Hind*III fragments of approximately 5.5
3 and 8.0 kbp and *Eco*RI fragments of approximately 1.8 kbp and 2.3 kbp wherein said fragment
4 hybridizes with a 700 to 800 bp DNA sequence produced by amplification of PS167P DNA
5 utilizing SEQ ID NO. 3 as a forward primer and SEQ ID NO. 4 as a reverse primer.

1 18. A purified toxin which is active against corn rootworms, wherein said toxin is encoded
2 by a polynucleotide from a *Bacillus thuringiensis* isolate selected from the group consisting of
3 PS80JJ1, having the identifying characteristics of NRRL B-18679; PS158D5, having the identifying
4 characteristics of NRRL B-18680; PS167P, having the identifying characteristics of NRRL B-
5 18681; PS169E, having the identifying characteristics of NRRL B-18682; PS177F1, having the
6 identifying characteristics of NRRL B-18683; PS177G, having the identifying characteristics of
7 NRRL B-18684; PS204G4, having the identifying characteristics of NRRL B-18685; and PS204G6,
8 having the identifying characteristics of NRRL B-18686; and mutants thereof which retain the
9 property of activity against corn rootworms.

1 19. The toxin, according to claim 18, wherein said toxin is encoded by a polynucleotide
2 of claim 14.

1 20. The toxin, according to claim 18, wherein said toxin has the amino acid sequence of
2 SEQ ID NO. 6.

1 21. The toxin, according to claim 18, wherein said toxin is encoded by a polynucleotide
2 of claim 16.

1 22. A microbe transformed to express a polynucleotide encoding a toxin active against
2 corn rootworms, said polynucleotide from a *Bacillus thuringiensis* isolate selected from the group
3 consisting of PS80JJ1, having the identifying characteristics of NRRL B-18679; PS158D5, having
4 the identifying characteristics of NRRL B-18680; PS167P, having the identifying characteristics
5 of NRRL B-18681; PS169E, having the identifying characteristics of NRRL B-18682; PS177F1,
6 having the identifying characteristics of NRRL B-18683; PS177G, having the identifying
7 characteristics of NRRL B-18684; PS204G4, having the identifying characteristics of NRRL B-
8 18685; and PS204G6, having the identifying characteristics of NRRL B-18686; and mutants thereof
9 which retain activity to corn rootworms.