Compositions and methods for conferring insecticidal activity to host cells are provided. Compositions comprising a coding sequence for a delta-endotoxin polypeptide are provided. The coding sequences can be used in DNA constructs or expression cassettes for transformation and expression in host cells. Compositions also comprise transformed host cells. In particular, isolated delta-endotoxin nucleic acid molecules are provided. Additionally, amino acid sequences corresponding to the polynucleotides are encompassed, and antibodies specifically binding to those amino acid sequences. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequence shown in SEQ ID NO: 4, 5, 6, 13, or 14, or the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 11, or 12, as well as variants and fragments thereof.
AXMI-1 15, AXMI-1 13, AXMI-005, AXMI-163 AND AXMI-184: INSECTICIDAL PROTEINS AND METHODS FOR THEIR USE

FIELD OF THE INVENTION

This invention relates to the field of molecular biology. Provided are novel genes that encode insecticidal proteins. These proteins and the nucleic acid sequences that encode them are useful in preparing insecticidal formulations and in the production of transgenic insect-resistant plants.

BACKGROUND OF THE INVENTION

*Bacillus thuringiensis* is a Gram-positive spore forming soil bacterium characterized by its ability to produce crystalline inclusions that are specifically toxic to certain orders and species of insects, but are harmless to plants and other non-targeted organisms. For this reason, compositions including *Bacillus thuringiensis* strains or their insecticidal proteins can be used as environmentally-acceptable insecticides to control agricultural insect pests or insect vectors for a variety of human or animal diseases.

Crystal (Cry) proteins (delta-endotoxins) from *Bacillus thuringiensis* have potent insecticidal activity against predominantly Lepidopteran, Dipteran, and Coleopteran larvae. These proteins also have shown activity against *Hymenoptera*, *Homoptera*, *Phthiraptera*, *Mallophaga*, *Acarina* pest orders, as well as other invertebrate orders such as *Nemathelminthes*, *Platyhelminthes*, and *Sarcomastigophora* (Feitelson (1993) The Bacillus Thuringiensis family tree. In *Advanced Engineered Pesticides*, Marcel Dekker, Inc., New York, N.Y.) These proteins were originally classified as Cry1 to CryV based primarily on their insecticidal activity. The major classes were *Lepidoptera-specific* (I), *Lepidoptera*–*and Diptera-specific* (II), *Coleoptera-specific* (III), *Diptera-specific* (IV), and nematode-specific (V) and (VI). The proteins were further classified into subfamilies; more highly related proteins within each family were assigned divisional letters such
as CryIA, CryIB, CryIC, etc. Even more closely related proteins within each division were given names such as CryICl, CryIC2, etc.

A new nomenclature was recently described for the Cry genes based upon amino acid sequence homology rather than insect target specificity (Crickmore et al. (1998) *Microbiol. Mol. Biol. Rev.* 62:807-813). In the new classification, each toxin is assigned a unique name incorporating a primary rank (an Arabic number), a secondary rank (an uppercase letter), a tertiary rank (a lowercase letter), and a quaternary rank (another Arabic number). In the new classification, Roman numerals have been exchanged for Arabic numerals in the primary rank. Proteins with less than 45% sequence identity have different primary ranks, and the criteria for secondary and tertiary ranks are 78% and 95%, respectively.

The crystal protein does not exhibit insecticidal activity until it has been ingested and solubilized in the insect midgut. The ingested protoxin is hydrolyzed by proteases in the insect digestive tract to an active toxic molecule. (Höfte and Whiteley (1989) *Microbiol. Rev.* 53:242-255). This toxin binds to apical brush border receptors in the midgut of the target larvae and inserts into the apical membrane creating ion channels or pores, resulting in larval death.

Delta-endotoxins generally have five conserved sequence domains, and three conserved structural domains (see, for example, de Maagd et al. (2001) *Trends Genetics* 17:193-199). The first conserved structural domain consists of seven alpha helices and is involved in membrane insertion and pore formation. Domain II consists of three beta-sheets arranged in a "Greek key" configuration, and domain III consists of two antiparallel beta-sheets in "jelly-roll" formation (de Maagd et al., 2001, supra). Domains II and III are involved in receptor recognition and binding, and are therefore considered determinants of toxin specificity.

Aside from delta-endotoxins, there are several other known classes of pesticidal protein toxins. The VIP1/VIP2 toxins (see, for example, U.S. Patent 5,770,696) are binary pesticidal toxins that exhibit strong activity on insects by a mechanism believed to involve receptor-mediated endocytosis followed by cellular toxification, similar to the mode of action of other binary ("A/B") toxins. A/B toxins such as VIP, C2, CDT, CST, or the *B. anthracis* edema and lethal toxins initially interact with target cells via a specific, receptor-mediated binding of "B" components as monomers. These monomers then form homoheptamers. The "B" heptamer-receptor complex then acts as a docking platform that subsequently binds and allows
the translocation of an enzymatic "A" component(s) into the cytosol via receptor-mediated endocytosis. Once inside the cell's cytosol, "A" components inhibit normal cell function by, for example, ADP-ribosylation of G-actin, or increasing intracellular levels of cyclic AMP (cAMP). See Barth et al. (2004) Microbiol Mol Biol Rev 68:373-402.

The intensive use of B. thuringiensis-based insecticides has already given rise to resistance in field populations of the diamondback moth, Plutella xylostella (Ferre and Van Rie (2002) Annu. Rev. Entomol. 47:501-533). The most common mechanism of resistance is the reduction of binding of the toxin to its specific midgut receptor(s). This may also confer cross-resistance to other toxins that share the same receptor (Ferre and Van Rie (2002)).

SUMMARY OF INVENTION
Compositions and methods for conferring insect resistance to bacteria, plants, plant cells, tissues and seeds are provided. Compositions include nucleic acid molecules encoding sequences for delta-endotoxin polypeptides, vectors comprising those nucleic acid molecules, and host cells comprising the vectors. Compositions also include the polypeptide sequences of the endotoxin, and antibodies to those polypeptides. The nucleotide sequences can be used in DNA constructs or expression cassettes for transformation and expression in organisms, including microorganisms and plants. The nucleotide or amino acid sequences may be synthetic sequences that have been designed for expression in an organism including, but not limited to, a microorganism or a plant. Compositions also comprise transformed bacteria, plants, plant cells, tissues, and seeds.

In particular, isolated nucleic acid molecules corresponding to delta-endotoxin nucleic acid sequences are provided. Additionally, amino acid sequences corresponding to the polynucleotides are encompassed. In particular, the present invention provides for an isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in any of SEQ ID NO:4, 5, 6, 13, or 14, or a nucleotide sequence set forth in any of SEQ ID NO: 1, 2, 3, 11, or 12, as well as variants and fragments thereof. Nucleotide sequences that are complementary to a nucleotide sequence of the invention, or that hybridize to a sequence of the invention are also encompassed.
The compositions and methods of the invention are useful for the production of organisms with insecticide resistance, specifically bacteria and plants. These organisms and compositions derived from them are desirable for agricultural purposes. The compositions of the invention are also useful for generating altered or improved delta-endotoxin proteins that have insecticidal activity, or for detecting the presence of delta-endotoxin proteins or nucleic acids in products or organisms.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show an alignment of AXMI-1 13 (SEQ ID NO:5), AXMI-005 (SEQ ID NO:4), and AXMI-1 15 (SEQ ID NO:6). The left and right arrows mark the boundaries of the C-terminal 1/3rd region of the proteins.

Figure 2 depicts domains within AXMI-005 and AXMI-1 15 that are swapped to generate new toxins.

DETAILED DESCRIPTION

The present invention is drawn to compositions and methods for regulating insect resistance in organisms, particularly plants or plant cells. The methods involve transforming organisms with a nucleotide sequence encoding a delta-endotoxin protein of the invention. In particular, the nucleotide sequences of the invention are useful for preparing plants and microorganisms that possess insecticidal activity. Thus, transformed bacteria, plants, plant cells, plant tissues and seeds are provided. Compositions are delta-endotoxin nucleic acids and proteins of *Bacillus thuringiensis*. The sequences find use in the construction of expression vectors for subsequent transformation into organisms of interest, as probes for the isolation of other delta-endotoxin genes, and for the generation of altered insecticidal proteins by methods known in the art, such as domain swapping or DNA shuffling. See, for example, Table 2 and Figure 2. The proteins find use in controlling or killing lepidopteran, coleopteran, and other insect populations, and for producing compositions with insecticidal activity.

By "delta-endotoxin" is intended a toxin from *Bacillus thuringiensis* that has toxic activity against one or more pests, including, but not limited to, members of the *Lepidoptera, Diptera, and Coleoptera* orders, or a protein that has homology to such a
protein. In some cases, delta-endotoxin proteins have been isolated from other organisms, including *Clostridium bifermentans* and *Paenibacillus popilliae*. Delta-endotoxin proteins include amino acid sequences deduced from the full-length nucleotide sequences disclosed herein, and amino acid sequences that are shorter than the full-length sequences, either due to the use of an alternate downstream start site, or due to processing that produces a shorter protein having insecticidal activity. Processing may occur in the organism the protein is expressed in, or in the pest after ingestion of the protein.

Delta-endotoxins include proteins identified as *cry1* through *cry43*, *cyt1* and *cyt2*, and Cyt-like toxin. There are currently over 250 known species of delta-endotoxins with a wide range of specificities and toxicities. For an expansive list see Crickmore *et al.* (1998), *Microbiol. Mol. Biol. Rev.* 62:807-813, and for regular updates see Crickmore *et al.* (2003) "*Bacillus thuringiensis* toxin nomenclature," at www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.

Provided herein are novel isolated nucleotide sequences that confer insecticidal activity. Also provided are the amino acid sequences of the delta-endotoxin proteins. The protein resulting from translation of this gene allows cells to control or kill insects that ingest it.

Isolated Nucleic Acid Molecules, and Variants and Fragments Thereof

One aspect of the invention pertains to isolated or recombinant nucleic acid molecules comprising nucleotide sequences encoding delta-endotoxin proteins and polypeptides or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify delta-endotoxin encoding nucleic acids. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., recombinant DNA, cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that
naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For purposes of the invention, "isolated" when used to refer to nucleic acid molecules excludes isolated chromosomes. For example, in various embodiments, the isolated delta-endotoxin encoding nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A delta-endotoxin protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-delta-endotoxin protein (also referred to herein as a "contaminating protein").

Nucleotide sequences encoding the proteins of the present invention include the sequence set forth in SEQ ID NO:1, 2, 3, 11, or 12, and variants, fragments, and complements thereof. By "complement" is intended a nucleotide sequence that is sufficiently complementary to a given nucleotide sequence such that it can hybridize to the given nucleotide sequence to thereby form a stable duplex. The corresponding amino acid sequence for the delta-endotoxin protein encoded by this nucleotide sequence are set forth in SEQ ID NO:4, 5, 6, 13, or 14.

Nucleic acid molecules that are fragments of these delta-endotoxin encoding nucleotide sequences are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence encoding a delta-endotoxin protein. A fragment of a nucleotide sequence may encode a biologically active portion of a delta-endotoxin protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. Nucleic acid molecules that are fragments of a delta-endotoxin nucleotide sequence comprise at least about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, 2000, 2050, 2100, 2150, 2200, 2250, 2300, 2350, 2400, 2450, 2500, 2550, 2600, 2650, 2700, 2750, 2800, 2850, 2900, 2950, 3000, 3050, 3100, 3150, 3200, 3250, 3300, 3350 contiguous nucleotides, or up to the number of nucleotides present in a full-length delta-endotoxin encoding nucleotide sequence disclosed herein depending upon the intended use. By "contiguous" nucleotides is intended nucleotide residues that are immediately adjacent to one another. Fragments of the nucleotide sequences of the present invention will encode protein fragments that retain the biological
activity of the delta-endotoxin protein and, hence, retain insecticidal activity. By "retains activity" is intended that the fragment will have at least about 30%, at least about 50%, at least about 70%, 80%, 90%, 95% or higher of the insecticidal activity of the delta-endotoxin protein. Methods for measuring insecticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83:2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by reference in their entirety.

A fragment of a delta-endotoxin encoding nucleotide sequence that encodes a biologically active portion of a protein of the invention will encode at least about 15, 25, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100 contiguous amino acids, or up to the total number of amino acids present in a full-length delta-endotoxin protein of the invention.

Preferred delta-endotoxin proteins of the present invention are encoded by a nucleotide sequence sufficiently identical to the nucleotide sequence of SEQ ID NO:1, 2, 3, 11, or 12. By "sufficiently identical" is intended an amino acid or nucleotide sequence that has at least about 60% or 65% sequence identity, about 70% or 75% sequence identity, about 80% or 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater sequence identity compared to a reference sequence using one of the alignment programs described herein using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., percent identity = number of identical positions/total number of positions) x 100. In one embodiment, the two sequences are the same length. In another embodiment, the comparison is across the entirety of the reference sequence (e.g., across the entirety of one of SEQ ID NO: 1, 2, 3, 11, or 12, or across the entirety of one of SEQ ID NO:4, 5, 6, 13, or 14). The percent identity between two sequences can be determined using
techniques similar to those described below, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A nonlimiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTX programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to delta-endotoxin-like nucleic acid molecules of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to delta-endotoxin protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., BLASTX and BLASTN) can be used. Alignment may also be performed manually by inspection.

Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the ClustalW algorithm (Higgins *et al.* (1994) *Nucleic Acids Res.* 22:4673-4680). ClustalW compares sequences and aligns the entirety of the amino acid or DNA sequence, and thus can provide data about the sequence conservation of the entire amino acid sequence. The ClustalW algorithm is used in several commercially available DNA/amin acid analysis software packages, such as the ALIGNX module of the Vector NTI Program Suite (Invitrogen Corporation, Carlsbad, CA). After alignment of amino acid sequences with ClustalW, the percent amino acid identity can be assessed. A non-limiting example of a software program useful for analysis of ClustalW alignments is GENEDOC™. GENEDOC™ (Karl Nicholas) allows assessment of amino acid (or DNA) similarity and identity between multiple proteins. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *CABIOS* 4:1 1-17. Such an algorithm is incorporated into the ALIGN program (version 2.0),
which is part of the GCG Wisconsin Genetics Software Package, Version 10
(available from Accelrys, Inc., 9685 Scranton Rd., San Diego, CA, USA). When
utilizing the ALIGN program for comparing amino acid sequences, a PAM 120 weight
residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.
5

Unless otherwise stated, GAP Version 10, which uses the algorithm of
Needleman and Wunsch (1970) J. Mol. Biol. 48(3):443-453, will be used to determine
sequence identity or similarity using the following parameters: % identity and %
similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3,
and the nwsgapdna.cmp scoring matrix; % identity or % similarity for an amino acid
sequence using GAP weight of 8 and length weight of 2, and the BLOSUM62 scoring
program. Equivalent programs may also be used. By "equivalent program" is
intended any sequence comparison program that, for any two sequences in question,
generates an alignment having identical nucleotide residue matches and an identical
percent sequence identity when compared to the corresponding alignment generated
by GAP Version 10. The invention also encompasses variant nucleic acid molecules.
"Variants" of the delta-endotoxin encoding nucleotide sequences include those
sequences that encode the delta-endotoxin proteins disclosed herein but that differ
conservatively because of the degeneracy of the genetic code as well as those that are
sufficiently identical as discussed above. Naturally occurring allelic variants can be
identified with the use of well-known molecular biology techniques, such as
polymerase chain reaction (PCR) and hybridization techniques as outlined below.
Variant nucleotide sequences also include synthetically derived nucleotide sequences
that have been generated, for example, by using site-directed mutagenesis but which
still encode the delta-endotoxin proteins disclosed in the present invention as
discussed below. Variant proteins encompassed by the present invention are
biologically active, that is they continue to possess the desired biological activity of
the native protein, that is, retaining insecticidal activity. By "retains activity" is
intended that the variant will have at least about 30%, at least about 50%, at least
about 70%, or at least about 80% of the insecticidal activity of the native protein.
Methods for measuring insecticidal activity are well known in the art. See, for
example, Czapla and Lang (1990) J. Econ. Entomol. 83: 2480-2485; Andrews et al.
78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by
reference in their entirety.
The skilled artisan will further appreciate that changes can be introduced by mutation of the nucleotide sequences of the invention thereby leading to changes in the amino acid sequence of the encoded delta-endotoxin proteins, without altering the biological activity of the proteins. Thus, variant isolated nucleic acid molecules can be created by introducing one or more nucleotide substitutions, additions, or deletions into the corresponding nucleotide sequence disclosed herein, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Such variant nucleotide sequences are also encompassed by the present invention.

For example, conservative amino acid substitutions may be made at one or more predicted, nonessential amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of a delta-endotoxin protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Delta-endotoxins generally have five conserved sequence domains, and three conserved structural domains (see, for example, de Maagd et al. (2001) Trends Genetics 17:193-199). The first conserved structural domain consists of seven alpha helices and is involved in membrane insertion and pore formation. Domain II consists of three beta-sheets arranged in a Greek key configuration, and domain III consists of two antiparallel beta-sheets in "jelly-roll" formation (de Maagd et al., 2001, supra). Domains II and III are involved in receptor recognition and binding, and are therefore considered determinants of toxin specificity.

Amino acid substitutions may be made in nonconserved regions that retain function. In general, such substitutions would not be made for conserved amino acid
residues, or for amino acid residues residing within a conserved motif, where such residues are essential for protein activity. Examples of residues that are conserved and that may be essential for protein activity include, for example, residues that are identical between all proteins contained in an alignment of the amino acid sequences of the present invention and known delta-endotoxin sequences. Examples of residues that are conserved but that may allow conservative amino acid substitutions and still retain activity include, for example, residues that have only conservative substitutions between all proteins contained in an alignment of the amino acid sequences of the present invention and known delta-endotoxin sequences. However, one of skill in the art would understand that functional variants may have minor conserved or nonconserved alterations in the conserved residues.

Alternatively, variant nucleotide sequences can be made by introducing mutations randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for ability to confer delta-endotoxin activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly, and the activity of the protein can be determined using standard assay techniques.


In a hybridization method, all or part of the delta-endotoxin nucleotide sequence can be used to screen cDNA or genomic libraries. Methods for construction of such cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook and Russell, 2001, supra. The so-called hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as $^{32}$P, or any other detectable marker, such as other radioisotopes, a fluorescent compound, an enzyme, or an enzyme co-factor. Probes for hybridization can be made by labeling synthetic oligonucleotides based on the known delta-endotoxin-encoding nucleotide sequence disclosed herein. Degenerate primers designed on the basis of conserved nucleotides or amino acid residues in the nucleotide sequence or encoded amino acid sequence
can additionally be used. The probe typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, at least about 25, at least about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, or 400 consecutive nucleotides of delta-endotoxin encoding nucleotide sequence of the invention or a fragment or variant thereof. Methods for the preparation of probes for hybridization are generally known in the art and are disclosed in Sambrook and Russell, 2001, supra herein incorporated by reference.

For example, an entire delta-endotoxin sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding delta-endotoxin-like sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, or at least about 20 nucleotides in length. Such probes may be used to amplify corresponding delta-endotoxin sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in depending upon circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes.
(e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in IX to 2X SSC (2OX SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to IX SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the $T_m$ can be approximated from the equation of Meinkoth and Wahl (1984) Anal. Biochem. 138:267-284: $T_m = 81.5°C + 16.6 \times \log M + 0.41 \times (%GC) - 0.61 \times (%\text{form}) - 500/L$; where $M$ is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and $L$ is the length of the hybrid in base pairs. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. $T_m$ is reduced by about 1°C for each 1% of mismatching; thus, $T_m$, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ≥90% identity are sought, the $T_m$ can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point ($T_m$) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point ($T_m$); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point ($T_m$); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point ($T_m$). Using the equation, hybridization and wash compositions, and desired $T_m$, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of
mismatching results in a $T_m$ of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology*—


**Isolated Proteins and Variants and Fragments Thereof**

Delta-endotoxin proteins are also encompassed within the present invention. By "delta-endotoxin protein" is intended a protein having the amino acid sequence set forth in SEQ ID NO:4, 5, 6, 13, or 14. Fragments, biologically active portions, and variants thereof are also provided, and may be used to practice the methods of the present invention.

"Fragments" or "biologically active portions" include polypeptide fragments comprising amino acid sequences sufficiently identical to the amino acid sequence set forth in any of SEQ ID NO:4, 5, 6, 13, or 14 and that exhibit insecticidal activity. A biologically active portion of a delta-endotoxin protein can be a polypeptide that is, for example, 10, 25, 50, 100 or more amino acids in length. Such biologically active portions can be prepared by recombinant techniques and evaluated for insecticidal activity. Methods for measuring insecticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83:2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by reference in their entirety. As used here, a fragment comprises at least 8 contiguous amino acids of SEQ ID NO:4, 5, 6, 13, or 14. The invention encompasses other fragments, however, such as any fragment in the protein greater than about 10, 20, 30, 50, 100, 150, 200, 250, 300, 350, 400, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, or 1300 amino acids.

By "variants" is intended proteins or polypeptides having an amino acid sequence that is at least about 60%, 65%, about 70%, 75%, about 80%, 85%, about
90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14. Variants also include polypeptides encoded by a nucleic acid molecule that hybridizes to the nucleic acid molecule of SEQ ID NO:1, 2, 3, 11, or 12, or a complement thereof, under stringent conditions.

Variants include polypeptides that differ in amino acid sequence due to mutagenesis. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, retaining insecticidal activity. Methods for measuring insecticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83:2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by reference in their entirety.

Bacterial genes, such as the *axmi* genes of this invention, quite often possess multiple methionine initiation codons in proximity to the start of the open reading frame. Often, translation initiation at one or more of these start codons will lead to generation of a functional protein. These start codons can include ATG codons. However, bacteria such as *Bacillus sp.* also recognize the codon GTG as a start codon, and proteins that initiate translation at GTG codons contain a methionine at the first amino acid. Furthermore, it is not often determined *apriori* which of these codons are used naturally in the bacterium. Thus, it is understood that use of one of the alternate methionine codons may also lead to generation of delta-endotoxin proteins that encode insecticidal activity. These delta-endotoxin proteins are encompassed in the present invention and may be used in the methods of the present invention.

Antibodies to the polypeptides of the present invention, or to variants or fragments thereof, are also encompassed. Methods for producing antibodies are well known in the art (see, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; U.S. Patent No. 4,196,265).

**Altered or Improved Variants**

It is recognized that DNA sequences of a delta-endotoxin may be altered by various methods, and that these alterations may result in DNA sequences encoding proteins with amino acid sequences different than that encoded by a delta-endotoxin of the present invention. This protein may be altered in various ways including amino...
acid substitutions, deletions, truncations, and insertions of one or more amino acids of
SEQ ID NO: 4, 5, 6, 13, or 14, including up to about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 100, about 105, about 110, about 115, about 120, about 125, about 130 or more amino acid substitutions, deletions or insertions.

Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of a delta-endotoxin protein can be prepared by mutations in the DNA. This may also be accomplished by one of several forms of mutagenesis and/or in directed evolution. In some aspects, the changes encoded in the amino acid sequence will not substantially affect the function of the protein. Such variants will possess the desired insecticidal activity. However, it is understood that the ability of a delta-endotoxin to confer insecticidal activity may be improved by the use of such techniques upon the compositions of this invention. For example, one may express a delta-endotoxin in host cells that exhibit high rates of base misincorporation during DNA replication, such as XL-I Red (Stratagene). After propagation in such strains, one can isolate the delta-endotoxin DNA (for example by preparing plasmid DNA, or by amplifying by PCR and cloning the resulting PCR fragment into a vector), culture the delta-endotoxin mutations in a non-mutagenic strain, and identify mutated delta-endotoxin genes with insecticidal activity, for example by performing an assay to test for insecticidal activity. Generally, the protein is mixed and used in feeding assays. See, for example Marrone et al. (1985) J. of Economic Entomology 78:290-293. Such assays can include contacting plants with one or more insects and determining the plant’s ability to survive and/or cause the death of the insects.

Examples of mutations that result in increased toxicity are found in Schnepf et al. (1998) Microbiol. Mol. Biol. Rev. 62:775-806.

Alternatively, alterations may be made to the protein sequence of many proteins at the amino or carboxy terminus without substantially affecting activity. This can include insertions, deletions, or alterations introduced by modern molecular methods, such as PCR, including PCR amplifications that alter or extend the protein coding sequence by virtue of inclusion of amino acid encoding sequences in the oligonucleotides utilized in the PCR amplification. Alternatively, the protein sequences added can include entire protein-coding sequences, such as those used commonly in the art to generate protein fusions. Such fusion proteins are often used to
(1) increase expression of a protein of interest (2) introduce a binding domain, enzymatic activity, or epitope to facilitate either protein purification, protein detection, or other experimental uses known in the art (3) target secretion or translation of a protein to a subcellular organelle, such as the periplasmic space of Gram-negative bacteria, or the endoplasmic reticulum of eukaryotic cells, the latter of which often results in glycosylation of the protein.

Variant nucleotide and amino acid sequences of the present invention also encompass sequences derived from mutagenic and recombinogenic procedures such as DNA shuffling. With such a procedure, one or more different delta-endotoxin protein coding regions can be used to create a new delta-endotoxin protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between a delta-endotoxin gene of the invention and other known delta-endotoxin genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased insecticidal activity. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) Proc. Natl. Acad. Sci. USA 91:10747-10751; Stemmer (1994) Nature 370:389-391; Cramer et al. (1997) Nature Biotech. 15:436-438; Moore et al. (1997) J. Mol. Biol. 272:336-347; Zhang et al. (1997) Proc. Natl. Acad. Sci. USA 94:4504-4509; Cramer et al. (1998) Nature 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

Vectors

A delta-endotoxin sequence of the invention may be provided in an expression cassette for expression in a plant of interest. By "plant expression cassette" is intended a DNA construct that is capable of resulting in the expression of a protein from an open reading frame in a plant cell. Typically these contain a promoter and a coding sequence. Often, such constructs will also contain a 3' untranslated region. Such constructs may contain a "signal sequence" or "leader sequence" to facilitate cotranslational or post-translational transport of the peptide to certain intracellular structures such as the chloroplast (or other plastid), endoplasmic reticulum, or Golgi apparatus.

By "signal sequence" is intended a sequence that is known or suspected to result in cotranslational or post-translational peptide transport across the cell membrane. In eukaryotes, this typically involves secretion into the Golgi apparatus, with some resulting glycosylation. By "leader sequence" is intended any sequence that when translated, results in an amino acid sequence sufficient to trigger cotranslational transport of the peptide chain to a sub-cellular organelle. Thus, this includes leader sequences targeting transport and/or glycosylation by passage into the endoplasmic reticulum, passage to vacuoles, plastids including chloroplasts, mitochondria, and the like.

By "plant transformation vector" is intended a DNA molecule that is necessary for efficient transformation of a plant cell. Such a molecule may consist of one or more plant expression cassettes, and may be organized into more than one "vector" DNA molecule. For example, binary vectors are plant transformation vectors that utilize two non-contiguous DNA vectors to encode all requisite cis- and trans-acting functions for transformation of plant cells (Hellens and Mullineaux (2000) Trends in Plant Science 5:446-451). "Vector" refers to a nucleic acid construct designed for transfer between different host cells. "Expression vector" refers to a vector that has the ability to incorporate, integrate and express heterologous DNA sequences or fragments in a foreign cell. The cassette will include 5' and 3' regulatory sequences operably linked to a sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two
protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

"Promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream coding sequence. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") are necessary for the expression of a DNA sequence of interest.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the delta-endotoxin sequence to be under the transcriptional regulation of the regulatory regions.

The expression cassette will include in the 5′-3′ direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a DNA sequence of the invention, and a translational and transcriptional termination region (i.e., termination region) functional in plants. The promoter may be native, or analogous, or foreign or heterologous, to the plant host and/or to the DNA sequence of the invention. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. Where the promoter is "native" or "homologous" to the plant host, it is intended that the promoter is found in the native plant into which the promoter is introduced. Where the promoter is "foreign" or "heterologous" to the DNA sequence of the invention, it is intended that the promoter is not the native or naturally occurring promoter for the operably linked DNA sequence of the invention.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the DNA sequence of interest, the plant host, or any combination thereof). Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.
Where appropriate, the gene(s) may be optimized for increased expression in the transformed host cell. That is, the genes can be synthesized using host cell-preferred codons for improved expression, or may be synthesized using codons at a host-preferred codon usage frequency. Generally, the GC content of the gene will be increased. See, for example, Campbell and Gowri (1990) Plant Physiol. 92: 1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray et al. (1989) Nucleic Acids Res. 17:477-498, herein incorporated by reference.

In one embodiment, the delta-endotoxin is targeted to the chloroplast for expression. In this manner, where the delta-endotoxin is not directly inserted into the chloroplast, the expression cassette will additionally contain a nucleic acid encoding a transit peptide to direct the delta-endotoxin to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne et al. (1991) Plant Mol. Biol. Rep. 9:104-126; Clark et al. (1989) J. Biol. Chem. 264:17544-17550; Della-Cioppa et al. (1987) Plant Physiol. 84:965-968; Romer et al. (1993) Biochem. Biophys. Res. Commun. 196:1414-1421; and Shah et al. (1986) Science 233:478-481.

The delta-endotoxin gene to be targeted to the chloroplast may be optimized for expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the nucleic acids of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

**Plant Transformation**

Methods of the invention involve introducing a nucleotide construct into a plant. By "introducing" is intended to present to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not require that a particular method for introducing a nucleotide construct to a plant is used, only that the nucleotide construct gains access to the interior of at least one cell of the plant. Methods for introducing nucleotide constructs into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.
By "plant" is intended whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, propagules, embryos and progeny of the same. Plant cells can be differentiated or undifferentiated (e.g. callus, suspension culture cells, protoplasts, leaf cells, root cells, phloem cells, pollen).

"Transgenic plants" or "transformed plants" or "stably transformed" plants or cells or tissues refers to plants that have incorporated or integrated exogenous nucleic acid sequences or DNA fragments into the plant cell. These nucleic acid sequences include those that are exogenous, or not present in the untransformed plant cell, as well as those that may be endogenous, or present in the untransformed plant cell.

"Heterologous" generally refers to the nucleic acid sequences that are not endogenous to the cell or part of the native genome in which they are present, and have been added to the cell by infection, transfection, microinjection, electroporation, microprojection, or the like.

Transformation of plant cells can be accomplished by one of several techniques known in the art. The delta-endotoxin gene of the invention may be modified to obtain or enhance expression in plant cells. Typically a construct that expresses such a protein would contain a promoter to drive transcription of the gene, as well as a 3' untranslated region to allow transcription termination and polyadenylation. The organization of such constructs is well known in the art. In some instances, it may be useful to engineer the gene such that the resulting peptide is secreted, or otherwise targeted within the plant cell. For example, the gene can be engineered to contain a signal peptide to facilitate transfer of the peptide to the endoplasmic reticulum. It may also be preferable to engineer the plant expression cassette to contain an intron, such that mRNA processing of the intron is required for expression.

Typically this "plant expression cassette" will be inserted into a "plant transformation vector". This plant transformation vector may be comprised of one or more DNA vectors needed for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that are comprised of more than one contiguous DNA segment. These vectors are often referred to in the art as "binary vectors". Binary vectors as well as vectors with helper plasmids are most often used for Agrobacterium-mediated transformation, where the size and complexity of DNA segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules.
Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a "gene of interest" (a gene engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired). Also present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker gene and the delta-endotoxin are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from Agrobacterium to plant cells. This plasmid often contains the virulence functions (Vir genes) that allow infection of plant cells by Agrobacterium, and transfer of DNA by cleavage at border sequences and vir-mediated DNA transfer, as is understood in the art (Hellens and Mullineaux (2000) Trends in Plant Science 5:446-451). Several types of Agrobacterium strains (e.g. LBA4404, GV3101, EHA101, EHA105, etc.) can be used for plant transformation. The second plasmid vector is not necessary for transforming the plants by other methods such as microprojection, microinjection, electroporation, polyethylene glycol, etc.

In general, plant transformation methods involve transferring heterologous DNA into target plant cells (e.g. immature or mature embryos, suspension cultures, undifferentiated callus, protoplasts, etc.), followed by applying a maximum threshold level of appropriate selection (depending on the selectable marker gene) to recover the transformed plant cells from a group of untransformed cell mass. Explants are typically transferred to a fresh supply of the same medium and cultured routinely. Subsequently, the transformed cells are differentiated into shoots after placing on regeneration medium supplemented with a maximum threshold level of selecting agent. The shoots are then transferred to a selective rooting medium for recovering rooted shoot or plantlet. The transgenic plantlet then grows into a mature plant and produces fertile seeds (e.g. Hiei et al. (1994) The Plant Journal 6:271-282; Ishida et al. (1996) Nature Biotechnology 14:745-750). Explants are typically transferred to a fresh supply of the same medium and cultured routinely. A general description of the techniques and methods for generating transgenic plants are found in Ayres and Park (1994) Critical Reviews in Plant Science 13:219-239 and Bommineni and Jauhar (1997) Maydica 42: 107-120. Since the transformed material contains many cells;
both transformed and non-transformed cells are present in any piece of subjected
target callus or tissue or group of cells. The ability to kill non-transformed cells and
allow transformed cells to proliferate results in transformed plant cultures. Often, the
ability to remove non-transformed cells is a limitation to rapid recovery of
transformed plant cells and successful generation of transgenic plants.

Transformation protocols as well as protocols for introducing nucleotide
sequences into plants may vary depending on the type of plant or plant cell, i.e.,
monocot or dicot, targeted for transformation. Generation of transgenic plants may be
performed by one of several methods, including, but not limited to, microinjection,
electroporation, direct gene transfer, introduction of heterologous DNA by
Agrobacterium into plant cells (Agrobacterium-mQdiatQd transformation),
bombardment of plant cells with heterologous foreign DNA adhered to particles,
ballistic particle acceleration, aerosol beam transformation (U.S. Published
Application No. 20010026941; U.S. Patent No. 4,945,050; International Publication
No. WO 91/00915; U.S. Published Application No. 2002015066), Lecl
transformation, and various other non-particle direct-mediated methods to transfer
DNA.

Methods for transformation of chloroplasts are known in the art. See, for
example, Svab et al. (1990) Proc. Natl. Acad. Sci. USA 87:8526-8530; Svab and
EMBO J. 12:601-606. The method relies on particle gun delivery of DNA containing
a selectable marker and targeting of the DNA to the plastid genome through
homologous recombination. Additionally, plastid transformation can be
accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred
expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a
system has been reported in McBride et al. (1994) Proc. Natl. Acad. Sci. USA
91:7301-7305.

Following integration of heterologous foreign DNA into plant cells, one then
applies a maximum threshold level of appropriate selection in the medium to kill the
untransformed cells and separate and proliferate the putatively transformed cells that
survive from this selection treatment by transferring regularly to a fresh medium. By
continuous passage and challenge with appropriate selection, one identifies and
proliferates the cells that are transformed with the plasmid vector. Molecular and
biochemical methods can then be used to confirm the presence of the integrated heterologous gene of interest into the genome of the transgenic plant.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al. (1986) Plant Cell Reports 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a nucleotide construct of the invention, for example, an expression cassette of the invention, stably incorporated into their genome.

Evaluation of Plant Transformation

Following introduction of heterologous foreign DNA into plant cells, the transformation or integration of heterologous gene in the plant genome is confirmed by various methods such as analysis of nucleic acids, proteins and metabolites associated with the integrated gene.

PCR analysis is a rapid method to screen transformed cells, tissue or shoots for the presence of incorporated gene at the earlier stage before transplanting into the soil (Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). PCR is carried out using oligonucleotide primers specific to the gene of interest or Agrobacterium vector background, etc.

Plant transformation may be confirmed by Southern blot analysis of genomic DNA (Sambrook and Russell, 2001, supra). In general, total DNA is extracted from the transformant, digested with appropriate restriction enzymes, resolved in an agarose gel and transferred to a nitrocellulose or nylon membrane. The membrane or "blot" is then probed with, for example, radiolabeled 32P target DNA fragment to confirm the integration of introduced gene into the plant genome according to standard techniques (Sambrook and Russell, 2001, supra).

In Northern blot analysis, RNA is isolated from specific tissues of transformant, fractionated in a formaldehyde agarose gel, and blotted onto a nylon
filter according to standard procedures that are routinely used in the art (Sambrook and Russell, 2001, supra). Expression of RNA encoded by the delta-endotoxin is then tested by hybridizing the filter to a radioactive probe derived from a delta-endotoxin, by methods known in the art (Sambrook and Russell, 2001, supra).

Western blot, biochemical assays and the like may be carried out on the transgenic plants to confirm the presence of protein encoded by the delta-endotoxin gene by standard procedures (Sambrook and Russell, 2001, supra) using antibodies that bind to one or more epitopes present on the delta-endotoxin protein.

**Insecticidal Activity in Plants**

In another aspect of the invention, one may generate transgenic plants expressing a delta-endotoxin that has insecticidal activity. Methods described above by way of example may be utilized to generate transgenic plants, but the manner in which the transgenic plant cells are generated is not critical to this invention. Methods known or described in the art such as Agrobacterium-mediated transformation, biolistic transformation, and non-particle-mediated methods may be used at the discretion of the experimenter. Plants expressing a delta-endotoxin may be isolated by common methods described in the art, for example by transformation of callus, selection of transformed callus, and regeneration of fertile plants from such transgenic callus. In such process, one may use any gene as a selectable marker so long as its expression in plant cells confers ability to identify or select for transformed cells.

A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Other genes that encode a product involved in chloroplast metabolism may also be used as selectable markers. For example, genes that provide resistance to plant herbicides such as glyphosate, bromoxynil, or imidazolinone may find particular use. Such genes have been reported (Stalker et al. (1985) J. Biol. Chem. 263:6310-6314 (bromoxynil resistance nitrilase gene); and Sathasivan et al. (1990) Nucl. Acids Res. 18:2188 (AHAS imidazolinone resistance gene). Additionally, the genes disclosed herein are useful as markers to assess transformation of bacterial or plant cells.

Methods for detecting the presence of a transgene in a plant, plant organ (e.g., leaves, stems, roots, etc.), seed, plant cell, propagule, embryo or progeny of the same are well known in the art. In one embodiment, the presence of the transgene is detected by testing for insecticidal activity.
Fertile plants expressing a delta-endotoxin may be tested for insecticidal activity, and the plants showing optimal activity selected for further breeding. Methods are available in the art to assay for insect activity. Generally, the protein is mixed and used in feeding assays. See, for example Marrone et al. (1985) *J. of Economic Entomology* 78:290-293.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (maize), sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape, *Brassica* sp., alfalfa, rye, millet, safflower, peanuts, sweet potato, cassava, coffee, coconut, pineapple, citrus trees, cocoa, tea, banana, avocado, fig, guava, mango, olive, papaya, cashew, macadamia, almond, oats, vegetables, ornamentals, and conifers.

Vegetables include, but are not limited to, tomatoes, lettuce, green beans, lima beans, peas, and members of the genus *Curcuma* such as cucumber, cantaloupe, and musk melon. Ornamentals include, but are not limited to, azalea, hydrangea, hibiscus, roses, tulips, daffodils, petunias, carnation, poinsettia, and chrysanthemum. Preferably, plants of the present invention are crop plants (for example, maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, oilseed rape, etc.).

**Use in Insect Control**

General methods for employing strains comprising a nucleotide sequence of the present invention, or a variant thereof, in insect control or in engineering other organisms as insecticidal agents are known in the art. See, for example U.S. Patent No. 5,039,523 and EP 0480762A2.

The *Bacillus* strains containing a nucleotide sequence of the present invention, or a variant thereof, or the microorganisms that have been genetically altered to contain an insecticidal gene and protein may be used for protecting agricultural crops and products from insects. In one aspect of the invention, whole, i.e., unlysed, cells of a toxin (insecticide)-producing organism are treated with reagents that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target insect(s).
Alternatively, the insecticide is produced by introducing a delta-endotoxin gene into a cellular host. Expression of the delta-endotoxin gene results, directly or indirectly, in the intracellular production and maintenance of the insecticide. In one aspect of this invention, these cells are then treated under conditions that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target insect(s). The resulting product retains the toxicity of the toxin. These naturally encapsulated insecticides may then be formulated in accordance with conventional techniques for application to the environment hosting a target insect, e.g., soil, water, and foliage of plants. See, for example EPA 0192319, and the references cited therein. Alternatively, one may formulate the cells expressing a gene of this invention such as to allow application of the resulting material as an insecticide.

*Insecticidal compositions*

The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. These compounds can be fertilizers, weed killers, cryoprotectants, surfactants, detergents, insecticidal soaps, dormant oils, polymers, and/or time-release or biodegradable carrier formulations that permit long-term dosing of a target area following a single application of the formulation. They can also be selective herbicides, chemical insecticides, virucides, microbicides, amoebicides, pesticides, fungicides, bacteriocides, nematocides, molluscicides or mixtures of several of these preparations, if desired, together with further agriculturally acceptable carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g. natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders or fertilizers. Likewise the formulations may be prepared into edible "baits" or fashioned into pest "traps" to permit feeding or ingestion by a target pest of the insecticidal formulation.

Methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention that contains at least one of the insecticidal proteins produced by the bacterial strains of the present invention include leaf application, seed coating and soil application. The number of applications and
the rate of application depend on the intensity of infestation by the corresponding insect.

The composition may be formulated as a powder, dust, pellet, granule, spray, emulsion, colloid, solution, or such like, and may be prepared by such conventional means as desiccation, lyophilization, homogenation, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of cells comprising the polypeptide. In all such compositions that contain at least one such insecticidal polypeptide, the polypeptide may be present in a concentration of from about 1% to about 99% by weight.

Lepidopteran, coleopteran, or other insects may be killed or reduced in numbers in a given area by the methods of the invention, or may be prophylactically applied to an environmental area to prevent infestation by a susceptible insect. Preferably the insect ingests, or is contacted with, an insecticidally-effective amount of the polypeptide. By "insecticidally-effective amount" is intended an amount of the insecticide that is able to bring about death to at least one insect, or to noticeably reduce insect growth, feeding, or normal physiological development. This amount will vary depending on such factors as, for example, the specific target insects to be controlled, the specific environment, location, plant, crop, or agricultural site to be treated, the environmental conditions, and the method, rate, concentration, stability, and quantity of application of the insecticidally-effective polypeptide composition. The formulations may also vary with respect to climatic conditions, environmental considerations, and/or frequency of application and/or severity of insect infestation.

The insecticide compositions described may be made by formulating either the bacterial cell, crystal and/or spore suspension, or isolated protein component with the desired agriculturally-acceptable carrier. The compositions may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, desiccated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well
known to those skilled in insecticide formulation. The formulations may be mixed
with one or more solid or liquid adjuvants and prepared by various means, e.g., by
homogeneously mixing, blending and/or grinding the insecticidal composition with
suitable adjuvants using conventional formulation techniques. Suitable formulations
and application methods are described in U.S. Patent No. 6,468,523, herein
incorporated by reference.

"Pest" includes but is not limited to, insects, fungi, bacteria, nematodes, mites,
ticks, and the like. Insect pests include insects selected from the orders Coleoptera,
Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera,
Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera,
Trichoptera, etc., particularly Coleoptera, Lepidoptera, and Diptera.

The order Coleoptera includes the suborders Adephaga and Polyphaga. Suborder Adephaga includes the superfamilies Caraboidea and Gyrinoidea, while
suborder Polyphaga includes the superfamilies Hydrophiloidae, Staphylinoidae,
Cantharoidea, Cleroidea, Elateroidea, Dascilloidea, Dryopoidea, Byrrhoidea,
Cucujoidea, Meloidea, Mordelloidea, Tenebrionoidea, Bostrichoidea, Scarabaeoidea,
Cerambycoidea, Chrysomeloidea, and Curculionoidea. Superfamily Caraboidea
includes the families Cicindelidae, Carabidae, and Dytiscidae. Superfamily
Gyrinoidea includes the family Gyrinidae. Superfamily Hydrophiloidae includes the
family Hydrophilidae. Superfamily Staphylinoidae includes the families Silphidae
and Staphylinidae. Superfamily Cantharoidea includes the families Cantharidae and
Lampyridae. Superfamily Cleroidea includes the families Cleridae and Dermentidae.
Superfamily Elateroidea includes the families Elateridae and Buprestidae.
Superfamily Cucujoidea includes the family Coccinellidae. Superfamily Meloidea
includes the family Meloidae. Superfamily Tenebrionoidea includes the family
Tenebrionidae. Superfamily Scarabaeoidea includes the families Passalidae and
Scarabaeidae. Superfamily Cerambycoidea includes the family Cerambycidae.
Superfamily Chrysomeloidea includes the family Chrysomelidae. Superfamily
Curculionoidea includes the families Curculionidae and Scolytidae.

The order Diptera includes the Suborders Nematocera, Brachycera, and
Cyclorrhapha. Suborder Nematocera includes the families Tipulidae, Psychodidae,
Culicidae, Ceratopogonidae, Chironomidae, Simuliidae, Bibionidae, and
Cecidomyiidae. Suborder Brachycera includes the families Stratiomyidae,
Tabanidae, Therevidae, Asilidae, Mydidae, Bombyliidae, and Dolichopodidae.
Suborder Cyclorrhapha includes the Divisions Aschiza and Aschiza. Division Aschiza includes the families Phoridae, Syrphidae, and Conopidae. Division Aschiza includes the Sections Acalyptratae and Calyptratae. Section Acalyptratae includes the families Otitidae, Tephritidae, Agromyzidae, and Drosophilidae. Section Calyptratae includes the families Hippoboscidae, Oestridae, Tachinidae, Anthomyiidae, Muscidae, Calliphoridae, and Sarcophagidae.

The order Lepidoptera includes the families Papilionidae, Pieridae, Lycaenidae, Nymphalidae, Satyridae, Hesperiidae, Sphingidae, Saturniidae, Geometridae, Arctiidae, Noctuidae, Lymantriidae, Sesiidae, Crambidae, and Tineidae.

Nematodes include parasitic nematodes such as root-knot, cyst, and lesion nematodes, including Heterodera spp., Meloidogyne spp., and Globodera spp.; particularly members of the cyst nematodes, including, but not limited to, Heterodera glycines (soybean cyst nematode); Heterodera schachtii (beet cyst nematode); Heterodera avenae (cereal cyst nematode); and Globodera rostochiensis and Globodera pallida (potato cyst nematodes). Lesion nematodes include Pratylenchus spp.

Insect pests of the invention for the major crops include: Maize: Ostrinia nubilalis, European corn borer; Agrotis ipsilon, black cutworm; Helicoverpa zea, corn earworm; Spodoptera frugiperda, fall armyworm; Diatraea grandiosella, southwestern corn borer; Elasmopalpus lignosellus, lesser cornstalk borer; Diatraea saccharalis, sugarcane borer; Diabrotica virgifera, western corn rootworm; Diabrotica longicorinis barberi, northern corn rootworm; Diabrotica undecimpunctata howardi, southern corn rootworm; Melanotus spp., wireworms; Cyclocephala borealis, northern masked chafer (white grub); Cyclocephala immaculata, southern masked chafer (white grub); Popillia japonica, Japanese beetle; Chaetocnema pulicaria, corn flea beetle; Sphenophorus maidis, maize billbug; Rhopalosiphum maidis, corn leaf aphid; Anuraphis maidiradicis, corn root aphid; Blissus leucopterus leucopterus, chinch bug; Melanoplus femurrubrum, redlegged grasshopper; Melanoplus sanguinipes, migratory grasshopper; Hylemya platura, seedcorn maggot; Agromyza parvicornis, corn blot leafminer; Anaphothrips obscurus, grass thrips; Solenopsis milesta, thief ant; Tetranychus urticae, twospotted spider mite; Sorghum: Chilo partellus, sorghum borer; Spodoptera frugiperda, fall armyworm; Helicoverpa zea, corn earworm; Elasmopalpus lignosellus, lesser cornstalk borer; Feltia
subterranea, granulate cutworm; Phyllophaga crinita, white grub; Eleodes, Conoderus, and Aeolus spp., wireworms; Oulema melanopus, cereal leaf beetle; Chaetocnema pulicaria, corn flea beetle; Sphenophorus maidis, maize billbug; Rhopalosipham maidis, corn leaf aphid; Siphaja, yellow sugarcane aphid; Blissus leucopterus leucopterus, chinch bug; Contarinia sorghicola, sorghum midge; Tetranychus cinnabarinus, carmine spider mite; Tetranychus urticae, twospotted spider mite; Wheat: Pseudaletia unipunctata, army worm; Spodoptera frugipen, fall armyworm; Elasmopalpus lignosellus, lesser cornstalk borer; Agrotis orthogonia, western cutworm; Elasmopalpus lignosellus, lesser cornstalk borer; Oulema melanopus, cereal leaf beetle; Hypera punctata, clover leaf weevil; Diabrotica undecimpunctata howardi, southern corn rootworm; Russian wheat aphid; Schizaphis graminum, greenbug; Macrosiphum avenae, English grain aphid; Melanoplus femurrubrum, redlegged grasshopper; Melanoplus differentialis, differential grasshopper; Melanoplus sanguinipes, migratory grasshopper; Mayetiola destructor, Hessian fly; Sitodiplosis mosellana, wheat midge; Meromyza americana, wheat stem maggot; Hylemya coarctata, wheat bulb fly; Frankliniella fusca, tobacco thrips; Cephus cinctus, wheat stem sawfly; Aceria tulipae, wheat curl mite; Sunflower: Suleima helianthana, sunflower bud moth; Homoeosoma electellum, sunflower moth; zygogramma exclamationis, sunflower beetle; Bothyris gibbosus, carrot beetle; Neolasioptera murtfeldtiana, sunflower seed midge; Cotton: Heliothis virescens, cotton budworm; Helicoverpa zea, cotton bollworm; Spodoptera exigua, beet armyworm; Pectinophora gossypiella, pink bollworm; Anthonomus grandis, boll weevil; Aphis gossypii, cotton aphid; Pseudatomoscelis seriatus, cotton fleahopper; Trialeurodes abutilonea, bandedwinged whitefly; Lygus lineolaris, tarnished plant bug; Melanoplus femurrubrum, redlegged grasshopper; Melanoplus differentialis, differential grasshopper; Thrips tabaci, onion thrips; Frankliniella fusca, tobacco thrips; Tetranychus cinnabarinus, carmine spider mite; Tetranychus urticae, twospotted spider mite; Rice: Diatracea saccharalis, sugarcane borer; Spodoptera frugipera, fall armyworm; Helicoverpa zea, corn earworm; Colaspis brunnea, grape colaspis; Lissorhoptrus oryzophilus, rice water weevil; Sitophilus oryzae, rice weevil; Nephotettix nigropictus, rice leafhopper; Blissus leucopterus leucopterus, chinch bug; Acrosternum hilare, green stink bug; Soybean: Pseudoplusia includens, soybean looper; Anticarsia gemmatalis, velvetbean caterpillar; Plathypena scabra, green cloverworm; Ostrinia nubilalis, European corn borer; Agrotis ipsilon, black cutworm.
Spodoptera exigua, beet armyworm; Heliothis virescens, cotton budworm; Helicoverpa zea, cotton bollworm; Epilachna varivestis, Mexican bean beetle; Myzus persicae, green peach aphid; Empoasca fabae, potato leafhopper; Acrosternum hilare, green stink bug; Melanoplus femur rubrum, redlegged grasshopper; Melanoplus differentialis, differential grasshopper; Hylemya platura, seedcorn maggot; Sericothrips variabilis, soybean thrips; Thrips tabaci, onion thrips; Tetranychus turkestanii, strawberry spider mite; Tetranychus urticae, twospotted spider mite; Barley: Ostrinia nubilalis, European corn borer; Agrotis ipsilon, black cutworm; Schizaphis graminum, greenbug; Blissus leucopterus leucopterus, chinch bug; Acrosternum hilare, green stink bug; Euschistus servus, brown stink bug; Delia platura, seedcorn maggot; Mayetiola destructor, Hessian fly; Petrobia latens, brown wheat mite; Oil Seed Rape: Brevicoryne brassicae, cabbage aphid; Phyllotreta cruciferae, Flea beetle; Mamestra brassicae, Bertha armyworm; Plutella xylostella, Diamond-back moth; Delia ssp., Root maggots.

Methods for increasing plant yield.

Methods for increasing plant yield are provided. The methods comprise introducing into a plant or plant cell a polynucleotide comprising an insecticidal sequence disclosed herein. As defined herein, the "yield" of the plant refers to the quality and/or quantity of biomass produced by the plant. By "biomass" is intended any measured plant product. An increase in biomass production is any improvement in the yield of the measured plant product. Increasing plant yield has several commercial applications. For example, increasing plant leaf biomass may increase the yield of leafy vegetables for human or animal consumption. Additionally, increasing leaf biomass can be used to increase production of plant-derived pharmaceutical or industrial products. An increase in yield can comprise any statistically significant increase including, but not limited to, at least a 1% increase, at least a 3% increase, at least a 5% increase, at least a 10% increase, at least a 20% increase, at least a 30%, at least a 50%, at least a 70%, at least a 100% or a greater increase in yield compared to a plant not expressing the insecticidal sequence.

In specific methods, plant yield is increased as a result of improved insect resistance of a plant expressing an insecticidal protein disclosed herein. Expression of the insecticidal protein results in a reduced ability of an insect to infest or feed on the plant, thus improving plant yield.
The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1. Discovery of a novel toxin gene Axmil 15 from the *Bacillus thuringiensis* strain ATX12983.

The complete gene sequence was identified from the selected strain via the MiDAS genomics approach as follows:

- Preparation of extrachromosomal DNA from the strain.
  Extrachromosomal DNA contains a mixture of some or all of the following: plasmids of various size; phage chromosomes; genomic DNA fragments not separated by the purification protocol; other uncharacterized extrachromosomal molecules.

- Mechanical or enzymatic shearing of the extrachromosomal DNA to generate size-distributed fragments.
- Sequencing of the fragmented DNA
- Identification of putative toxin genes via homology and/or other computational analyses.

- When required, sequence finishing of the gene of interest by one of several PCR or cloning strategies (e.g. TAIL-PCR).

The novel gene is referred to herein as *axmi-115* (SEQ ID NO:3), and the encoded amino acid referred to as AXMI-1 15 (SEQ ID NO:6). Synthetic nucleotide sequences encoding AXMI-1 15 is set forth in SEQ ID NO: 15 and 16.

Gene and Protein Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene length, DNA base pairs</td>
<td>2409</td>
</tr>
<tr>
<td>Protein length, amino acid residues</td>
<td>803</td>
</tr>
<tr>
<td>Estimated protein molecular weight, Da</td>
<td>90877</td>
</tr>
</tbody>
</table>

Known homologs and approximate percent identity:

- Vip3Afl - 70.7%
- Axmi005 - 70.4%
AxmiO26 - 70.4%
Vip3Aa7 - 70.1

Example 2. Novel insecticidal protein AXMI-005 from the *Bacillus thuringiensis*
strain ATX1 3002

The AXMI-005 insecticidal gene was identified from the strain ATX1 3002 using the MiDAS approach as described in U.S. Patent Publication No. 20040014091, which is herein incorporated by reference in its entirety, using the following steps:

Steps taken in the current strategy to gene discovery:

Step 1: Culture from the strain was grown in large quantities. The plasmid DNA was then separated from the chromosomal DNA by a cesium chloride gradient spun in an ultracentrifuge. The purified plasmid DNA was then nebulized to a 5-10kb size range appropriate for coverage of an average sized coding region. The fragment ends were polished then ligated overnight into a vector cut with a restriction enzyme producing blunt ends.

Step 3: Once the shotgun library quality was checked and confirmed, colonies were grown, prepped and sequenced in a 96-well format. The library plates were end sequenced off of the vector backbone for initial screening.

Step 5: All of the reads were compiled into an assembly project and aligned together to form contigs. These contigs, along with any individual read that may not have been added to a contig, were analyzed using BLAST, using a batch format, against an internal database made up of all classes of known delta-endotoxin genes. Any contigs or individual reads that pulled up any homology to a known gene were analyzed further by selecting a single clone from the library that covered the entire hypothesized coding region.

Step 6: The individual clone covering the area of interest was then walked over read by read by designing primers to extend the sequence. This was done until both end reads of the clone were joined and the coverage was at least 2X. The completed contig of the single clone was then analyzed using BLAST (both blastn and blastx) against a public database of all known insecticidal genes. Hits from both searches were then pulled from an internal database of all the genes (clipped to coding sequence only) and aligned with the completed library clone sequence to determine the percentage of divergence from the known gene.
A novel gene, referred to herein as axmi-005 (SEQ ID NO:1), and the encoded amino acid referred to as AXMI-005 (SEQ ID NO:4) was identified by this approach. Searching of public sequence databases, including the GENBANK® databases, showed that AXMI-005 is a unique protein, that has highest homology (94.9%) to the vip3Aa insecticidal protein (GenePept ID L48841).

A synthetic sequence encoding the AXMI-005 protein was designed and termed optaxmi-005. The nucleotide sequence is set forth in SEQ ID NO:7, and encodes the amino acid sequence set forth in SEQ ID NO:9 (with the addition of a C-terminal histidine tag). The optaxmi-005 gene disclosed herein can be used with or without the C-terminal histidine tag.

Example 3. Discovery of a novel toxin gene Axmi-113 from the Bacillus thuringiensis strain ATX12987.

The complete gene sequence was identified from the selected strain via the MiDAS genomics approach as follows:

- Preparation of extrachromosomal DNA from the strain. Extrachromosomal DNA contains a mixture of some or all of the following: plasmids of various size; phage chromosomes; genomic DNA fragments not separated by the purification protocol; other uncharacterized extrachromosomal molecules.
- Mechanical or enzymatic shearing of the extrachromosomal DNA to generate size-distributed fragments.
- Sequencing of the fragmented DNA
- Identification of putative toxin genes via homology and/or other computational analyses.
- When required, sequence finishing of the gene of interest by one of several PCR or cloning strategies (e.g. TAIL-PCR).

The novel gene is referred to herein as axmi-113 (SEQ ID NO:2), and the encoded amino acid referred to as AXMI-113 (SEQ ID NO:5).

Gene and Protein Characteristics

| Gene length, DNA base pairs: | 2385 |
Protein length, amino acid residues: 795
Estimated protein molecular weight, Da: 89475

Known homologs and approximate percent identity:

Vip3Ah - 99%
Vip3Aal8 - 79.8%
Axmi005 - 79%

A synthetic sequence encoding the AXMI-1 13 protein was designed and termed optaxmi-113. The nucleotide sequence is set forth in SEQ ID NO:8, and encodes the amino acid sequence set forth in SEQ ID NO:4 or 14 (with the addition of a C-terminal histidine tag). The optaxmi-113 gene disclosed herein can be used with or without the C-terminal histidine tag.

Example 4. Discovery of novel toxin genes Axmi-163 and Axmi-184 from the Bacillus thuringiensis strain ATX 14775.

The complete gene sequence for each was identified from the selected strain via the MiDAS genomics approach as follows:

- Preparation of extrachromosomal DNA from the strain.
  Extrachromosomal DNA contains a mixture of some or all of the following: plasmids of various size; phage chromosomes; genomic DNA fragments not separated by the purification protocol; other uncharacterized extrachromosomal molecules.
- Mechanical or enzymatic shearing of the extrachromosomal DNA to generate size-distributed fragments.
- Sequencing of the fragmented DNA
- Identification of putative toxin genes via homology and/or other computational analyses.
- When required, sequence finishing of the gene of interest by one of several PCR or cloning strategies (e.g. TAIL-PCR).

The novel gene referred to herein as axmi-163 is set forth in SEQ ID NO:6, and the encoded amino acid referred to as AXMI-163 is set forth in SEQ ID NO: 13.
Gene and Protein Characteristics

Gene length, DNA base pairs: 2370
Protein length, amino acid residues: 790
Estimated protein molecular weight, Da: 88,700

5 Known homologs and approximate percent identity:

SEQ ID NO:17 from U.S. Patent 7,129,212 - 98%
Axmi005 - 78%

The novel gene referred to herein as axmi-184 is set forth in SEQ ID NO: 12, and the
encoded amino acid referred to as AXMI-184 is set forth in SEQ ID NO: 14.

Synthetic nucleotide sequences encoding AXMI-184 are set forth in SEQ ID NO: 17
and 18.

Gene and Protein Characteristics

Gene length, DNA base pairs: 2370
Protein length, amino acid residues: 790
Estimated protein molecular weight, Da: 88,300

Known homologs and approximate percent identity:

Vip3Afl -93%
Axmi005 - 86%

Example 5. Construction of synthetic sequences

In one aspect of the invention, synthetic axmi sequences are generated. These
synthetic sequences have an altered DNA sequence relative to the parent axmi
sequence, and encode a protein that is collinear with the parent AXMI protein to
which it corresponds, but lacks the C-terminal "crystal domain" present in many
delta-endotoxin proteins.

In another aspect of the invention, modified versions of synthetic genes are
designed such that the resulting peptide is targeted to a plant organelle, such as the
endoplasmic reticulum or the apoplast. Peptide sequences known to result in targeting
of fusion proteins to plant organelles are known in the art. For example, the N-
termsinal region of the acid phosphatase gene from the White Lupin Lupinus albus
(Genebank ID GI: 14276838; Miller et al. (2001) Plant Physiology 127: 594-606) is
known in the art to result in endoplasmic reticulum targeting of heterologous proteins.
If the resulting fusion protein also contains an endoplasmic retention sequence comprising the peptide N-terminus-lysine-aspartic acid-glutamic acid-leucine (i.e. the "KDEL" motif (SEQ ID NO: 19) at the C-terminus, the fusion protein will be targeted to the endoplasmic reticulum. If the fusion protein lacks an endoplasmic reticulum targeting sequence at the C-terminus, the protein will be targeted to the endoplasmic reticulum, but will ultimately be sequestered in the apoplast.

Example 6. Expression in Bacillus

As an example of the expression of the genes and proteins of the invention in Bacillus species, the insecticidal gene disclosed herein is amplified by PCR, and the PCR product is cloned into the Bacillus expression vector pAX916, or another suitable vector, by methods well known in the art. The resulting Bacillus strain, containing the vector with axmi gene is cultured on a conventional growth media, such as CYS media (10 g/l Bacto-casitone; 3 g/l yeast extract; 6 g/l KH₂PO₄; 14 g/l K₂HPO₄; 0.5 mM MgSO₄; 0.05 mM MnCl₂; 0.05 mM FeSO₄), until sporulation is evident by microscopic examination. Samples are prepared and tested for activity in bioassays.

Example 7. Expression in E. coli

As an example of a method of expression of the genes and proteins of the invention in E. coli based systems, the complete ORF of each axmi gene is cloned into an E. coli expression vector based on pRSFlb. The resulting clones are confirmed by restriction analysis and finally, by complete sequencing of the cloned gene.

For expression in E. coli, BL21 *DE3 is transformed with the vector expressing the axmi gene. Single colonies are inoculated in LB supplemented with kanamycin and grown overnight at 37°C. The following day, fresh medium is inoculated in duplicate with 1% of overnight culture and grown at 37°C to logarithmic phase. Subsequently, cultures are induced with ImM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 37°C or overnight at 20°C. Each cell pellet is suspended in 5OmM sodium carbonate buffer, pH 10.5 supplemented with ImM DTT dithiothreitol and sonicated. Samples are prepared and tested for activity in bioassays.
Example 8. Expression of AXMI-115, AXMI-113, and AXMI-005 in E. coli

*E. coli* clones were generated that contained DNA segments containing the complete open reading frame as well as a portion of the DNA region naturally occurring upstream and adjacent to each gene. This DNA segment for each of *axmi-113* (SEQ ID:5), *axmi-115* (SEQ ID NO:6) or *axmi-005* (SEQ ID NO:4) was amplified and cloned into the vector pAX916, to yield the clones pAX5463, pAX5464 and pAX5465 respectively. The resulting clones were confirmed by restriction analysis and by complete sequencing of the cloned fragments.

*E. coli* cells were transformed with each of the clones pAX5463, pAX5464 and pAX5465.

*axmi005*, *axmill3* and *axmill5* genes that had their codons optimized for expression in corn, and had a C-terminal his6-tag added, were also expressed from *E. coli* expression vector utilizing T7 promoter. In addition, constructs were also generated that expressed N-terminal hisβ-tagged or untagged versions of *optaxmi005* (pAX5475, pAX5478) and *optaxmill5* (pAX5476, pAX5477).

Single *E. coli* colonies of the *axmi-115*, *axmi-113*, and *axmi-005* expressing clones were then grown overnight at 37°C in LB medium. The following day, fresh medium was inoculated in duplicate with 1% of overnight culture and grown at 37°C to logarithmic phase. Subsequently, cultures were induced with ImM IPTG overnight at 20°C. The resulting cells were collected by centrifugation, and suspended in either 50mM sodium carbonate buffer, pH 10.5 supplemented with ImM DTT or 50mM Tris Cl buffer, pH 8 with ImM DTT prior to sonication. SDS-PAGE analysis showed expression of a ~ 90kD protein in all samples.

Example 9. Insect Bioassays of *E. coli* expressed proteins

Soluble extracts containing AXMI-005, AXMI-113, or AXMI-115 were tested in insect assays with appropriate controls. Twenty four well tissue culture plates (Corning) were filled with 1 ml of multi-species diet (Bio-Serv) and allowed to solidify. Once solidified, 40 µl of protein sample was placed on the diet surface of each well and allowed to soak in/dry at room temperature. Depending upon the experiment, either egg masses or neonate larvae were placed in each well. Plates were sealed with gas-permeable membranes (Research Products International) and incubated at 25 °C and 90% relative humidity. After five or seven days, samples were scored visually compared to a buffer only or non-transformed extract control.
Strong activity of AXMI-005 extracts was observed on *Helicoverpa zea* (HZ), *Heliothis virescens* (HV), Fall Armyworm (FAW), Black cutworm (BCW), Sugarcane borer (SCB), and Velvet Bean caterpillar (VBC). AXMI-005 also showed activity on Southwestern Corn Borer (SWCB).

Strong activity of AXMI-1 15 extracts was observed on *Heliothis virescens*, Fall Armyworm, Black cutworm, and Velvet Bean caterpillar. AXMI-1 15 also exhibited activity on the European Corn Borer (ECB), SCB, SWCB, and Diamondback moth (DBM). Activity of AXMI-1 15 on *Helicoverpa zea* (HZ) was less pronounced than for the other insects tested, but was still significant.

Activity of each of the AXMI-005 and AXMI-1 15 extracts was scored, and assigned a number from 1 to 5 based on relative activity in the assays. A summary of the scores in a particular assay is shown in Table 1.

AXMI-005 showed some activity on SWCB (score of 2) and high levels of activity on HZ, HV, FAW, BCW and VBC (scores of 4 to 5). AXMI-1 15 showed high levels of activity on SWCB (80% mortality), ECB, FAW and VBC (scores of 4 to 5) and lesser activity on HZ and HV. AXMI-1 13 also showed high activity on SWCB (score of 4 with 20% mortality) and on SCB. No activity was seen on the other insects tested.

Table 1. Insecticidal activity of AXMI-115, AXMI-113, and AXMI-005 *

<table>
<thead>
<tr>
<th></th>
<th>Axmi115 (pH 10.5)</th>
<th>Axmi115 (pH 8)</th>
<th>Axmi005 (pH 10.5)</th>
<th>Axmi005 (pH 8)</th>
<th>Axmi113 (pH 10.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hz</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>ECB live infest</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hv</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4/5</td>
<td>0</td>
</tr>
<tr>
<td>FAW</td>
<td>4</td>
<td>2</td>
<td>4/5</td>
<td>4/5</td>
<td>0</td>
</tr>
<tr>
<td>BCW</td>
<td>0</td>
<td>0</td>
<td>3/4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>VBC</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4/5</td>
<td>0</td>
</tr>
<tr>
<td>SWCB</td>
<td>4; 80% mortality</td>
<td>3; 50% mortality</td>
<td>2</td>
<td>ND</td>
<td>4; 20% mortality</td>
</tr>
<tr>
<td>SCB</td>
<td>ND</td>
<td>3; 25% mortality</td>
<td>ND</td>
<td>4; 100% mortality</td>
<td>3; 50% mortality</td>
</tr>
<tr>
<td>Score</td>
<td>Definition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>------------------------------------------------</td>
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<td></td>
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<td>0</td>
<td>No Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Slight, non-uniform stunt</td>
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<td></td>
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</tr>
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<td>2</td>
<td>Non-uniform stunt</td>
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<td>3</td>
<td>Uniform stunt</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>Uniform stunt with mortality (expressed as a percentage)</td>
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<tr>
<td>5</td>
<td>Uniform stunt with 100% mortality</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Example 10. Bioassay of Axmil84

Gene Expression and Purification

- The DNA region encoding the toxin domain of Axmil 84 was cloned into an E. coli expression vector pMAL-C4x behind the malE gene coding for Maltose binding protein (MBP). This in-frame fusion resulted in MBP-AxmiO84 fusion protein expression in E. coli.

- For expression in E. coli, BL21 *DE3 was transformed with individual plasmids. Single colony was inoculated in LB supplemented with carbenicillin and glucose, and grown overnight at 37°C. The following day, fresh medium was inoculated with 1% of overnight culture and grown at 37°C to logarithmic phase. Subsequently, cultures were induced with 0.3mM IPTG for overnight at 20°C. Each cell pellet was suspended in 20mM Tris-Cl buffer, pH 7.4 +200mM NaCl+1mM DTT+ protease inhibitors and sonicated. Analysis by SDS-PAGE confirmed expression of fusion proteins.

- Total cell free extracts were run over amylose column attached to FPLC for affinity purification of MBP-AXMI 184 fusion proteins. Bound fusion protein was eluted from the resin with 10mM maltose solution. Purified fusion proteins were then cleaved with either Factor Xa or trypsin to remove the amino terminal MBP tag from the AXMI 84 protein. Cleavage and solubility of the proteins was determined by SDS-PAGE.

- Cleaved proteins were tested in insect assays with appropriate controls. A 5- day read of the plates showed following activities of AXMI-184 against Diamondback moth.

* = represented as stunt and mortality percent where stunting is scored according to the following scale:

<table>
<thead>
<tr>
<th>DBM</th>
<th>ND</th>
<th>2/3</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
</table>
Example 11. Domain swapping

*axmi005, axmill3* and *axmill5* genes that had their codons optimized for expression in corn were used in this example. Plasmids expressing untagged versions of *optaxmi005* (pAX5478), *optaxmilB* (pAX5493) and *optaxmill5* (pAX5477) were used to design DNA swap constructs as describe here.

AXMI-005, AXMI-1 13 and AXMI-1 15 have significant sequence identity/similarity in their N-terminal 2/3\textsuperscript{rd} region. The remaining 1/3\textsuperscript{rd} region in their C-termini (CT) shows substantial sequence divergence as seen in the protein sequence alignment provided as Figures 1A and IB.

The protein region of AXMI-1 13 between the forward and reverse arrows shown in Figure 1 was replaced with the corresponding fragment of either AXMI-005 (to give pAX5492) or AXMI-1 15 (pAX5494).

For expression in *E. coli*, BL21\*DE3 was transformed with individual constructs. A single colony was inoculated in LB supplemented with kanamycin and grown overnight at 37\textdegree C. The following day, fresh medium was inoculated in duplicate with 1\% of overnight culture and grown at 37\textdegree C to logarithmic phase. Subsequently, cultures were induced with ImM IPTG overnight at 20\textdegree C. Cell pellet was suspended in 50mM sodium carbonate buffer, pH 10.5 supplemented with ImM DTT, and sonicated. Analysis by SDS-PAGE showed extremely good soluble expression of all proteins.

Filter sterilized, soluble extracts expressing OptAxmi005, 113, 115, Optaxmil 13+CT of Optaxmi005 and Optaxmil 13+CT of Optaxmil 15 were tested in insect assays with appropriate controls. As shown in Example 9, AXMI-1 13 showed high activity on SWCB (25\% mortality). It showed an additional activity on SCB (50\% mortality).

Also as shown in Example 9, AXMI-005 showed activity on SWCB, Hz, Hv, FAW, BCW and VBC. It showed an additional activity on SCB (25\% mortality). AXMI-1 15 was also found to have some activity on SCB.

The fusion of AXMI-1 13+CT of AXMI-005 showed all of the insect activities seen with AXMI-005. In other words, replacement of the C-terminal fragment of AXMI-1 13 with that of AXMI-005 bestowed upon it the insect activities that were otherwise missing in its naturally occurring form.
Additional toxin protein sequences can be generated by swapping domains from one protein into another. For example, one or more of the AXMI-005 domains shown in Figure 2 are introduced into AXMI-1 15. The domains are introduced using the sense ("s") and antisense ("a") oligonucleotides shown in Table 2. The portion of the axmi-005 sequence that is being introduced into the axmi-115 sequence is shown in bold print. The flanking sequences in each oligonucleotide are axmi-115 sequences that are used for annealing the oligonucleotides to the axmi-115 template. The number following the term "sub" in each primer name corresponds to the numbered boxes in Figure 2. Similar oligonucleotides can be designed to swap domains between multiple sequences, for example, between the AXMI-005, AXMI-1 13, AXMI-1 15, AXMI-163 and AXMI-184 sequences described herein.

Table 2.

<table>
<thead>
<tr>
<th>Oligonucleotide primer</th>
<th>Sequence</th>
<th>SEQ ID NO:</th>
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<tbody>
<tr>
<td>axmi115sub1 s</td>
<td>AAC ACC GGC GGC GTC AAT GGA ACA AGG GCG CTC TTC ACC CA</td>
<td>20</td>
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<tr>
<td>axmi115sub1 a</td>
<td>TGG GTG AAG AGC GCC CTT GTT CCA TTG ACG CCG CCG GTG TT</td>
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<tr>
<td>axmi115sub10 s</td>
<td>GCC CGG AGC TCA TCA ATG TCA ACA ACT GGA TCA GAA CTG GCA CCA CCT ACA TCA C</td>
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<td>axmi115sub10 a</td>
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<tr>
<td>axmi115sub11 s</td>
<td>ATG ATT GGG AGA GGT TCG GAA GCA CCC ACA TCA GCG GCA ATG AGC TGA GG</td>
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<td>axmi115sub11 a</td>
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<td>axmi115sub12 s</td>
<td>CTA CAT CAC CGG CAA TAC CTT GAC GCT CTA CCA AGG AGG AGG AGG CTA CTT CCG C</td>
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<tr>
<td>axmi115sub2 a</td>
<td>CGA TGA ACT GCC TGA AGC CTC CAT CCT TGT GAA CAT AGA GCC CCT TGC TTC</td>
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<tr>
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<td>axmi115sub6 s</td>
<td>GAG GAG TTC CAA ACA ATT ACC AAG AGG TTC ACC ACC GCC ACA GAT TTG AGC CAG ACC</td>
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</tbody>
</table>
Example 12. Additional Assays for Pesticidal Activity

The ability of an insecticidal protein to act as a pesticide upon a pest is often assessed in a number of ways. One way well known in the art is to perform a feeding assay. In such a feeding assay, one exposes the pest to a sample containing either compounds to be tested, or control samples. Often this is performed by placing the material to be tested, or a suitable dilution of such material, onto a material that the pest will ingest, such as an artificial diet. The material to be tested may be in a liquid, solid, or slurry form. The material to be tested may be placed upon the surface and then allowed to dry or incorporate into the diet. Alternatively, the material to be tested may be mixed with a molten artificial diet, then dispensed into the assay chamber. The assay chamber may be, for example, a cup, a dish, or a well of a microtiter plate.

Assays for sucking pests (for example aphids) may involve separating the test material from the insect by a partition, ideally a portion that can be pierced by the sucking mouth parts of the sucking insect, to allow ingestion of the test material. Often the test material is mixed with a feeding stimulant, such as sucrose, to promote ingestion of the test compound.

Other types of assays can include microinjection of the test material into the mouth, or gut of the pest, as well as development of transgenic plants, followed by test of the ability of the pest to feed upon the transgenic plant. Plant testing may involve isolation of the plant parts normally consumed, for example, small cages attached to a leaf, or isolation of entire plants in cages containing insects.

Other methods and approaches to assay pests are known in the art, and can be found, for example in Robertson, J. L. & H. K. Preisler. 1992. *Pesticide bioassays with arthropods*. CRC, Boca Raton, FL. Alternatively, assays are commonly described in the journals "Arthropod Management Tests" and "Journal of Economic Entomology" or by discussion with members of the Entomological Society of America (ESA).

Example 13. Vectoring of the Insecticidal genes of the invention for Plant Expression

Each of the coding regions of the genes of the invention is connected independently with appropriate promoter and terminator sequences for expression in plants. Such sequences are well known in the art and may include the rice actin promoter or maize ubiquitin promoter for expression in monocots, the *Arabidopsis*
UBQ3 promoter or CaMV 35S promoter for expression in dicots, and the nos or pinII terminators. Techniques for producing and confirming promoter - gene - terminator constructs also are well known in the art.

Example 14. Transformation of the genes of the invention into Plant Cells by Agrobacterium-MQdiatQd Transformation

Ears are collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are used for transformation. Embryos are plated scutellum side-up on a suitable incubation media, and incubated overnight at 25°C in the dark. However, it is not necessary per se to incubate the embryos overnight. Embryos are contacted with an Agrobacterium strain containing the appropriate vectors for Ti plasmid mediated transfer for 5-10 min, and then plated onto co-cultivation media for 3 days (25°C in the dark). After co-cultivation, explants are transferred to recovery period media for five days (at 25°C in the dark). Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated as known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants.

Example 15. Transformation of Maize Cells with the insecticidal genes of the invention

Maize ears are collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are used for transformation. Embryos are plated scutellum side-up on a suitable incubation media, such as DN62A5S media (3.98 g/L N6 Salts; 1 mL/L (of 100x Stock) N6 Vitamins; 800 mg/L L-Asparagine; 100 mg/L Myo-inositol; 1.4 g/L L-Proline; 100 mg/L Casaminoacids; 50 g/L sucrose; 1 mL/L (of 1 mg/mL Stock) 2,4-D), and incubated overnight at 25°C in the dark.

The resulting explants are transferred to mesh squares (30-40 per plate), transferred onto osmotic media for 30-45 minutes, then transferred to a beaming plate (see, for example, PCT Publication No. WO/0138514 and U.S. Patent No. 5,240,842).
DNA constructs designed to express the genes of the invention in plant cells are accelerated into plant tissue using an aerosol beam accelerator, using conditions essentially as described in PCT Publication No. WO/0138514. After beaming, embryos are incubated for 30 min on osmotic media, then placed onto incubation media overnight at 25°C in the dark. To avoid unduly damaging beamed explants, they are incubated for at least 24 hours prior to transfer to recovery media. Embryos are then spread onto recovery period media, for 5 days, 25°C in the dark, then transferred to a selection media. Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated by methods known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants.

Materials

<table>
<thead>
<tr>
<th>Components</th>
<th>per liter</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chu'S N6 Basal Salt Mixture (Prod. No. C 416)</td>
<td>3.98 g/L</td>
<td>Phytotechnology Labs</td>
</tr>
<tr>
<td>Chu’s N6 Vitamin Solution (Prod. No. C 149)</td>
<td>1 mL/L (of 1000x Stock)</td>
<td>Phytotechnology Labs</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>800 mg/L</td>
<td>Phytotechnology Labs</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100 mg/L</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.4 g/L</td>
<td>Phytotechnology Labs</td>
</tr>
<tr>
<td>Casaminoacids</td>
<td>100 mg/L</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50 g/L</td>
<td>Phytotechnology Labs</td>
</tr>
<tr>
<td>2,4-D (Prod. No. D-7299)</td>
<td>1 mL/L (of 1 mg/mL Stock)</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Adjust the pH of the solution to pH to 5.8 with IN KOH/1N KCl, add Gelrite (Sigma) to 3g/L, and autoclave. After cooling to 50°C, add 2 ml/1 of a 5 mg/ml stock solution of Silver Nitrate (Phytotechnology Labs). This recipe yields about 20 plates.
All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.
THAT WHICH IS CLAIMED:

1. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

   a) the nucleotide sequence of any of SEQ ID NO:1, 2, 3, 11, or 12, or a complement thereof;

   b) a nucleotide sequence having at least 96% sequence identity to the nucleotide sequence of SEQ ID NO:1 or 12, wherein said nucleotide sequence encodes a protein having insecticidal activity, or a complement thereof;

   c) a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3, wherein said nucleotide sequence encodes a protein having insecticidal activity, or a complement thereof;

   d) a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14;

   e) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 96% sequence identity to the amino acid sequence of SEQ ID NO:4 or 14, wherein said amino acid sequence has insecticidal activity;

   f) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:6, wherein said amino acid sequence has insecticidal activity; and

   g) a nucleotide sequence encoding an insecticidal polypeptide that is a variant of SEQ ID NO:4, 5, 6, 13, or 14, wherein said variant is the result of one or more domain(s) of SEQ ID NO:4, 5, 6, 13, or 14 being exchanged with the corresponding domain(s) of SEQ ID NO:4, 5, 6, 13, or 14.

2. The isolated nucleic acid molecule of claim 1, wherein said nucleotide sequence is a synthetic sequence that has been designed for expression in a plant.

3. The nucleic acid molecule of claim 2, wherein said synthetic sequence is selected from any of SEQ ID NO:15, 16, 17, or 18.
4. An expression cassette comprising the nucleic acid molecule of claim 1.

5. The expression cassette of claim 4, further comprising a nucleic acid molecule encoding a heterologous polypeptide.

6. A plant or a bacterial host cell that contains the expression cassette of claim 4.

7. An isolated polypeptide with insecticidal activity, selected from the group consisting of:
   a) a polypeptide comprising the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14;
   b) a polypeptide comprising an amino acid sequence having at least 96% sequence identity to the amino acid sequence of SEQ ID NO:4 or 14, wherein said amino acid sequence has insecticidal activity;
   c) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:6, wherein said amino acid sequence has insecticidal activity;
   d) a polypeptide that is encoded by the nucleotide sequence of any of SEQ ID NO:1, 2, 3, 11, or 12;
   e) a polypeptide that is encoded by a nucleotide sequence that is at least 96% identical to the nucleotide sequence of SEQ ID NO:1 or 12, wherein said polypeptide has insecticidal activity;
   f) a polypeptide that is encoded by a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO:3, wherein said polypeptide has insecticidal activity; and
   g) a polypeptide that is a variant of SEQ ID NO:4, 5, 6, 13, or 14, wherein said variant is the result of one or more domain(s) of SEQ ID NO:4, 5, 6, 13, or 14 being exchanged with the corresponding domain(s) of SEQ ID NO:4, 5, 6, 13, or 14, wherein said polypeptide has insecticidal activity.

8. The polypeptide of claim 7 further comprising heterologous amino acid sequences.
9. An antibody that selectively binds to the polypeptide of claim 7.

10. A composition comprising the polypeptide of claim 7.

11. The composition of claim 10, wherein said composition is selected from the group consisting of a powder, dust, pellet, granule, spray, emulsion, colloid, and solution, and wherein optionally said composition is prepared by desiccation, lyophilization, homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of Bacillus thuringiensis cells.

12. A method for controlling or killing a lepidopteran or coleopteran pest population comprising contacting said population with an insecticidally-effective amount of the polypeptide of claim 7.

13. A method for producing a polypeptide with insecticidal activity, comprising culturing the host cell of claim 6 under conditions in which the nucleic acid molecule encoding the polypeptide is expressed.

14. A plant having stably incorporated into its genome a DNA construct comprising a nucleotide sequence that encodes a protein having insecticidal activity, wherein said nucleotide sequence is selected from the group consisting of:

a) the nucleotide sequence of any of SEQ ID NO: 1, 2, 3, 11, or 12;

b) a nucleotide sequence having at least 96% sequence identity to the nucleotide sequence of SEQ ID NO:1 or 12, wherein said nucleotide sequence encodes a protein having insecticidal activity;

c) a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3, wherein said nucleotide sequence encodes a protein having insecticidal activity;

d) a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14;

e) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 96% sequence identity to the amino acid
sequence of SEQ ID NO:4 or 14, wherein said amino acid sequence has insecticidal activity;

f) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:6, wherein said amino acid sequence has insecticidal activity; and

g) a nucleotide sequence encoding an insecticidal polypeptide that is a variant of SEQ ID NO:4, 5, 6, 13, or 14, wherein said variant is the result of one or more domain(s) of SEQ ID NO:4, 5, 6, 13, or 14 being exchanged with the corresponding domain(s) of SEQ ID NO:4, 5, 6, 13, or 14; wherein said nucleotide sequence is operably linked to a promoter that drives expression of a coding sequence in a plant cell.

15. The plant of claim 14, wherein said plant is a plant cell.

16. A transgenic seed of the plant of claim 14, wherein said seed comprises a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence of any of SEQ ID NO: 1, 2, 3, 11, or 12;

b) a nucleotide sequence having at least 96% sequence identity to the nucleotide sequence of SEQ ID NO:1 or 12, wherein said nucleotide sequence encodes a protein having insecticidal activity;

c) a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3, wherein said nucleotide sequence encodes a protein having insecticidal activity;

d) a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14;

e) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 96% sequence identity to the amino acid sequence of SEQ ID NO:4 or 14, wherein said amino acid sequence has insecticidal activity;

f) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:4 or 14.
sequence of SEQ ID NO:6, wherein said amino acid sequence has insecticidal activity; and
g) a nucleotide sequence encoding an insecticidal polypeptide that is a variant of SEQ ID NO:4, 5, 6, 13, or 14, wherein said variant is the result of one or more domain(s) of SEQ ID NO:4, 5, 6, 13, or 14 being exchanged with the corresponding domain(s) of SEQ ID NO:4, 5, 6, 13, or 14.

17. A method for protecting a plant from an insect pest, comprising introducing into said plant or cell thereof at least one expression vector comprising a nucleotide sequence that encodes a insecticidal polypeptide, wherein said nucleotide sequence is selected from the group consisting of:
   a) the nucleotide sequence of any of SEQ ID NO: 1, 2, 3, 11, or 12;
   b) a nucleotide sequence having at least 96% sequence identity to the nucleotide sequence of SEQ ID NO:1 or 12, wherein said nucleotide sequence encodes a protein having insecticidal activity;
   c) a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:3, wherein said nucleotide sequence encodes a protein having insecticidal activity;
   d) a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence of any of SEQ ID NO:4, 5, 6, 13, or 14;
   e) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 96% sequence identity to the amino acid sequence of SEQ ID NO:4 or 14, wherein said amino acid sequence has insecticidal activity; and,
   f) a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:6, wherein said amino acid sequence has insecticidal activity;
   g) a nucleotide sequence encoding an insecticidal polypeptide that is a variant of SEQ ID NO:4, 5, 6, 13, or 14, wherein said variant is the result of one or more domain(s) of SEQ ID NO:4, 5, 6, 13, or 14 being exchanged with the corresponding domain(s) of SEQ ID NO:4, 5, 6, 13, or 14.
18. The isolated nucleic acid sequence of claim 1, the polypeptide of claim 7, the plant of claim 14, the plant cell of claim 15, the seed of claim 16, or the method of claim 17, wherein said one or more domains is selected from the domains outlined in Figure 2.

19. The plant of claim 14, the plant cell of claim 17, the plant seed of claim 18, or the method of claim 19, wherein said plant is selected from the group consisting of maize, sorghum, wheat, cabbage, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape.