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(54) Title: DIG-3 INSECTICIDAL CRY TOXINS

(57) Abstract: DIG-3 Cry toxins, polynucleotides encoding such toxins, and transgenic plants that produce such toxins are useful to control insect pests.



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DIG-3 INSECTICIDAL CRY TOXINS

Cross Reference to Related Applications

[0001] This application claims benefit to U.S. Provisional Patent Application No. 61/170,189, filed April 17, 2009, which is expressly incorporated by reference herein.

Field of the Invention

[0002] This invention concerns new insecticidal Cry toxins and their use to control insects.

Background of the Invention

[0003] *Bacillus thuringiensis* (*B.t.*) is a soil-borne bacterium that produces pesticidal crystal proteins known as delta endotoxins or Cry proteins. Cry proteins are oral intoxicants that function by acting on midgut cells of susceptible insects. An extensive list of delta endotoxins is maintained and regularly updated at

http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html.

[0004] European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is the most damaging insect pest of corn throughout the United States and Canada, and causes an estimated \$1 billion revenue loss each year due to crop yield loss and expenditures for insect management (Witkowski *et al.*, 2002). Transgenic corn expressing genes encoding Cry proteins, most notably Cry1Ab, Cry1Ac, or Cry1F, provide commercial levels of efficacy against ECB.

[0005] Despite the success of ECB-resistant transgenic corn, the possibility of the development of resistant insect populations threatens the long-term durability of Cry proteins in ECB control and creates the need to discover and develop new Cry proteins to control ECB and other pests. Insect resistance to *B.t.* Cry proteins can develop through several mechanisms (Heckel *et al.*, 2007, Pigott and Ellar, 2007). Multiple receptor protein classes for Cry proteins have been identified within insects, and multiple examples exist within each receptor class. Resistance to a particular Cry protein may develop, for example, by means of a mutation within the toxin-binding portion of a cadherin domain of a receptor protein. A further means of resistance may be mediated through a protoxin-processing protease. Thus, resistance to Cry toxins in species of Lepidoptera has a complex genetic basis, with at least four distinct, major resistance genes. Lepidopteran insects resistant to Cry proteins have developed in the field within the species *Plutella xylostella* (Tabashnik, 1994), *Trichoplusia ni* (Janmaat and Myers 2003, 2005), and *Helicoverpa zea* (Tabashnik *et al.*, 2008). Development of new high potency

Cry proteins would provide additional tools for management of ECB and other insect pests. Cry proteins with different modes of action produced in combination in transgenic corn would prevent the development ECB insect resistance and protect the long term utility of *B.t.* technology for insect pest control.

Brief Summary of the Invention

[0006] The present invention provides insecticidal Cry toxins, including the toxin designated herein as DIG-3 as well as variants of DIG-3, nucleic acids encoding these toxins, methods of controlling pests using the toxins, methods of producing the toxins in transgenic host cells, and transgenic plants that produce the toxins. The predicted amino acid sequence of the wild type DIG-3 toxin is given in SEQ ID NO:2.

[0007] As described in Example 1, a nucleic acid encoding the DIG-3 protein was isolated from a *B.t.* strain internally designated by Dow AgroSciences LLC as PS46L. The nucleic acid sequence for the full length coding region was determined, and the full length protein sequence was deduced from the nucleic acid sequence. The DIG-3 toxin has some similarity to Cry1BII (Genbank Accession No. AAM93496) and other *B. thuringiensis* Cry1B-type proteins (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html).

[0008] Insecticidally active variants of the DIG-3 toxin are also described herein, and are referred to collectively as DIG-3 toxins.

[0009] DIG-3 toxins may also be used in combination with RNAi methodologies for control of other insect pests. For example, DIG-3 can be used in transgenic plants in combination with a dsRNA for suppression of an essential gene in corn rootworm or an essential gene in an insect pest. Such target genes include, for example, vacuolar ATPase, ARF-1, Act42A, CHD3, EF-1 α , and TFIIB. An example of a suitable target gene is vacuolar ATPase, as disclosed in WO2007/035650.

[0010] A surprising finding reported herein is that DIG-3 toxins are active against populations of European corn borer and diamond back moth that are resistant to Cry1F and Cry1A toxins. Accordingly, DIG-3 toxins are ideal candidates for use to control of Lepidopteran pests. The toxins can be used alone or in combination with other Cry toxins, such as Cry1F, Cry1Ab, and Cry1Ac, to control development of resistant insect populations.

[0011] Insecticidally active fragments of SEQ ID NO:2, and nucleotides encoding such fragments, are another aspect of the invention.

[0012] In one embodiment the invention provides an isolated DIG-3 toxin polypeptide comprising a core toxin segment selected from the group consisting of

- (a) a polypeptide comprising the amino acid sequence of residues 113 to 643 of SEQ ID NO:2;
- (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 113 to 643 of SEQ ID NO:2;
- (c) a polypeptide comprising an amino acid sequence of residues 113 to 643 of SEQ ID NO:2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO:2.

[0013] In one embodiment the invention provides an isolated DIG-3 toxin polypeptide comprising a core toxin segment selected from the group consisting of

- (a) a polypeptide comprising the amino acid sequence of residues 73 to 643 of SEQ ID NO:2;
- (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 73 to 643 of SEQ ID NO:2;
- (c) a polypeptide comprising an amino acid sequence of residues 73 to 643 of SEQ ID NO:2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO:2.

[0014] In another embodiment the invention provides an isolated DIG-3 toxin polypeptide comprising a DIG-3 core toxin segment selected from the group consisting of

- (a) a polypeptide comprising the amino acid sequence of residues 1 to 643 of SEQ ID NO:2;
- (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 1 to 643 of SEQ ID NO:2;
- (c) a polypeptide comprising an amino acid sequence of residues 1 to 643 of SEQ ID NO:2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO:2.

[0015] By “isolated” applicants mean that the polypeptide or DNA molecules have been removed from their native environment and have been placed in a different environment by the hand of man.

[0016] In another embodiment the invention provides a plant comprising a DIG-3 toxin.

[0017] In another embodiment the invention provides a method for controlling a pest population comprising contacting said population with a pesticidally effective amount of a DIG-3 toxin.

[0018] In another embodiment the invention provides an isolated nucleic acid that encodes a DIG-3 toxin.

[0019] In another embodiment the invention provides a DNA construct comprising a nucleotide sequence that encodes a DIG-3 toxin operably linked to a promoter that is not derived from *Bacillus thuringiensis* and is capable of driving expression in a plant. The invention also provides a transgenic plant that comprises the DNA construct stably incorporated into its genome and a method for protecting a plant from a pest comprising introducing the construct into said plant.

Brief Description of the Sequences

[0020] SEQ ID NO:1 DNA sequence encoding full-length DIG-3 toxin; 3771 nt.

[0021] SEQ ID NO:2 Full-length DIG-3 protein sequence; 1256 aa.

[0022] SEQ ID NO:3 Plant-optimized full length DIG-3 DNA sequence; 3771 nt.

[0023] SEQ ID NO:4 Cry1Ab protoxin segment; 545 aa.

[0024] SEQ ID NO:5 Chimeric toxin: DIG-3 Core toxin segment/Cry1Ab protoxin segment; 1188 aa.

[0025] SEQ ID NO:6 Dicot-optimized DNA sequence encoding the Cry1Ab protoxin segment; 1635 nt

[0026] SEQ ID NO:7 Maize-optimized DNA sequence encoding the Cry1Ab protoxin segment; 1635 nt

Detailed Description of the Invention

[0027] DIG-3 Toxins, and insecticidally active variants. In addition to the full length DIG-3 toxin of SEQ ID NO:2, the invention encompasses insecticidally active variants. By the term “variant”, applicants intend to include fragments, certain deletion and insertion mutants, and certain fusion proteins. DIG-3 is a classic three-domain Cry toxin. As a preface to describing

variants of the DIG-3 toxin that are included in the invention, it will be useful to briefly review the architecture of three-domain Cry toxins in general and of the DIG-3 protein toxin in particular.

[0028] A majority of *Bacillus thuringiensis* delta-endotoxin crystal protein molecules are composed of two functional segments. The protease-resistant core toxin is the first segment and corresponds to about the first half of the protein molecule. The full ~130 kDa protoxin molecule is rapidly processed to the resistant core segment by proteases in the insect gut. The segment that is deleted by this processing will be referred to herein as the "protoxin segment". The protoxin segment is believed to participate in toxin crystal formation (Arvidson *et al.*, 1989). The protoxin segment may thus convey a partial insect specificity for the toxin by limiting the accessibility of the core to the insect by reducing the protease processing of the toxin molecule (Haider *et al.*, 1986) or by reducing toxin solubility (Aronson *et al.*, 1991). *B.t.* toxins, even within a certain class, vary to some extent in length and in the precise location of the transition from the core toxin segment to protoxin segment. The transition from core toxin segment to protoxin segment will typically occur at between about 50% to about 60% of the full length toxin. SEQ ID NO:2 discloses the 1256 amino acid sequence of the full-length DIG-3 polypeptide, of which the N-terminal 643 amino acids comprise the DIG-3 core toxin segment. The 5'-terminal 1929 nucleotides of SEQ ID NO:1 comprise the coding region for the core toxin segment.

[0029] Three dimensional crystal structures have been determined for Cry1Aa1, Cry2Aa1, Cry3Aa1, Cry3Bb1, Cry4Aa, Cry4Ba and Cry8Ea1. These structures for the core toxins are remarkably similar and are comprised of three distinct domains with the features described below (reviewed in de Maagd *et al.*, 2003).

[0030] Domain I is a bundle of seven alpha helices where α -helix 5 is surrounded by six amphipathic helices. This domain has been implicated in pore formation and shares homology with other pore forming proteins including hemolysins and colicins. Domain I of the DIG-3 protein comprises amino acid residues 56 to 278 of SEQ ID NO:2.

[0031] Domain II is formed by three anti-parallel beta sheets packed together in a beta prism. The loops of this domain play important roles in binding insect midgut receptors. In Cry1A proteins, surface exposed loops at the apices of Domain II beta sheets are involved in binding to Lepidopteran cadherin receptors. Cry3Aa Domain II loops bind a membrane-associated metalloprotease of *Leptinotarsa decemlineata* (Say) (Colorado potato beetle) in a similar fashion

(Ochoa-Campuzano *et al.*, 2007). Domain II shares homology with certain carbohydrate-binding proteins including vitelline and jacaline. Domain II of the DIG-3 protein comprises amino acid residues 283 to 493 of SEQ ID NO:2.

[0032] Domain III is a beta sandwich of two anti-parallel beta sheets. Structurally this domain is related to carbohydrate-binding domains of proteins such as glucanases, galactose oxidase, sialidase and others. Domain III binds certain classes of receptor proteins and perhaps participates in insertion of an oligomeric toxin pre-pore that interacts with a second class of receptors, examples of which are aminopeptidase and alkaline phosphatase in the case of Cry1A proteins (Pigott and Ellar, 2007). Analogous Cry Domain III receptors have yet to be identified in Coleoptera. Conserved *B.t.* sequence blocks 2 and 3 map near the N-terminus and C-terminus of Domain 2, respectively. Hence, these conserved sequence blocks 2 and 3 are approximate boundary regions between the three functional domains. These regions of conserved DNA and protein homology have been exploited for engineering recombinant *B.t.* toxins (US Patent No. 6090931, WO 91/01087, WO 95/06730, WO 1998022595). Domain III of the DIG-3 protein comprises amino acid residues 503 to 641 of SEQ ID NO:2.

[0033] It has been reported that α -helix 1 of Domain I is removed following receptor binding. Aronson *et al.* (1999) demonstrated that Cry1Ac bound to BBMV was protected from proteinase K cleavage beginning at residue 59, just after α -helix 1; similar results were cited for Cry1Ab. Gomez *et al.* (2002) found that Cry1Ab oligomers formed upon BBMV receptor binding lacked the α -helix 1 portion of Domain I. Also, Soberon *et al.* (2007) have shown that N-terminal deletion mutants of Cry1Ab and Cry1Ac which lack approximately 60 amino acids encompassing α -helix 1 on the three dimensional Cry structure are capable of assembling monomers of molecular weight about 60 kDa into pre-pores in the absence of cadherin binding. These N-terminal deletion mutants were reported to be active on Cry-resistant insect larvae. Furthermore, Diaz-Mendoza *et al.* (2007) described Cry1Ab fragments of 43 kDa and 46 kDa that retained activity on Mediterranean corn borer (*Sesamia nonagrioides*). These fragments were demonstrated to include amino acid residues 116 to 423; however the precise amino acid sequences were not elucidated and the mechanism of activity of these proteolytic fragments is unknown. The results of Gomez *et al.* (2002), Soberon *et al.* (2007), and Diaz-Mendoza *et al.* (2007) contrast with those of Hofte *et al.* (1986), who reported that deletion of 36 amino acids from the N-terminus of Cry1Ab resulted in loss of insecticidal activity.

[0034] We have deduced the beginnings and ends of α -helix 1, α -helix 2A, α -helix 2B, and α -helix 3, and the location of the spacer regions between them in Domain I of the DIG-3 toxin by comparing the DIG-3 protein sequence with the protein sequence for Cry8Ea1, for which the structure is known. These locations are described in Table 1.

Table 1

Amino acid coordinates of projected α -helices of DIG-3 protein.

	α -helix 1	spacer	α -helix 2A	spacer	α -helix 2B	spacer	α -helix 3
Residues of SEQ ID NO:2	53-70	71-76	77-91	92-99	100-108	109-113	114-138

[0035] Amino terminal deletion variants of DIG-3. In one of its aspects the invention provides DIG-3 variants in which all or part of α -helix 1, α -helix 2A, and α -helix 2B are deleted to improve insecticidal activity and avoid development of resistance by insects. These modifications are made to provide DIG-3 variants with improved attributes, such as improved target pest spectrum, potency, and insect resistance management. In some embodiments of the invention, the subject modifications may affect the efficiency of protoxin activation and pore formation, leading to insect intoxication. More specifically, to provide DIG-3 variants with improved attributes, step-wise deletions are described that remove part of the nucleic acid sequence encoding the N-terminus of the DIG-3 protein. The deletions remove all of α -helix 1 and all or part of α -helix 2 in Domain I, while maintaining the structural integrity of α -helices 3 through 7. The subject invention therefore relates in part to improvements to Cry protein efficacy made by engineering the α -helical components of Domain 1 for more efficient pore formation. More specifically, the subject invention relates in part to improved DIG-3 proteins designed to have N-terminal deletions in regions with putative secondary structure homology to α -helix 1 and α -helix 2 in Domain I of Cry1 proteins.

[0036] Deletions to improve the insecticidal properties of the DIG-3 toxins may initiate before the predicted α -helix 2A start, and may terminate after the α -helix 2B end, but preferably do not extend into α -helix 3.

[0037] In designing coding sequences for the N-terminal deletion variants, an ATG start codon, encoding methionine, is inserted at the 5' end of the nucleotide sequence designed to encode the deletion variant. For sequences designed for use in transgenic plants, it may be of benefit to adhere to the "N-end rule" of Varshavsky (1997). It is taught that some amino acids

may contribute to protein instability and degradation in eukaryotic cells when displayed as the N-terminal residue of a protein. For example, data collected from observations in yeast and mammalian cells indicate that the N-terminal destabilizing amino acids are F, L, W, Y, R, K, H, I, N, Q, D, E and possibly P. While the specifics of protein degradation mechanisms may differ somewhat between organisms, the conservation of identity of N-terminal destabilizing amino acids seen above suggests that similar mechanisms may function in plant cells. For instance, Worley *et al.* (1998) found that in plants, the N-end rule includes basic and aromatic residues. It is a possibility that proteolytic cleavage by plant proteases near the start of α -helix 3 of subject *B.t.* insecticidal proteins may expose a destabilizing N-terminal amino acid. Such processing may target the cleaved proteins for rapid decay and limit the accumulation of the *B.t.* insecticidal proteins to levels insufficient for effective insect control. Accordingly, for N-terminal deletion variants that begin with one of the destabilizing amino acids, applicants prefer to add a codon that specifies a G (glycine) amino acid between the translational initiation methionine and the destabilizing amino acid.

[0038] Example 2 gives specific examples of amino-terminal deletion variants of DIG-3 in accordance with the invention. Additional useful fragments that retain toxicity can be identified by trypsin or chymotrypsin digestion of the full length solubilized crystal protein. Further examples of toxic DIG-3 protein fragments may be encoded by fragments of the DIG-3 coding region. Insect active DIG-3 variants will mostly have a short N-terminal truncation and a long C-terminal truncation. The N-terminal end of the smallest toxic fragment is conveniently determined by N-terminal amino acid sequence determination of trypsin- or chymotrypsin-treated soluble crystal protein by techniques routinely available in the art.

[0039] Chimeric Toxins. Chimeric proteins utilizing the core toxin segment of one Cry toxin fused to the protoxin segment of another Cry toxin have previously been reported. DIG-3 variants include toxins comprising an N-terminal core toxin segment of a DIG-3 toxin (which may be full length or have the N-terminal deletions described above) fused to a heterologous protoxin segment at some point past the end of the core toxin segment. The transition to the heterologous protoxin segment can occur at approximately the native core toxin/protoxin junction or, in the alternative, a portion of the native protoxin (extending past the core toxin segment) can be retained with the transition to the heterologous protoxin occurring downstream. As an example, a chimeric toxin of the subject invention has the full core toxin segment of DIG-3 (amino acids 1-

643) and a heterologous protoxin segment (amino acids 643 to the C-terminus). In a preferred embodiment, the heterologous protoxin segment is derived from a Cry1Ab delta-endotoxin, as illustrated in SEQ ID NO:5.

[0040] SEQ ID NO:4 discloses the 545 amino acid sequence of a Cry1Ab protoxin segment useful in DIG-3 variants of the invention. Attention is drawn to the last about 100 to 150 amino acids of this protoxin segment, which it is most critical to include in the chimeric toxin of the subject invention.

[0041] Protease sensitivity variants. Insect gut proteases typically function in aiding the insect in obtaining needed amino acids from dietary protein. The best understood insect digestive proteases are serine proteases, which appear to be the most common type (Englemann and Geraerts (1980), particularly in Lepidopteran species. Coleopteran insects have guts that are more neutral to acidic than are Lepidopteran guts. The majority of Coleopteran larvae and adults, for example Colorado potato beetle, have slightly acidic midguts, and cysteine proteases provide the major proteolytic activity (Wolfson and Murdock, 1990). More precisely, Thie and Houseman (1990) identified and characterized the cysteine proteases, cathepsin B-like and cathepsin H-like, and the aspartyl protease, cathepsin D-like, in Colorado potato beetle. Gillikin *et al.* (1992) characterized the proteolytic activity in the guts of western corn rootworm larvae and found primarily cysteine proteases. US Patent No. 7230167 disclosed that a protease activity attributed to cathepsin G exists in western corn rootworm. The diversity and different activity levels of the insect gut proteases may influence an insect's sensitivity to a particular *B.t.* toxin.

[0042] In another embodiment of the invention, protease cleavage sites may be engineered at desired locations to affect protein processing within the midgut of susceptible larvae of certain insect pests. These protease cleavage sites may be introduced by methods such as chemical gene synthesis or splice overlap PCR (Horton *et al.*, 1989). Serine protease recognition sequences, for example, can optionally be inserted at specific sites in the Cry protein structure to effect protein processing at desired deletion points within the midgut of susceptible larvae. Serine proteases that can be exploited in such fashion include Lepidopteran midgut serine proteases such as trypsin or trypsin-like enzymes, chymotrypsin, elastase, *etc.* (Christeller *et al.*, 1992). Further, deletion sites identified empirically by sequencing Cry protein digestion products generated with unfractionated larval midgut protease preparations or by binding to brush border membrane vesicles can be engineered to effect protein activation. Modified Cry proteins generated either by gene deletion or by introduction of protease cleavage sites have improved activity on

Lepidopteran pests including *Ostrinia nubilalis*, *Diatraea grandiosella*, *Helicoverpa zea*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Diatraea saccharalis*, *Loxagrotis albicosta*, and other target pests.

[0043] Coleopteran serine proteases such as trypsin, chymotrypsin and cathepsin G-like protease, Coleopteran cysteine proteases such as cathepsins (B-like, L-like, O-like, and K-like proteases) (Koiwa *et al.*, (2000) and Bown *et al.*, (2004)], Coleopteran metalloproteases such as ADAM10 [Ochoa-Campuzano *et al.*, (2007)], and Coleopteran aspartic acid proteases such as cathepsins D-like and E-like, pepsin, plasmepsin, and chymosin may further be exploited by engineering appropriate recognition sequences at desired processing sites to affect Cry protein processing within the midgut of susceptible larvae of certain insect pests.

[0044] A preferred location for the introduction of such protease cleavage sites may be within the "spacer" region between α -helix 2B and α -helix 3, for example within amino acids 109 to 113 of the full length DIG-3 protein (SEQ ID NO:2 and Table 1). Modified Cry proteins generated either by gene deletion or by introduction of protease cleavage sites have improved activity on insect pests including but not limited to western corn rootworm, southern corn rootworm, northern corn rootworm, and the like.

[0045] Various technologies exist to enable determination of the sequence of the amino acids which comprise the N-terminal or C-terminal residues of polypeptides. For example, automated Edman degradation methodology can be used in sequential fashion to determine the N-terminal amino acid sequence of up to 30 amino acid residues with 98% accuracy per residue. Further, determination of the sequence of the amino acids comprising the carboxy end of polypeptides is also possible [Bailey *et al.*, (1992); US Patent No. 6046053]. Thus, in some embodiments, *B.t.* Cry proteins which have been activated by means of proteolytic processing, for example, by proteases prepared from the gut of an insect, may be characterized and the N-terminal or C-terminal amino acids of the activated toxin fragment identified. DIG-3 variants produced by introduction or elimination of protease processing sites at appropriate positions in the coding sequence to allow, or eliminate, proteolytic cleavage of a larger variant protein by insect, plant or microorganism proteases are within the scope of the invention. The end result of such manipulation is understood to be the generation of toxin fragment molecules having the same or better activity as the intact (full length) toxin protein.

[0046] Domains of the DIG-3 toxin. The separate domains of the DIG-3 toxin, (and variants that are 90%, 95%, or 97% identical to such domains) are expected to be useful in forming combinations with domains from other Cry toxins to provide new toxins with increased spectrum of pest toxicity, improved potency, or increased protein stability. Domain I of the DIG-3 protein consists of amino acid residues 56 to 278 of SEQ ID NO:2. Domain II of the DIG-3 protein consists of amino acid residues 283 to 493 of SEQ ID NO:2. Domain III of the DIG-3 protein consists of amino acid residues 503 to 641 of SEQ ID NO:2. Domain swapping or shuffling is a mechanism for generating altered delta-endotoxin proteins. Domains II and III may be swapped between delta-endotoxin proteins, resulting in hybrid or chimeric toxins with improved pesticidal activity or target spectrum. Domain II is involved in receptor binding, and the DIG-3 Domain II is very divergent from other Cry1B toxins. Domain III binds certain classes of receptor proteins and perhaps participates in insertion of an oligomeric toxin pre-pore. Some Domain III substitutions in other toxins have been shown to produce superior toxicity against *Spodoptera exigua* (de Maagd *et al.*, 1996), and guidance exists on the design of the Cry toxin domain swaps (Knight *et al.*, 2004).

[0047] Methods for generating recombinant proteins and testing them for pesticidal activity are well known in the art (see, for example, Naimov *et al.*, (2001), de Maagd *et al.*, (1996), Ge *et al.*, (1991), Schnepf *et al.*, (1990), Rang *et al.*, (1999)). Domain I from Cry1A and Cry3A proteins has been studied for the ability to insert and form pores in membranes. α -helix 4 and α -helix 5 of Domain I play key roles in membrane insertion and pore formation [Walters *et al.*, (1993), Gazit *et al.*, (1998); Nunez-Valdez *et al.*, (2001)], with the other alpha helices proposed to contact the membrane surface like the ribs of an umbrella (Bravo *et al.*, (2007); Gazit *et al.*, (1998)).

[0048] DIG-3 variants created by making a limited number of amino acid deletions, substitutions, or additions. Amino acid deletions, substitutions, and additions to the amino acid sequence of SEQ ID NO:2 can readily be made in a sequential manner and the effects of such variations on insecticidal activity can be tested by bioassay. Provided the number of changes is limited in number, such testing does not involve unreasonable experimentation. The invention includes insecticidally active variants of the core toxin segment (amino acids 1-643 of SEQ ID NO:2, or amino acids 73-643 of SEQ ID NO:2) in which up to 10, up to 15, or up to 20 independent amino acid additions, deletions, or substitutions have been made.

[0049] The invention includes DIG-3 variants having a core toxin segment that is 90%, 95% or 97% identical to amino acids 1-643 of SEQ ID NO:2 or amino acids 73-643 of SEQ ID NO:2.

[0050] Variants may be made by making random mutations or the variants may be designed. In the case of designed mutants, there is a high probability of generating variants with similar activity to the native toxin when amino acid identity is maintained in critical regions of the toxin which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. A high probability of retaining activity will also occur if substitutions are conservative. Amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type are least likely to materially alter the biological activity of the variant. Table 2 provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar Side Chains	Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Met (M), Phe (F), Trp (W)
Uncharged Polar Side Chains	Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q)
Acidic Side Chains	Asp (D), Glu (E)
Basic Side Chains	Lys (K), Arg (R), His (H)
Beta-branched Side Chains	Thr, Val, Ile
Aromatic Side Chains	Tyr, Phe, Trp, His

[0051] In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin. 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, namely, retaining pesticidal activity.

[0052] Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See *e.g.* U.S. Patent No. 7058515; Larson *et al.* (2002); Stemmer (1994a, 1994b, 1995); and Cramer *et al.* (1996a, 1996b, 1997).

[0053] Nucleic Acids. Isolated nucleic acids encoding DIG-3 toxins are one aspect of the present invention. This includes nucleic acids encoding SEQ ID NO:2 and SEQ ID NO:5, and

complements thereof, as well as other nucleic acids that encode insecticidal variants of SEQ ID NO:2. Because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding the same, or essentially the same, toxins.

[0054] Gene synthesis. DNA sequences encoding the improved Cry proteins described herein can be made by a variety of methods well-known in the art. For example, synthetic gene segments and synthetic genes can be made by phosphite tri-ester and phosphoramidite chemistry (Caruthers *et al.*, 1987), and commercial vendors are available to perform DNA synthesis on demand. Sequences encoding full-length DIG-3 proteins can be assembled in a variety of ways including, for example, by ligation of restriction fragments or polymerase chain reaction assembly of overlapping oligonucleotides (Stewart and Burgin, 2005). Further, sequences encoding terminal deletions can be made by PCR amplification using site-specific terminal oligonucleotides.

[0055] Nucleic acids encoding DIG-3 toxins can be made for example, by synthetic construction by methods currently practiced by any of several commercial suppliers. (See for example, US Patent No. 7482119 B2). These nucleic acids, or portions or variants thereof, may also be constructed synthetically, for example, by use of a gene synthesizer and the design methods of, for example, US Patent No. 5380831. Alternatively, variations of synthetic or naturally occurring genes may be readily constructed using standard molecular biological techniques for making point mutations. Fragments of these genes can also be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal31* or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, gene fragments which encode active toxin fragments may be obtained using a variety of restriction enzymes.

[0056] Given the amino acid sequence for a DIG-3 toxin, a coding sequence can be designed by reverse translating the coding sequence using codons preferred by the intended host, and then refining the sequence using alternative codons to remove sequences that might cause problems and provide periodic stop codons to eliminate long open coding sequences in the non-coding reading frames.

[0057] Quantifying Sequence Identity. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, 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 (*e.g.* overlapping positions) x100). In one embodiment, the two sequences are the same length. 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.

[0058] The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A nonlimiting example of such an algorithm is that of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993), and incorporated into the BLASTN and BLASTX programs. BLAST searches may be conveniently used to identify sequences homologous (similar) to a query sequence in nucleic or protein databases. BLASTN searches can be performed, (score = 100, word length = 12) to identify nucleotide sequences having homology to claimed nucleic acid molecules of the invention. BLASTX searches can be performed (score = 50, word length = 3) to identify amino acid sequences having homology to claimed insecticidal protein molecules of the invention.

[0059] Gapped BLAST (Altschul *et al.*, 1997) can be utilized to obtain gapped alignments for comparison purposes. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules (Altschul *et al.*, 1997). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs can be used. See www.ncbi.nlm.nih.gov.

[0060] A non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the ClustalW algorithm (Thompson *et al.*, 1994). 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 or nucleotide sequence. The ClustalW algorithm is used in several commercially available DNA/amino acid analysis software packages, such as the ALIGNX module of the Vector NTI Program Suite (Invitrogen, Inc., Carlsbad, CA). When aligning amino acid sequences with ALIGNX, one may conveniently use the default settings with a Gap open penalty of 10, a Gap extend penalty of 0.1 and the blosum63mt2 comparison matrix to assess the percent amino acid similarity (consensus) or identity between

the two sequences. When aligning DNA sequences with ALIGNX, one may conveniently use the default settings with a Gap open penalty of 15, a Gap extend penalty of 6.6 and the swgapdnamt comparison matrix to assess the percent identity between the two sequences.

[0061] Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is that of Myers and Miller (1988). Such an algorithm is incorporated into the wSTRETCHER program, which is part of the wEMBOSS sequence alignment software package (available at <http://emboss.sourceforge.net/>). wSTRETCHER calculates an optimal global alignment of two sequences using a modification of the classic dynamic programming algorithm which uses linear space. The substitution matrix, gap insertion penalty and gap extension penalties used to calculate the alignment may be specified. When utilizing the wSTRETCHER program for comparing nucleotide sequences, a Gap open penalty of 16 and a Gap extend penalty of 4 can be used with the scoring matrix file EDNAFULL. When used for comparing amino acid sequences, a Gap open penalty of 12 and a Gap extend penalty of 2 can be used with the EBLOSUM62 scoring matrix file.

[0062] A further non-limiting example of a mathematical algorithm utilized for the comparison of sequences is that of Needleman and Wunsch (1970), which is incorporated in the sequence alignment software packages GAP Version 10 and wNEEDLE (<http://emboss.sourceforge.net/>). GAP Version 10 may be used to determine sequence identity or similarity using the following parameters: for a nucleotide sequence, % identity and % similarity are found using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna. cmp scoring matrix. For amino acid sequence comparison, % identity or % similarity are determined using GAP weight of 8 and length weight of 2, and the BLOSUM62 scoring program.

[0063] wNEEDLE reads two input sequences, finds the optimum alignment (including gaps) along their entire length, and writes their optimal global sequence alignment to file. The algorithm explores all possible alignments and chooses the best, using a scoring matrix that contains values for every possible residue or nucleotide match. wNEEDLE finds the alignment with the maximum possible score, where the score of an alignment is equal to the sum of the matches taken from the scoring matrix, minus penalties arising from opening and extending gaps in the aligned sequences. The substitution matrix and gap opening and extension penalties are user-specified. When amino acid sequences are compared, a default Gap open penalty of 10, a Gap extend penalty of 0.5, and the EBLOSUM62 comparison matrix are used. When DNA

sequences are compared using wNEEDLE, a Gap open penalty of 10, a Gap extend penalty of 0.5, and the EDNAFULL comparison matrix are used.

[0064] 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 or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by ALIGNX, wNEEDLE, or wSTRETCHER. The % identity is the percentage of identical matches between the two sequences over the reported aligned region (including any gaps in the length) and the % similarity is the percentage of matches between the two sequences over the reported aligned region (including any gaps in the length).

[0065] Alignment may also be performed manually by inspection.

[0066] Recombinant hosts. The toxin-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticidal protein. With suitable microbial hosts, *e.g.* *Pseudomonas*, the microbes can be applied to the environment of the pest, where they will proliferate and be ingested. The result is a control of the pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest.

[0067] Where the *B.t.* toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type indigenous microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

[0068] A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest

are microorganisms, such as bacteria, *e.g.* genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Sinorhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, *e.g.* genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, *Rhodopseudomonas spheroides*, *Xanthomonas campestris*, *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*), *Alcaligenes eutrophus*, and *Azotobacter vinelandii*; and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C. diffluens*, *C. laurentii*, *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odoratus*, *Kluyveromyces veronae*, and *Aureobasidium pollulans*. Of particular interest are the pigmented microorganisms.

Methods of controlling insect pests

[0069] When an insect comes into contact with an effective amount of toxin delivered via transgenic plant expression, formulated protein compositions(s), sprayable protein composition(s), a bait matrix or other delivery system, the results are typically death of the insect, or the insects do not feed upon the source which makes the toxins available to the insects.

[0070] The subject protein toxins can be “applied” or provided to contact the target insects in a variety of ways. For example, transgenic plants (wherein the protein is produced by and present in the plant) can be used and are well-known in the art. Expression of the toxin genes can also be achieved selectively in specific tissues of the plants, such as the roots, leaves, *etc.* This can be accomplished via the use of tissue-specific promoters, for example. Spray-on applications are another example and are also known in the art. The subject proteins can be appropriately formulated for the desired end use, and then sprayed (or otherwise applied) onto the plant and/or around the plant / to the vicinity of the plant to be protected – before an infestation is discovered, after target insects are discovered, both before and after, and the like. Bait granules, for example, can also be used and are known in the art.

Transgenic plants

[0071] The subject proteins can be used to protect practically any type of plant from damage by a Lepidopteran insect. Nonlimiting examples of such plants include maize, sunflower, soybean, cotton, canola, rice, sorghum, wheat, barley, vegetables, ornamentals, peppers

(including hot peppers), sugar beets, fruit, and turf, to name but a few. Methods for transforming plants are well known in the art, and illustrative transformation methods are described in the Examples.

[0072] A preferred embodiment of the subject invention is the transformation of plants with genes encoding the subject insecticidal protein or its variants. The transformed plants are resistant to attack by an insect target pest by virtue of the presence of controlling amounts of the subject insecticidal protein or its variants in the cells of the transformed plant. By incorporating genetic material that encodes the insecticidal properties of the *B.t.* insecticidal toxins into the genome of a plant eaten by a particular insect pest, the adult or larvae would die after consuming the food plant. Numerous members of the monocotyledonous and dicotyledonous classifications have been transformed. Transgenic agronomic crops as well as fruits and vegetables are of commercial interest. Such crops include, but are not limited to, maize, rice, soybeans, canola, sunflower, alfalfa, sorghum, wheat, cotton, peanuts, tomatoes, potatoes, and the like. Several techniques exist for introducing foreign genetic material into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (US Patent No. 4945050 and US Patent No. 5141131). Plants may be transformed using *Agrobacterium* technology, see US Patent No. 5177010, US Patent No. 5104310, European Patent Application No. 0131624B1, European Patent Application No. 120516, European Patent Application No. 159418B1, European Patent Application No. 176112, US Patent No. 5149645, US Patent No. 5469976, US Patent No. 5464763, US Patent No. 4940838, US Patent No. 4693976, European Patent Application No. 116718, European Patent Application No. 290799, European Patent Application No. 320500, European Patent Application No. 604662, European Patent Application No. 627752, European Patent Application No. 0267159, European Patent Application No. 0292435, US Patent No. 5231019, US Patent No. 5463174, US Patent No. 4762785, US Patent No. 5004863, and US Patent No. 5159135. Other transformation technology includes WHISKERS™ technology, see US Patent No. 5302523 and US Patent No. 5464765. Electroporation technology has also been used to transform plants, see WO 87/06614, US Patent No. 5472869, US Patent No. 5384253, WO 9209696, and WO 9321335. All of these transformation patents and publications are incorporated by reference. In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue

types I and II, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques within the skill of an artisan.

[0073] Genes encoding DIG-3 toxins can be inserted into plant cells using a variety of techniques which are well known in the art as disclosed above. For example, a large number of cloning vectors comprising a marker that permits selection of the transformed microbial cells and a replication system functional in *Escherichia coli* are available for preparation and modification of foreign genes for insertion into higher plants. Such manipulations may include, for example, the insertion of mutations, truncations, additions, or substitutions as desired for the intended use. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the Cry protein or variants can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation of cells of *E. coli*, the cells of which are cultivated in a suitable nutrient medium, then harvested and lysed so that workable quantities of the plasmid are recovered. Sequence analysis, restriction fragment analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each manipulated DNA sequence can be cloned in the same or other plasmids.

[0074] The use of T-DNA-containing vectors for the transformation of plant cells has been intensively researched and sufficiently described in EP 120516; Lee and Gelvin (2008), Fraley *et al.* (1986), and An *et al.* (1985), and is well established in the field.

[0075] Once the inserted DNA has been integrated into the plant genome, it is relatively stable throughout subsequent generations. The vector used to transform the plant cell normally contains a selectable marker gene encoding a protein that confers on the transformed plant cells resistance to a herbicide or an antibiotic, such as bialaphos, kanamycin, G418, bleomycin, or hygromycin, *inter alia*. The individually employed selectable marker gene should accordingly permit the selection of transformed cells while the growth of cells that do not contain the inserted DNA is suppressed by the selective compound.

[0076] A large number of techniques are available for inserting DNA into a host plant cell. Those techniques include transformation with T-DNA delivered by *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation agent. Additionally, fusion of plant protoplasts with liposomes containing the DNA to be delivered, direct injection of the DNA,

biolistics transformation (microparticle bombardment), or electroporation, as well as other possible methods, may be employed.

[0077] In a preferred embodiment of the subject invention, plants will be transformed with genes wherein the codon usage of the protein coding region has been optimized for plants. See, for example, US Patent No. 5380831, which is hereby incorporated by reference. Also, advantageously, plants encoding a truncated toxin will be used. The truncated toxin typically will encode about 55% to about 80% of the full length toxin. Methods for creating synthetic *B.t.* genes for use in plants are known in the art (Stewart, 2007).

[0078] Regardless of transformation technique, the gene is preferably incorporated into a gene transfer vector adapted to express the *B.t.* insecticidal toxin genes and variants in the plant cell by including in the vector a plant promoter. In addition to plant promoters, promoters from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, one may use promoters of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, and the mannopine synthase promoter. Promoters of plant virus origin may be used, for example, the 35S and 19S promoters of Cauliflower Mosaic Virus, a promoter from Cassava Vein Mosaic Virus, and the like. Plant promoters include, but are not limited to, ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, phaseolin promoter, ADH (alcohol dehydrogenase) promoter, heat-shock promoters, ADF (actin depolymerization factor) promoter, ubiquitin promoter, actin promoter, and tissue specific promoters. Promoters may also contain certain enhancer sequence elements that may improve the transcription efficiency. Typical enhancers include but are not limited to ADH1-intron 1 and ADH1-intron 6. Constitutive promoters may be used. Constitutive promoters direct continuous gene expression in nearly all cells types and at nearly all times (*e.g.*, actin, ubiquitin, CaMV 35S). Tissue specific promoters are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP (Acyl Carrier Protein) promoters), and these promoters may also be used. Promoters may also be used that are active during a certain stage of the plants' development as well as active in specific plant tissues and organs. Examples of such promoters include but are not limited to promoters that are root specific, pollen-specific, embryo specific, corn silk specific, cotton fiber specific, seed endosperm specific, phloem specific, and the like.

[0079] Under certain circumstances it may be desirable to use an inducible promoter. An inducible promoter is responsible for expression of genes in response to a specific signal, such

as: physical stimulus (*e.g.* heat shock genes); light (*e.g.* RUBP carboxylase); hormone (*e.g.* glucocorticoid); antibiotic (*e.g.* tetracycline); metabolites; and stress (*e.g.* drought). Other desirable transcription and translation elements that function in plants may be used, such as 5' untranslated leader sequences, RNA transcription termination sequences and poly-adenylate addition signal sequences. Numerous plant-specific gene transfer vectors are known to the art.

[0080] Transgenic crops containing insect resistance (IR) traits are prevalent in corn and cotton plants throughout North America, and usage of these traits is expanding globally. Commercial transgenic crops combining IR and herbicide tolerance (HT) traits have been developed by multiple seed companies. These include combinations of IR traits conferred by *B.t.* insecticidal proteins and HT traits such as tolerance to Acetolactate Synthase (ALS) inhibitors such as sulfonylureas, imidazolinones, triazolopyrimidine, sulfonanilides, and the like, Glutamine Synthetase (GS) inhibitors such as bialaphos, glufosinate, and the like, 4-HydroxyPhenylPyruvate Dioxygenase (HPPD) inhibitors such as mesotrione, isoxaflutole, and the like, 5-EnolPyruvylShikimate-3-Phosphate Synthase (EPSPS) inhibitors such as glyphosate and the like, and Acetyl-Coenzyme A Carboxylase (ACCase) inhibitors such as haloxyfop, quizalofop, diclofop, and the like. Other examples are known in which transgenically provided proteins provide plant tolerance to herbicide chemical classes such as phenoxy acids herbicides and pyridyloxyacetates auxin herbicides (see WO 2007/053482 A2), or phenoxy acids herbicides and aryloxyphenoxypropionates herbicides (see WO 2005107437 A2, A3). The ability to control multiple pest problems through IR traits is a valuable commercial product concept, and the convenience of this product concept is enhanced if insect control traits and weed control traits are combined in the same plant. Further, improved value may be obtained via single plant combinations of IR traits conferred by a *B.t.* insecticidal protein such as that of the subject invention, with one or more additional HT traits such as those mentioned above, plus one or more additional input traits (*e.g.* other insect resistance conferred by *B.t.*-derived or other insecticidal proteins, insect resistance conferred by mechanisms such as RNAi and the like, nematode resistance, disease resistance, stress tolerance, improved nitrogen utilization, and the like), or output traits (*e.g.* high oils content, healthy oil composition, nutritional improvement, and the like). Such combinations may be obtained either through conventional breeding (breeding stack) or jointly as a novel transformation event involving the simultaneous introduction of multiple genes (molecular stack). Benefits include the ability to manage insect pests and improved weed control in a crop plant that provides secondary benefits to the producer

and/or the consumer. Thus, the subject invention can be used in combination with other traits to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

Target Pests

[0081] The DIG-3 toxins of the invention are particularly suitable for use in control of Lepidopteran insects. Lepidopterans are an important group of agricultural, horticultural, and household pests which cause a very large amount of damage each year. This insect order encompasses foliar- and root-feeding larvae and adults. Lepidopteran insect pests include, but are not limited to: *Achoria grisella*, *Acleris gloverana*, *Acleris variana*, *Adoxophyes orana*, *Agrotis ipsilon* (black cutworm), *Alabama argillacea*, *Alsophila pometaria*, *Amyelois transitella*, *Anagasta kuehniella*, *Anarsia lineatella*, *Anisota senatoria*, *Antheraea pernyi*, *Anticarsia gemmatalis*, *Archips* sp., *Argyrotaenia* sp., *Athetis mindara*, *Bombyx mori*, *Bucculatrix thurberiella*, *Cadra cautella*, *Choristoneura* sp., *Cochylis hospes*, *Colias eurytheme*, *Corcyra cephalonica*, *Cydia latiferreanus*, *Cydia pomonella*, *Datana integerrima*, *Dendrolimus sibericus*, *Desmia fernalis*, *Diaphania hyalinata*, *Diaphania nitidalis*, *Diatraea grandiosella* (southwestern corn borer), *Diatraea saccharalis* (sugarcane borer), *Ennomos subsignaria*, *Eoreuma loftini*, *Esphestia elutella*, *Erannis tilaria*, *Estigmene acrea*, *Eulia salubricola*, *Eupocoellia ambiguella*, *Eupoecilia ambiguella*, *Euproctis chrysorrhoea*, *Euxoa messoria*, *Galleria mellonella*, *Grapholita molesta*, *Harrisina americana*, *Helicoverpa subflexa*, *Helicoverpa zea* (corn earworm), *Heliothis virescens* (tobacco budworm), *Hemileuca oliviae*, *Homoeosoma electellum* (sunflower head moth), *Hyphantia cunea*, *Keiferia lycopersicella*, *Lambdina fiscellaria fiscellaria*, *Lambdina fiscellaria lugubrosa*, *Leucoma salicis*, *Lobesia botrana*, *Loxagrotis albicosta* (western bean cutworm), *Loxostege sticticalis*, *Lymantria dispar*, *Macalla thyrisalis*, *Malacosoma* sp., *Mamestra brassicae*, *Mamestra configurata* (bertha armyworm), *Manduca quinquemaculata*, *Manduca sexta* (tobacco hornworm), *Maruca testulalis*, *Melanchra picta*, *Operophtera brumata*, *Orgyia* sp., *Ostrinia nubilalis* (European corn borer), *Paleacrita vernata*, *Papiapema nebris* (common stalk borer), *Papilio cresphontes*, *Pectinophora gossypiella*, *Phryganidia californica*, *Phyllonorycter blancardella*, *Pieris napi*, *Pieris rapae*, *Plathypena scabra*, *Platynota flouendana*, *Platynota stultana*, *Platyptilia carduidactyla*, *Plodia interpunctella*, *Plutella xylostella* (diamondback moth), *Pontia protodice*, *Pseudaletia unipuncta* (armyworm), *Pseudoplasia includens*, *Rachiplusia nu* (Argentine looper), *Sabulodes aegrotata*, *Schizura concinna*, *Sitotroga cerealella*, *Spilonta ocellana*, *Spodoptera*

frugiperda (fall armyworm), *Spodoptera exigua* (beet armyworm), *Thaurnstopoea pityocampa*, *Ensola bisselliella*, *Trichoplusia hi*, *Udea rubigalis*, *Xylomyges curiails*, and *Yponomeuta padella*.

[0082] Use of DIG-3 toxins to control Coleopteran pests of crop plants is also contemplated. In some embodiments, Cry proteins may be economically deployed for control of insect pests that include but are not limited to, for example, rootworms such as *Diabrotica undecimpunctata howardi* (southern corn rootworm), *Diabrotica longicornis barberi* (northern corn rootworm), and *Diabrotica virgifera* (western corn rootworm), and grubs such as the larvae of *Cyclocephala borealis* (northern masked chafer), *Cyclocephala immaculate* (southern masked chafer), and *Popillia japonica* (Japanese beetle).

[0083] Use of the DIG-3 toxins to control parasitic nematodes including, but not limited to, root knot nematode (*Meloidogyne icognita*) and soybean cyst nematode (*Heterodera glycines*) is also contemplated.

Antibody detection of DIG-3 toxins

[0084] Anti-toxin antibodies. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in this art. Such antibodies are useful to detect the presence of the DIG-3 toxins.

[0085] Once the *B.t.* insecticidal toxin has been isolated, antibodies specific for the toxin may be raised by conventional methods that are well known in the art. Repeated injections into a host of choice over a period of weeks or months will elicit an immune response and result in significant anti-*B.t.* toxin serum titers. Preferred hosts are mammalian species and more highly preferred species are rabbits, goats, sheep and mice. Blood drawn from such immunized animals may be processed by established methods to obtain antiserum (polyclonal antibodies) reactive with the *B.t.* insecticidal toxin. The antiserum may then be affinity purified by adsorption to the toxin according to techniques known in the art. Affinity purified antiserum may be further purified by isolating the immunoglobulin fraction within the antiserum using procedures known in the art. The resulting material will be a heterogeneous population of immunoglobulins reactive with the *B.t.* insecticidal toxin.

[0086] Anti-*B.t.* toxin antibodies may also be generated by preparing a semi-synthetic immunogen consisting of a synthetic peptide fragment of the *B.t.* insecticidal toxin conjugated to an immunogenic carrier. Numerous schemes and instruments useful for making peptide fragments are well known in the art. Many suitable immunogenic carriers such as bovine serum

albumin or keyhole limpet hemocyanin are also well known in the art, as are techniques for coupling the immunogen and carrier proteins. Once the semi-synthetic immunogen has been constructed, the procedure for making antibodies specific for the *B.t.* insecticidal toxin fragment is identical to those used for making antibodies reactive with natural *B.t.* toxin.

[0087] Anti-*B.t.* toxin monoclonal antibodies (MAbs) are readily prepared using purified *B.t.* insecticidal toxin. Methods for producing MAbs have been practiced for over 20 years and are well known to those of ordinary skill in the art. Repeated intraperitoneal or subcutaneous injections of purified *B.t.* insecticidal toxin in adjuvant will elicit an immune response in most animals. Hyperimmunized B-lymphocytes are removed from the animal and fused with a suitable fusion partner cell line capable of being cultured indefinitely. Preferred animals whose B-lymphocytes may be hyperimmunized and used in the production of MAbs are mammals. More preferred animals are rats and mice and most preferred is the BALB/c mouse strain.

[0088] Numerous mammalian cell lines are suitable fusion partners for the production of hybridomas. Many such lines are available from the American Type Culture Collection (ATCC, Manassas, VA) and commercial suppliers. Preferred fusion partner cell lines are derived from mouse myelomas and the HL-1® Friendly myeloma-653 cell line (Ventrex, Portland, ME) is most preferred. Once fused, the resulting hybridomas are cultured in a selective growth medium for one to two weeks. Two well known selection systems are available for eliminating unfused myeloma cells, or fusions between myeloma cells, from the mixed hybridoma culture. The choice of selection system depends on the strain of mouse immunized and myeloma fusion partner used. The AAT selection system, described by Taggart and Samloff, (1983), may be used; however, the HAT (hypoxanthine, aminopterin, thymidine) selection system, described by Littlefield, (1964), is preferred because of its compatibility with the preferred mouse strain and fusion partner mentioned above. Spent growth medium is screened for immunospecific MAb secretion. Enzyme linked immunosorbent assay (ELISA) procedures are best suited for this purpose; though, radioimmunoassays adapted for large volume screening are also acceptable. Multiple screens designed to consecutively pare down the considerable number of irrelevant or less desired cultures may be performed. Cultures that secrete MAbs reactive with the *B.t.* insecticidal toxin may be screened for cross-reactivity with known *B.t.* insecticidal toxins. MAbs that preferentially bind to the preferred *B.t.* insecticidal toxin may be isotyped using commercially available assays. Preferred MAbs are of the IgG class, and more highly preferred MAbs are of the IgG₁ and IgG_{2a} subisotypes.

[0089] Hybridoma cultures that secrete the preferred MAbs may be sub-cloned several times to establish monoclonality and stability. Well known methods for sub-cloning eukaryotic, non-adherent cell cultures include limiting dilution, soft agarose and fluorescence activated cell sorting techniques. After each subcloning, the resultant cultures preferably are re-assayed for antibody secretion and isotype to ensure that a stable preferred MAb-secreting culture has been established.

[0090] The anti-*B.t.* toxin antibodies are useful in various methods of detecting the claimed *B.t.* insecticidal toxin of the instant invention, and variants or fragments thereof. It is well known that antibodies labeled with a reporting group can be used to identify the presence of antigens in a variety of milieus. Antibodies labeled with radioisotopes have been used for decades in radioimmunoassays to identify, with great precision and sensitivity, the presence of antigens in a variety of biological fluids. More recently, enzyme labeled antibodies have been used as a substitute for radiolabeled antibodies in the ELISA assay. Further, antibodies immunoreactive to the *B.t.* insecticidal toxin of the present invention can be bound to an immobilizing substance such as a polystyrene well or particle and used in immunoassays to determine whether the *B.t.* toxin is present in a test sample.

Detection using nucleic acid probes

[0091] A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are detectable nucleotide sequences. These sequences may be rendered detectable by virtue of an appropriate radioactive label or may be made inherently fluorescent as described in, for example, US Patent No. 6268132. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming strong base-pairing bonds between the two molecules, it can be reasonably assumed that the probe and sample have substantial sequence homology. Preferably, hybridization is conducted under stringent conditions by techniques well-known in the art, as described, for example, in Keller and Manak (1993). Detection of the probe provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying toxin-encoding genes of the subject invention. The nucleotide segments which are used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Nucleic acid hybridization

[0092] As is well known to those skilled in molecular biology, similarity of two nucleic acids can be characterized by their tendency to hybridize. As used herein the terms “stringent conditions” or “stringent hybridization conditions” are intended to refer to conditions under which a probe will hybridize (anneal) 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 different 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.

[0093] 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 pH 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 sulfate) at 37°C and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50°C 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 1X SSC at 55°C 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°C 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.

[0094] 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 thermal melting point (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 conditions, and/or wash conditions can be adjusted to facilitate annealing of 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 T_m for the specific sequence and its complement at a defined ionic strength and pH. However, highly stringent conditions can utilize a hybridization and/or wash at 1°C, 2°C, 3°C, or 4°C lower than the T_m ; moderately stringent conditions can utilize a hybridization and/or wash at 6°C, 7°C, 8°C, 9°C, or 10°C lower than the T_m , and low stringency conditions can utilize a hybridization and/or wash at 11°C, 12°C, 13°C, 14°C, 15°C, or 20°C lower than the T_m .

[0095] T_m (in °C) may be experimentally determined or may be approximated by calculation. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984):

$$T_m(^{\circ}\text{C}) = 81.5^{\circ}\text{C} + 16.6(\log M) + 0.41(\% \text{GC}) - 0.61(\% \text{ formamide}) - 500/L;$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % formamide is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.

[0096] Alternatively, the T_m is described by the following formula (Beltz *et al.*, 1983).

$$T_m(^{\circ}\text{C}) = 81.5^{\circ}\text{C} + 16.6(\log[\text{Na}^+]) + 0.41(\% \text{GC}) - 0.61(\% \text{ formamide}) - 600/L$$

where [Na⁺] is the molarity of sodium ions, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % formamide is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.

[0097] Using the equations, 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) and Ausubel *et al.* (1995) Also see Sambrook *et al.* (1989).

[0098] Hybridization of immobilized DNA on Southern blots with radioactively labeled gene-specific probes may be performed by standard methods Sambrook *et al.*, *supra.*).

Radioactive isotopes used for labeling polynucleotide probes may include ³²P, ³³P, ¹⁴C, or ³H. Incorporation of radioactive isotopes into polynucleotide probe molecules may be done by any of several methods well known to those skilled in the field of molecular biology. (See, *e.g.*

Sambrook *et al.*, *supra*.) In general, hybridization and subsequent washes may be carried out under stringent conditions that allow for detection of target sequences with homology to the claimed toxin encoding genes. For double-stranded DNA gene probes, hybridization may be carried out overnight at 20°-25°C below the T_m of the DNA hybrid in 6X SSPE, 5X Denhardt's Solution, 0.1% SDS, 0.1 mg/mL denatured DNA [20X SSPE is 3M NaCl, 0.2 M NaHPO₄, and 0.02M EDTA (ethylenediamine tetra-acetic acid sodium salt); 100X Denhardt's Solution is 20 gm/L Polyvinylpyrrolidone, 20 gm/L Ficoll type 400 and 20 gm/L Bovine Serum Albumin (fraction V)].

[0100] Washes may typically be carried out as follows:

Twice at room temperature for 15 minutes in 1X SSPE, 0.1% SDS (low stringency wash).

Once at $T_m - 20^\circ\text{C}$ for 15 minutes in 0.2X SSPE, 0.1% SDS (moderate stringency wash).

[0101] For oligonucleotide probes, hybridization may be carried out overnight at 10°-20°C below the T_m of the hybrid in 6X SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/mL denatured DNA. T_m for oligonucleotide probes may be determined by the following formula (Suggs *et al.*, 1981).

$$T_m(^{\circ}\text{C}) = 2(\text{number of T/A base pairs}) + 4(\text{number of G/C base pairs})$$

[0102] Washes may typically be carried out as follows:

Twice at room temperature for 15 minutes 1X SSPE, 0.1% SDS (low stringency wash).

Once at the hybridization temperature for 15 minutes in 1X SSPE, 0.1% SDS (moderate stringency wash).

[0103] Probe molecules for hybridization and hybrid molecules formed between probe and target molecules may be rendered detectable by means other than radioactive labeling. Such alternate methods are intended to be within the scope of this invention.

[0104] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification. Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein. By the use of the term “genetic material” herein, it is meant to include all genes, nucleic acid, DNA and RNA.

[0105] For designations of nucleotide residues of polynucleotides, DNA, RNA, oligonucleotides, and primers, and for designations of amino acid residues of proteins, standard IUPAC abbreviations are employed throughout this document. Nucleic acid sequences are presented in the standard 5' to 3' direction, and protein sequences are presented in the standard amino (N) terminal to carboxy (C) terminal direction.

[0106] Following are examples that illustrate procedures for practicing the invention. It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. All temperatures are in degrees Celsius.

EXAMPLE 1

Isolation of a gene encoding DIG-3 toxin

[0107] Nucleic acid encoding the insecticidal Cry protein designated herein as DIG-3 was isolated from genomic DNA of *B.t.* strain PS46L by PCR using a degenerate forward primer that hybridized to bases 1286 to 1311 of SEQ ID NO:1, and a mismatched reverse primer that hybridized to the complement of bases 2480 to 2499 of SEQ ID NO:1. This pair of primers was used to amplify a fragment of 1214 bp, corresponding to nucleotides 1286 to 2499 of SEQ ID NO:1. This sequence was used as the anchor point to begin genome walking using methods adapted from the GenomeWalker™ Universal Kit (Clontech, Palo Alto, CA). The nucleic acid sequence of a fragment spanning the DIG-3 coding region was determined. SEQ ID NO:1 is the 3771 bp nucleotide sequence encoding the full length DIG-3 protein. SEQ ID NO:2 is the amino acid sequence of the full length DIG-3 protein deduced from SEQ ID NO:1. It is noted that in *Bacillus* species, protein coding regions such as that of SEQ ID NO:1 may initiate with the TTG codon, which translationally represents the amino acid methionine.

EXAMPLE 2

Deletion of Domain I α -helices from DIG-3

[0108] To improve the insecticidal properties of the DIG-3 toxin, serial, step-wise deletions are made, each of which removes part of the N-terminus of the DIG-3 protein. The deletions remove part or all of α -helix 1 and part or all of α -helix 2 in Domain I, while maintaining the structural integrity of α -helix 3 through α -helix 7.

[0109] Deletions were designed as follows. This example utilizes the full length chimeric DNA sequence encoding the full-length DIG-3 protein *e.g.* SEQ ID NO:1 and SEQ ID NO:2, respectively) to illustrate the design principles with 67 specific variants. It utilizes the chimeric sequence of SEQ ID NO:5 (DNA encoding DIG-3 core toxin segment fused to Cry1Ab protoxin segment) to provide an additional 67 specific variants. One skilled in the art will realize that other DNA sequences encoding all or an N-terminal portion of the DIG-3 protein may be similarly manipulated to achieve the desired result. To devise the first deleted variant coding sequence, all of the bases that encode α -helix 1 up to the codon for the proline residue near the beginning of α -helix 2A (*i.e.* P73 for the full length DIG-3 protein of SEQ ID NO:2), are removed. Thus, elimination of bases 1 to 216 of SEQ ID NO:1 removes the coding sequence for amino acids 1 to 72 of SEQ ID NO:2. Reintroduction of a translation initiating ATG (methionine) codon at the beginning (*i.e.* in front of the codon corresponding to amino acid 73 of the full length protein) provides for the deleted variant coding sequence comprising an open reading frame of 3555 bases which encodes a deleted variant DIG-3 protein comprising 1185 amino acids (*i.e.* methionine plus amino acids 73 to 1256 of the full-length DIG-3 protein). Serial, stepwise deletions that remove additional codons for a single amino acid corresponding to residues 73 to 112 of the full-length DIG-3 protein of SEQ ID NO:2 provide variants lacking part or all of α -helix 2A and α -helix 2B. Thus a second designed deleted variant coding sequence requires elimination of bases 1 to 219 of SEQ ID NO:1, thereby removing the coding sequence for amino acids 1-73. Restoration of a functional open reading frame is again accomplished by reintroduction of a translation initiation methionine codon at the beginning of the remaining coding sequence, thus providing for a second deleted variant coding sequence having an open reading frame of 3552 bases encoding a deleted variant DIG-3 protein comprising 1184 amino acids (*i.e.* methionine plus amino acids 74 to 1256 of the full-length DIG-3 protein). The last designed deleted variant coding sequence requires removal of bases 1 to 336 of SEQ ID NO:1,

thus eliminating the coding sequence for amino acids 1 to 112, and, after reintroduction of a translation initiation methionine codon, providing a deletion variant coding sequence having an open reading frame of 3435 bases which encodes a deletion variant DIG-3 protein of 1145 amino acids (*i.e.* methionine plus amino acids 113 to 1256 of the full-length DIG-3 protein). As exemplified, after elimination of the deletion sequence, an initiator methionine codon is added to the beginning of the remaining coding sequence to restore a functional open reading frame. Also as described, an additional glycine codon is to be added between the methionine codon and the codon for the instability-determining amino acid in the instance that removal of the deleted sequence leaves exposed at the N-terminus of the remaining portion of the full-length protein one of the instability-determining amino acids as provided above.

[0110] Table 3 describes specific variants designed in accordance with the strategy described above.

Table 3

Deletion variant protein sequences of the full-length DIG-3 protein of SEQ ID NO:2 and the fusion protein sequence of SEQ ID NO:5.

DIG-3 Deletion Variant	Residues added at NH2 terminus	Residues of SEQ ID NO:2	DIG-3 Deletion Variant	Residues added at NH2 terminus	Residues of SEQ ID NO:5
1	M	73-1256	68	M	73-1188
2	MG	73-1256	69	MG	73-1188
3	M	74-1256	70	M	74-1188
4	MG	74-1256	71	MG	74-1188
5	M	75-1256	72	M	75-1188
6	M	76-1256	73	M	76-1188
7	M	77-1256	74	M	77-1188
8	M	78-1256	75	M	78-1188
9	MG	78-1256	76	MG	78-1188
10	M	79-1256	77	M	79-1188
11	M	80-1256	78	M	80-1188
12	M	81-1256	79	M	81-1188
13	MG	81-1256	80	MG	81-1188
14	M	82-1256	81	M	82-1188
15	MG	82-1256	82	MG	82-1188
16	M	83-1256	83	M	83-1188
17	M	84-1256	84	M	84-1188
18	MG	84-1256	85	MG	84-1188
19	M	85-1256	86	M	85-1188

20	MG	85-1256	87	MG	85-1188
21	M	86-1256	88	M	86-1188
22	M	87-1256	89	M	87-1188
23	M	88-1256	90	M	88-1188
24	M	89-1256	91	M	89-1188
25	MG	89-1256	92	MG	89-1188
26	M	90-1256	93	M	90-1188
27	MG	90-1256	94	MG	90-1188
28	M	91-1256	95	M	91-1188
29	MG	91-1256	96	MG	91-1188
30	M	92-1256	97	M	92-1188
31	M	93-1256	98	M	93-1188
32	M	94-1256	99	M	94-1188
33	M	95-1256	100	M	95-1188
34	MG	95-1256	101	MG	95-1188
35	M	96-1256	102	M	96-1188
36	MG	96-1256	103	MG	96-1188
37	M	97-1256	104	M	97-1188
38	MG	97-1256	105	MG	97-1188
39	M	98-1256	106	M	98-1188
40	MG	98-1256	107	MG	98-1188
41	M	99-1256	108	M	99-1188
42	MG	99-1256	109	MG	99-1188
43	M	100-1256	110	M	100-1188
44	MG	100-1256	111	MG	100-1188
45	M	101-1256	112	M	101-1188
46	MG	101-1256	113	MG	101-1188
47	M	102-1256	114	M	102-1188
48	MG	102-1256	115	MG	102-1188
49	M	103-1256	116	M	103-1188
50	MG	103-1256	117	MG	103-1188
51	M	104-1256	118	M	104-1188
52	M	105-1256	119	M	105-1188
53	MG	105-1256	120	MG	105-1188
54	M	106-1256	121	M	106-1188
55	MG	106-1256	122	MG	106-1188
56	M	107-1256	123	M	107-1188
57	MG	107-1256	124	MG	107-1188
58	M	108-1256	125	M	108-1188
59	MG	108-1256	126	MG	108-1188
60	M	109-1256	127	M	109-1188
61	MG	109-1256	128	MG	109-1188
62	M	110-1256	129	M	110-1188
63	MG	110-1256	130	MG	110-1188
64	M	111-1256	131	M	111-1188

65	MG	111-1256	132	MG	111-1188
66	M	112-1356	133	M	112-1356
67	M	113-1256	134	M	113-1188

[0111] Nucleic acids encoding the toxins described in Table 3 are designed in accordance with the general principles for synthetic genes intended for expression in plants, as discussed above.

EXAMPLE 3

Design of a plant-optimized version of the coding sequence for the DIG-3 *B.t.* insecticidal protein

[0112] A DNA sequence having a plant codon bias was designed and synthesized to produce the DIG-3 protein in transgenic monocot and dicot plants. A codon usage table for maize (*Zea mays* L.) was calculated from 706 protein coding sequences (CDs) obtained from sequences deposited in GenBank. Codon usage tables for tobacco (*Nicotiana tabacum*, 1268 CDs), canola (*Brassica napus*, 530 CDs), cotton (*Gossypium hirsutum*, 197 CDs), and soybean (*Glycine max*; ca. 1000 CDs) were downloaded from data at the website <http://www.kazusa.or.jp/codon/>. A biased codon set that comprises highly used codons common to both maize and dicot datasets, in appropriate weighted average relative amounts, was calculated after omitting any redundant codon used less than about 10% of total codon uses for that amino acid in either plant type. To derive a plant optimized sequence encoding the DIG-3 protein, codon substitutions to the experimentally determined DIG-3 DNA sequence were made such that the resulting DNA sequence had the overall codon composition of the plant-optimized codon bias table. Further refinements of the sequence were made to eliminate undesirable restriction enzyme recognition sites, potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to introduce desired restriction enzyme recognition sites, and to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the plant-biased codon composition. Synthesis of the designed sequence was performed by a commercial vendor (DNA2.0, Menlo Park, CA).

[0113] Additional guidance regarding the production of synthetic genes can be found in, for example, WO 97/13402 and US Patent No. 5380831.

[0114] A plant-optimized DNA sequence encoding the full length DIG-3 toxin is given in SEQ ID NO:3. A dicot-optimized DNA sequence encoding the Cry1Ab protoxin segment is disclosed as SEQ ID NO:6. A maize- optimized DNA sequence encoding the Cry1Ab protoxin segment is disclosed as SEQ ID NO:7.

EXAMPLE 4

Construction of expression plasmids encoding DIG-3 insecticidal toxin and expression in bacterial hosts

[0115] Standard cloning methods were used in the construction of *Pseudomonas fluorescens* (*Pf*) expression plasmids engineered to produce full-length DIG-3 proteins encoded by plant-optimized coding regions. Restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA) and T4 DNA Ligase (Invitrogen) was used for DNA ligation. Plasmid preparations were performed using the NucleoBond® Xtra Kit (Macherey-Nagel Inc, Bethlehem, PA) or the Plasmid Midi Kit® (Qiagen), following the instructions of the suppliers. DNA fragments were purified using the Millipore Ultrafree®-DA cartridge (Billerica, MA) after agarose Tris-acetate gel electrophoresis.

[0116] The basic cloning strategy entailed subcloning the DIG-3 toxin coding sequence (CDS) into pDOW1169 at the *SpeI* and *XhoI* restriction sites, whereby it is placed under the expression control of the *Ptac* promoter and the *rrnBT1T2* terminator from plasmid pKK223-3 (PL Pharmacia, Milwaukee, WI). pDOW1169 is a medium copy plasmid with the RSF1010 origin of replication, a *pyrF* gene, and a ribosome binding site preceding the restriction enzyme recognition sites into which DNA fragments containing protein coding regions may be introduced, (US Application 20080193974). The expression plasmid, designated pDAB4171, was transformed by electroporation into DC454 (a near wild-type *P. fluorescens* strain having mutations $\Delta pyrF$ and $lsc::lacI^{QI}$), or its derivatives, recovered in SOC-Soy hydrolysate medium, and plated on selective medium (M9 glucose agar lacking uracil, Sambrook *et al.*, *supra*). Details of the microbiological manipulations are available in Squires *et al.*, (2004), US Patent Application 20060008877, US Patent Application 20080193974, and US Patent Application 20080058262, incorporated herein by reference. Colonies were first screened by PCR and positive clones were then analyzed by restriction digestion of miniprep plasmid DNA. Plasmid DNA of selected clones containing inserts was sequenced, either by using Big Dye® Terminator version 3.1 as recommended by the supplier (Applied Biosystems/Invitrogen), or by contract with

a commercial sequencing vendor (MWG Biotech, Huntsville, AL). Sequence data were assembled and analyzed using the Sequencher™ software (Gene Codes Corp., Ann Arbor, MI).

[0117] Growth and Expression Analysis in Shake Flasks. Production of DIG-3 toxin for characterization and insect bioassay was accomplished by shake-flask-grown *P. fluorescens* strains harboring expression constructs (*e.g.* clone DP2826). Seed cultures grown in M9 medium supplemented with 1% glucose and trace elements were used to inoculate 50 mL of defined minimal medium with 5% glycerol (Teknova Catalog No. 3D7426, Hollister, CA). Expression of the DIG-3 toxin gene via the *Ptac* promoter was induced by addition of isopropyl- β -D-1-thiogalactopyranoside (IPTG) after an initial incubation of 24 hours at 30° with shaking. Cultures were sampled at the time of induction and at various times post-induction. Cell density was measured by optical density at 600 nm (OD₆₀₀). Other culture media suitable for growth of *Pseudomonas fluorescens* may also be utilized, for example, as described in Huang *et al.* (2007) and US Patent Application 20060008877.

[0118] Cell Fractionation and SDS-PAGE Analysis of Shake Flask Samples. At each sampling time, the cell density of samples was adjusted to OD₆₀₀ = 20 and 1 mL aliquots were centrifuged at 14000 x g for five minutes. The cell pellets were frozen at -80°. Soluble and insoluble fractions from frozen shake flask cell pellet samples were generated using EasyLyse™ Bacterial Protein Extraction Solution (EPICENTRE® Biotechnologies, Madison, WI). Each cell pellet was resuspended in 1 mL EasyLyse™ solution and further diluted 1:4 in lysis buffer and incubated with shaking at room temperature for 30 minutes. The lysate was centrifuged at 14,000 rpm for 20 minutes at 4° and the supernatant was recovered as the soluble fraction. The pellet (insoluble fraction) was then resuspended in an equal volume of phosphate buffered saline (PBS; 11.9 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, pH7.4).

[0119] Samples were mixed 1:1 with 2X Laemmli sample buffer containing β -mercaptoethanol (Sambrook *et al.*, *supra.*) and boiled for 5 minutes prior to loading onto Criterion XT® Bis-Tris 12% gels (Bio-Rad Inc., Hercules, CA.) Electrophoresis was performed in the recommended XT MOPS buffer. Gels were stained with Bio-Safe Coomassie Stain according to the manufacturer's (Bio-Rad) protocol and imaged using the Alpha Innotech Imaging system (San Leandro, CA).

[0120] Inclusion body preparation. Cry protein inclusion body (IB) preparations were performed on cells from *P. fluorescens* fermentations that produced insoluble *B.t.* insecticidal

protein, as demonstrated by SDS-PAGE and MALDI-MS (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry). *P. fluorescens* fermentation pellets were thawed in a 37° water bath. The cells were resuspended to 25% w/v in lysis buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 20 mM EDTA disodium salt (Ethylenediaminetetraacetic acid), 1% Triton X-100, and 5 mM Dithiothreitol (DTT); 5 mL/L of bacterial protease inhibitor cocktail (P8465 Sigma-Aldrich, St. Louis, MO) were added just prior to use). The cells were suspended using a hand-held homogenizer at lowest setting (Tissue Tearor, BioSpec Products, Inc., Bartlesville, OK). Lysozyme (25 mg of Sigma L7651, from chicken egg white) was added to the cell suspension by mixing with a metal spatula, and the suspension was incubated at room temperature for one hour. The suspension was cooled on ice for 15 minutes, then sonicated using a Branson Sonifier 250 (two 1- minute sessions, at 50% duty cycle, 30% output). Cell lysis was checked by microscopy. An additional 25 mg of lysozyme were added if necessary, and the incubation and sonication were repeated. When cell lysis was confirmed via microscopy, the lysate was centrifuged at 11,500 x g for 25 minutes (4°) to form the IB pellet, and the supernatant was discarded. The IB pellet was resuspended with 100 mL lysis buffer, homogenized with the hand-held mixer and centrifuged as above. The IB pellet was repeatedly washed by resuspension (in 50 mL lysis buffer), homogenization, sonication, and centrifugation until the supernatant became colorless and the IB pellet became firm and off-white in color. For the final wash, the IB pellet was resuspended in sterile-filtered (0.22 µm) distilled water containing 2 mM EDTA, and centrifuged. The final pellet was resuspended in sterile-filtered distilled water containing 2 mM EDTA, and stored in 1 mL aliquots at -80°.

[0121] SDS-PAGE analysis and quantitation of protein in IB preparations was done by thawing a 1 mL aliquot of IB pellet and diluting 1:20 with sterile-filtered distilled water. The diluted sample was then boiled with 4X reducing sample buffer [250 mM Tris, pH6.8, 40% glycerol (v/v), 0.4% Bromophenol Blue (w/v), 8% SDS (w/v) and 8% β-Mercapto-ethanol (v/v)] and loaded onto a Novex® 4-20% Tris-Glycine, 12+2 well gel (Invitrogen) run with 1X Tris/Glycine/SDS buffer (BioRad). The gel was run for 60 min at 200 volts then stained with Coomassie Blue (50% G-250/50% R-250 in 45% methanol, 10% acetic acid), and destained with 7% acetic acid, 5% methanol in distilled water. Quantification of target bands was done by comparing densitometric values for the bands against Bovine Serum Albumin (BSA) samples run on the same gel to generate a standard curve.

[0122] Solubilization of Inclusion Bodies. Six mL of inclusion body suspension from Pf clone DP2826 (containing 32 mg/mL of DIG-3 protein) were centrifuged on the highest setting of an Eppendorf model 5415C microfuge (approximately 14,000 x g) to pellet the inclusions. The storage buffer supernatant was removed and replaced with 25 mL of 100 mM sodium carbonate buffer, pH11, in a 50 mL conical tube. Inclusions were resuspended using a pipette and vortexed to mix thoroughly. The tube was placed on a gently rocking platform at 4° overnight to extract the target protein. The extract was centrifuged at 30,000 x g for 30 min at 4°, and the resulting supernatant was concentrated 5-fold using an Amicon Ultra-15 regenerated cellulose centrifugal filter device (30,000 Molecular Weight Cutoff; Millipore). The sample buffer was then changed to 10 mM CAPS [3-(cyclohexamino)1-propanesulfonic acid] pH 10, using disposable PD-10 columns (GE Healthcare, Piscataway, NJ).

[0123] Gel electrophoresis. The concentrated extract was prepared for electrophoresis by diluting 1:50 in NuPAGE® LDS sample buffer (Invitrogen) containing 5 mM dithiothreitol as a reducing agent and heated at 95° for 4 minutes. The sample was loaded in duplicate lanes of a 4-12% NuPAGE® gel alongside five BSA standards ranging from 0.2 to 2 µg/lane (for standard curve generation). Voltage was applied at 200V using MOPS SDS running buffer (Invitrogen) until the tracking dye reached the bottom of the gel. The gel was stained with 0.2% Coomassie Blue G-250 in 45% methanol, 10% acetic acid, and destained, first briefly with 45% methanol, 10% acetic acid, and then at length with 7% acetic acid, 5% methanol until the background cleared. Following destaining, the gel was scanned with a Biorad Fluor-S MultiImager. The instrument's Quantity One v.4.5.2 Software was used to obtain background-subtracted volumes of the stained protein bands and to generate the BSA standard curve that was used to calculate the concentration of DIG-3 protein in the stock solution.

EXAMPLE 5

Insecticidal activity of modified DIG-3 protein produced in *Pseudomonas fluorescens*

[0124] DIG-3 *B.t.* insecticidal toxin was demonstrated to be active on Lepidopteran species including the European corn borer (ECB; *Ostrinia nubilalis* (Hübner)), cry1F-resistant ECB (rECB), diamondback moth (DBM; *Plutella xylostella* (Linnaeus)), cry1A-resistant DBM (rDBM), corn earworm (CEW; *Helicoverpa zea* (Boddie)), black cutworm (BCW; *Agrotis ipsilon* (Hufnagel)), tobacco budworm (TBW; *Heliothis virescens* (Fabricius)), and cabbage looper (CL; *Trichoplusia ni* (Hübner)). DIG-3 protein was also tested for activity on fall

armyworm (FAW, *Spodoptera frugiperda*), Cry1F-resistant FAW (rFAW) and western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte).

[0125] Sample preparation and bioassays. Inclusion body preparations in 10 mM CAPS pH10 were diluted appropriately in 10 mM CAPS pH 10, and all bioassays contained a control treatment consisting of this buffer, which served as a background check for mortality or growth inhibition.

[0126] Protein concentrations in bioassay buffer were estimated by gel electrophoresis using BSA to create a standard curve for gel densitometry, which was measured using a BioRad imaging system (Fluor-S MultiImager with Quantity One software version 4.5.2). Proteins in the gel matrix were stained with Coomassie Blue-based stain and destained before reading.

[0127] Purified proteins were tested for insecticidal activity in bioassays conducted with neonate Lepidopteran larvae on artificial insect diet. Larvae of BCW, CEW, CL, DBM, rDBM, ECB, FAW and TBW were hatched from eggs obtained from a colony maintained by a commercial insectary (Benzon Research Inc., Carlisle, PA). WCR eggs were obtained from Crop Characteristics, Inc. (Farmington, MN). Larvae of rECB and rFAW were hatched from eggs harvested from proprietary colonies (Dow AgroSciences LLC, Indianapolis, IN).

[0128] The bioassays were conducted in 128-well plastic trays specifically designed for insect bioassays (C-D International, Pitman, NJ). Each well contained 1.0 mL of Multi-species Lepidoptera diet (Southland Products, Lake Village, AR). A 40 μL aliquot of protein sample was delivered by pipette onto the 1.5 cm^2 diet surface of each well (26.7 $\mu\text{L}/\text{cm}^2$). Diet concentrations were calculated as the amount (ng) of DIG-3 protein per square centimeter (cm^2) of surface area in the well. The treated trays were held in a fume hood until the liquid on the diet surface had evaporated or was absorbed into the diet.

[0129] Within a few hours of eclosion, individual larvae were picked up with a moistened camel hair brush and deposited on the treated diet, one larva per well. The infested wells were then sealed with adhesive sheets of clear plastic, vented to allow gas exchange (C-D International, Pitman, NJ). Bioassay trays were held under controlled environmental conditions (28°C, ~40% Relative Humidity, 16:8 [Light:Dark]) for 5 days, after which the total number of insects exposed to each protein sample, the number of dead insects, and the weight of surviving insects were recorded. Percent mortality and percent growth inhibition were calculated for each treatment. Growth inhibition (GI) was calculated as follows:

$$GI = [1 - (TWIT/TNIT)/(TWIBC/TNIBC)]$$

where TWIT is the Total Weight of Insects in the Treatment,

TNIT is the Total Number of Insects in the Treatment

TWIBC is the Total Weight of Insects in the Background Check (Buffer control), and

TNIBC is the Total Number of Insects in the Background Check (Buffer control).

[0130] The GI_{50} was determined to be the concentration of DIG-3 protein in the diet at which the GI value was 50%. The LC_{50} (50% Lethal Concentration) was recorded as the concentration of DIG-3 protein in the diet at which 50% of test insects were killed. Statistical analysis (One-way ANOVA) was done using JMP software (SAS, Cary, NC)

[0131] Table 6 presents the results of bioassay tests of DIG-3 protein on European corn borer and cry1F-resisant European corn borer (rECB). An unexpected and surprising finding is that the rECB test insects were as susceptible to the action of DIG-3 protein as were the wild type ECB insects.

Table 6

LC_{50} and GI_{50} values calculated for ECB and rECB, with Confidence Intervals (CI) of 95%

Insect	LC_{50} (ng/cm ²)	95% CI	GI_{50} (ng/cm ²)	95% CI
ECB	591.9	308.1-1315.3	122.6	45.6-328.4
rECB	953.6	534.1-1953.6	270.9	53.0-1382.2

[0132] Table 7 presents the results of bioassays on a broad spectrum of Lepidopteran and a Coleopteran pest (WCR). The DIG-3 protein has unexpected and surprising activity on diamondback moth as well as rDBM. Further the DIG-3 Cry protein is effective in controlling the growth of several other Lepidopteran insects.

Table 7

Insecticidal and growth inhibitory effects of DIG-3 protein ingested by test insects

Test Insect	Response at 9000 ng/cm ²	Statistical Analysis*
DBM	100% mortality	
rDBM	100% mortality	
CL	75% mortality, significant GI	(GI) $P < 0.001$, $df = 1$, $\alpha = 0.05$
CEW	Significant GI	(GI) $P = 0.02$, $df = 1$, $\alpha = 0.05$
TBW	Visible GI, some mortality	Not available
BCW	Visible GI	Not available
FAW	No activity observed	
rFAW	No activity observed	
WCR	No activity observed	

*GI = Growth Inhibition. P-value = test statistic. df = Degrees of Freedom, α (alpha) level 0.05 = the level of test significance.

EXAMPLE 6

Agrobacterium transformation

[0133] Standard cloning methods are used in the construction of binary plant transformation and expression plasmids. Restriction endonucleases and T4 DNA Ligase are obtained from NEB. Plasmid preparations are performed using the NucleoSpin® Plasmid Preparation kit or the NucleoBond® AX Xtra Midi kit (both from Macherey-Nagel), following the instructions of the manufacturers. DNA fragments are purified using the QIAquick® PCR Purification Kit or the QIAEX II® Gel Extraction Kit (both from Qiagen) after gel isolation.

[0134] DNA fragments comprising nucleotide sequences that encode the DIG-3 protein in native or modified forms, or fragments thereof, may be synthesized by a commercial vendor (*e.g.* DNA2.0, Menlo Park, CA) and supplied as cloned fragments in standard plasmid vectors, or may be obtained by standard molecular biology manipulation of other constructs containing appropriate nucleotide sequences. Unique restriction sites internal to a DIG-3 coding region may be identified and DNA fragments comprising the sequences between the restriction sites of the DIG-3 coding region may be synthesized, each such fragment encoding a specific deletion, insertion or other DIG-3 variation. The DNA fragments encoding the modified DIG-3 fragments may be joined to other DIG-3 coding region fragments or other Cry coding region fragments at appropriate restriction sites to obtain a coding region encoding the desired full-length DIG-3 protein, deleted or variant DIG-3 protein, or fused protein. For example, one may identify an appropriate restriction recognition site at the start of a first DIG-3 coding region, and a second restriction site internal to the DIG-3 coding region. Cleavage of this first DIG-3 coding region at these restriction sites would generate a DNA fragment comprising part of the first DIG-3 coding region. A second DNA fragment flanked by analogously-situated compatible restriction sites specific for another DIG-3 coding region or other Cry coding region may be used in combination with the first DNA restriction fragment to construct a variant or fused clone.

[0135] In a non-limiting example, a basic cloning strategy may be to subclone full length or modified DIG-3 coding sequences (CDS) into a plant expression plasmid at *NcoI* and *SacI* restriction sites. The resulting plant expression cassettes containing the appropriate DIG-3 coding region under the control of plant expression elements, (*e.g.*, plant expressible promoters,

3' terminal transcription termination and polyadenylate addition determinants, and the like) are subcloned into a binary vector plasmid, utilizing, for example, Gateway[®] technology or standard restriction enzyme fragment cloning procedures. LR Clonase[™] (Invitrogen) for example, may be used to recombine the full length and modified gene plant expression cassettes into a binary plant transformation plasmid if the Gateway[®] technology is utilized. It is convenient to employ a binary plant transformation vector that harbors a bacterial gene that confers resistance to the antibiotic spectinomycin when the plasmid is present in *E. coli* and *Agrobacterium* cells. It is also convenient to employ a binary vector plasmid that contains a plant-expressible selectable marker gene that is functional in the desired host plants. Examples of plant-expressible selectable marker genes include but are not limited to those that encode the aminoglycoside phosphotransferase gene (*aphII*) of transposon Tn5, which confers resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which code for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos), imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorosulfuron, bromoxynil, dalapon and the like.

[0136] Electro-competent cells of *Agrobacterium tumefaciens* strain Z707S (a streptomycin-resistant derivative of Z707; Hepburn *et al.*, 1985) are prepared and transformed using electroporation (Weigel and Glazebrook, 2002). After electroporation, 1 mL of YEP broth (gm/L: yeast extract, 10; peptone, 10; NaCl, 5) are added to the cuvette and the cell-YEP suspension is transferred to a 15 mL culture tube for incubation at 28° in a water bath with constant agitation for 4 hours. The cells are plated on YEP plus agar (25 gm/L) with spectinomycin (200 µg/mL) and streptomycin (250 µg/mL) and the plates are incubated for 2-4 days at 28°. Well separated single colonies are selected and streaked onto fresh YEP + agar plates with spectinomycin and streptomycin as before, and incubated at 28° for 1-3 days.

[0137] The presence of the DIG-3 gene insert in the binary plant transformation vector is performed by PCR analysis using vector-specific primers with template plasmid DNA prepared from selected *Agrobacterium* colonies. The cell pellet from a 4 mL aliquot of a 15 mL overnight culture grown in YEP with spectinomycin and streptomycin as before is extracted using Qiagen Spin[®] Mini Preps, performed per manufacturer's instructions. Plasmid DNA from the binary vector used in the *Agrobacterium* electroporation transformation is included as a control. The PCR reaction is completed using Taq DNA polymerase from Invitrogen per manufacture's instructions at 0.5X concentrations. PCR reactions are carried out in a MJ Research Peltier

Thermal Cycler programmed with the following conditions: Step 1) 94° for 3 minutes; Step 2) 94° for 45 seconds; Step 3) 55° for 30 seconds; Step 4) 72° for 1 minute per kb of expected product length; Step 5) 29 times to Step 2; Step 6) 72° for 10 minutes. The reaction is maintained at 4° after cycling. The amplification products are analyzed by agarose gel electrophoresis (*e.g.* 0.7 % to 1% agarose, w/v) and visualized by ethidium bromide staining. A colony is selected whose PCR product is identical to the plasmid control.

[0138] Alternatively, the plasmid structure of the binary plant transformation vector containing the DIG-3 gene insert is performed by restriction digest fingerprint mapping of plasmid DNA prepared from candidate *Agrobacterium* isolates by standard molecular biology methods well known to those skilled in the art of *Agrobacterium* manipulation.

[0139] Those skilled in the art of obtaining transformed plants *via Agrobacterium*-mediated transformation methods will understand that other *Agrobacterium* strains besides Z707S may be used to advantage, and the choice of strain may depend upon the identity of the host plant species to be transformed.

EXAMPLE 7

Production of DIG-3 *B.t.* insecticidal proteins and variants in dicot plants

[0140] *Arabidopsis* Transformation. *Arabidopsis thaliana* Col-01 is transformed using the floral dip method (Weigel and Glazebrook, 2002). The selected *Agrobacterium* colony is used to inoculate 1 mL to 15 mL cultures of YEP broth containing appropriate antibiotics for selection. The culture is incubated overnight at 28° with constant agitation at 220 rpm. Each culture is used to inoculate two 500 mL cultures of YEP broth containing appropriate antibiotics for selection and the new cultures are incubated overnight at 28° with constant agitation. The cells are pelleted at approximately 8700 x g for 10 minutes at room temperature, and the resulting supernatant is discarded. The cell pellet is gently resuspended in 500 mL of infiltration media containing: 1/2x Murashige and Skoog salts (Sigma-Aldrich)/Gamborg's B5 vitamins (Gold BioTechnology, St. Louis, MO), 10% (w/v) sucrose, 0.044 µM benzylaminopurine (10 µL/liter of 1 mg/mL stock in DMSO) and 300 µL/liter Silwet L-77. Plants approximately 1 month old are dipped into the media for 15 seconds, with care taken to assure submergence of the newest inflorescence. The plants are then laid on their sides and covered (transparent or opaque) for 24 hours, washed with water, and placed upright. The plants are grown at 22°, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds are harvested.

[0141] Arabidopsis Growth and Selection. Freshly harvested T1 seed is allowed to dry for at least 7 days at room temperature in the presence of desiccant. Seed is suspended in a 0.1% agar/water (Sigma-Aldrich) solution and then stratified at 4° for 2 days. To prepare for planting, Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) in 10.5 inch x 21 inch germination trays (T.O. Plastics Inc., Clearwater, MN) is covered with fine vermiculite, sub-irrigated with Hoagland's solution (Hoagland and Arnon, 1950) until wet, then allowed to drain for 24 hours. Stratified seed is sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days. Seeds are germinated and plants are grown in a Conviron (Models CMP4030 or CMP3244; Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu\text{mol}/\text{m}^2\text{sec}$ under constant temperature (22°) and humidity (40-50%). Plants are initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

[0142] The domes are removed 5-6 days post sowing and plants are sprayed with a chemical selection agent to kill plants germinated from nontransformed seeds. For example, if the plant expressible selectable marker gene provided by the binary plant transformation vector is a *pat* or *bar* gene (Wehrmann *et al.*, 1996), transformed plants may be selected by spraying with a 1000X solution of Finale (5.78% glufosinate ammonium, Farnam Companies Inc., Phoenix, AZ.). Two subsequent sprays are performed at 5-7 day intervals. Survivors (plants actively growing) are identified 7-10 days after the final spraying and transplanted into pots prepared with Sunshine Mix LP5. Transplanted plants are covered with a humidity dome for 3-4 days and placed in a Conviron under the above-mentioned growth conditions.

[0143] Those skilled in the art of dicot plant transformation will understand that other methods of selection of transformed plants are available when other plant expressible selectable marker genes (*e.g.* herbicide tolerance genes) are used.

[0144] Insect Bioassays of transgenic *Arabidopsis*. Transgenic *Arabidopsis* lines expressing modified Cry proteins are demonstrated to be active against sensitive insect species in artificial diet overlay assays. Protein extracted from transgenic and non-transgenic *Arabidopsis* lines is quantified by appropriate methods and sample volumes are adjusted to normalize protein concentration. Bioassays are conducted on artificial diet as described above. Non-transgenic *Arabidopsis* and/or buffer and water are included in assays as background check treatments.

EXAMPLE 8

Agrobacterium transformation for generation of superbinary vectors

[0145] The *Agrobacterium* superbinary system is conveniently used for transformation of monocot plant hosts. Methodologies for constructing and validating superbinary vectors are well disclosed and incorporated herein by reference (Operating Manual for Plasmid pSB1, Version 3.1, available from Japan Tobacco, Inc., Tokyo, Japan). Standard molecular biological and microbiological methods are used to generate superbinary plasmids. Verification/validation of the structure of the superbinary plasmid is done using methodologies as described above for binary vectors, and may be modified as suggested in the Operating Manual for Plasmid pSB1.

EXAMPLE 9

Production of DIG-3 *B.t.* insecticidal proteins and variants in monocot plants

[0146] Agrobacterium-Mediated Transformation of Maize. Seeds from a High II F₁ cross (Armstrong *et al.*, 1991) are planted into 5-gallon-pots containing a mixture of 95% Metro-Mix 360 soilless growing medium (Sun Gro Horticulture, Bellevue, WA) and 5% clay/loam soil. The plants are grown in a greenhouse using a combination of high pressure sodium and metal halide lamps with a 16:8 hour Light:Dark photoperiod. For obtaining immature F₂ embryos for transformation, controlled sib-pollinations are performed. Immature embryos are isolated at 8-10 days post-pollination when embryos are approximately 1.0 to 2.0 mm in size.

[0147] Infection and co-cultivation. Maize ears are surface sterilized by scrubbing with liquid soap, immersing in 70% ethanol for 2 minutes, and then immersing in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes before being rinsed with sterile water. A suspension of *Agrobacterium* cells containing a superbinary vector is prepared by transferring 1-2 loops of bacteria grown on YEP solid medium containing 100 mg/L spectinomycin, 10 mg/L tetracycline, and 250 mg/L streptomycin at 28° for 2-3 days into 5 mL of liquid infection medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins (Chu *et al.*, 1975), 1.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 68.5 gm/L sucrose, 36.0 gm/L glucose, 6 mM L-proline, pH 5.2) containing 100 µM acetosyringone. The solution was vortexed until a uniform suspension was achieved, and the concentration is adjusted to a final density of about 200 Klett units, using a Klett-Summerson colorimeter with a purple filter, or an optical density of approximately 0.4 at 550 nm. Immature embryos are isolated directly into a micro centrifuge tube containing 2 mL of the infection medium. The medium is removed and replaced with 1 mL

of the *Agrobacterium* solution with a density of 200 Klett units, and the *Agrobacterium* and embryo solution is incubated for 5 minutes at room temperature and then transferred to co-cultivation medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 gm/L sucrose, 6 mM L-proline, 0.85 mg/L AgNO₃, 100 µM acetosyringone, 3.0 gm/L Gellan gum (*PhytoTechnology Laboratories*., Lenexa, KS), pH 5.8) for 5 days at 25° under dark conditions.

[0148] After co-cultivation, the embryos are transferred to selective medium after which transformed isolates are obtained over the course of approximately 8 weeks. For selection of maize tissues transformed with a superbinary plasmid containing a plant expressible *pat* or *bar* selectable marker gene, an LS based medium (LS Basal medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 gm/L MES (2-(N-morpholino)ethanesulfonic acid monohydrate; *PhytoTechnologies Labr.*), 30.0 gm/L sucrose, 6 mM L-proline, 1.0 mg/L AgNO₃, 250 mg/L cefotaxime, 2.5 gm/L Gellan gum, pH 5.7) is used with Bialaphos (Gold BioTechnology). The embryos are transferred to selection media containing 3 mg/L Bialaphos until embryogenic isolates were obtained. Recovered isolates are bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

[0149] Those skilled in the art of maize transformation will understand that other methods of selection of transformed plants are available when other plant expressible selectable marker genes (*e.g.* herbicide tolerance genes) are used.

[0150] Regeneration and seed production. For regeneration, the cultures are transferred to “28” induction medium (MS salts and vitamins, 30 gm/L sucrose, 5 mg/L Benzylaminopurine, 0.25 mg/L 2, 4-D, 3 mg/L Bialaphos, 250 mg/L cefotaxime, 2.5 gm/L Gellan gum, pH 5.7) for 1 week under low-light conditions (14 µEm⁻²s⁻¹) then 1 week under high-light conditions (approximately 89 µEm⁻²s⁻¹). Tissues are subsequently transferred to “36” regeneration medium (same as induction medium except lacking plant growth regulators). When plantlets grow to 3-5 cm in length, they were transferred to glass culture tubes containing SHGA medium (Schenk and Hildebrandt salts and vitamins (1972); *PhytoTechnologies Labr.*), 1.0 gm/L *myo*-inositol, 10 gm/L sucrose and 2.0 gm/L Gellan gum, pH 5.8) to allow for further growth and development of the shoot and roots. Plants are transplanted to the same soil mixture as described earlier herein and grown to flowering in the greenhouse. Controlled pollinations for seed production are conducted.

EXAMPLE 10

Bioassay of transgenic maize

[0151] Bioactivity of the DIG-3 protein and variants produced in plant cells is demonstrated by conventional bioassay methods (see, for example Huang *et al.*, 2006). One is able to demonstrate efficacy, for example, by feeding various plant tissues or tissue pieces derived from a plant producing a DIG-3 toxin to target insects in a controlled feeding environment. Alternatively, protein extracts may be prepared from various plant tissues derived from a plant producing the DIG-3 toxin and incorporate the extracted proteins in an artificial diet bioassay as previously described herein. It is to be understood that the results of such feeding assays are to be compared to similarly conducted bioassays that employ appropriate control tissues from host plants that do not produce the DIG-3 protein or variants, or to other control samples.

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Claims

1. An isolated polypeptide comprising a core toxin segment selected from the group consisting of
 - (a) a polypeptide comprising the amino acid sequence of residues 113 to 643 of SEQ ID NO: 2;
 - (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 113 to 643 of SEQ ID NO:2;
 - (c) a polypeptide comprising an amino acid sequence of residues 113 to 643 of SEQ ID NO: 2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO: 2;or an insecticidally active fragment thereof.
2. The isolated polypeptide of claim 1 comprising a core toxin segment selected from the group consisting of
 - (a) a polypeptide comprising the amino acid sequence of residues 73 to 643 of SEQ ID NO: 2;
 - (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 73 to 643 of SEQ ID NO:2;
 - (c) a polypeptide comprising an amino acid sequence of residues 73 to 643 of SEQ ID NO: 2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO: 2;or an insecticidally active fragment thereof.
3. The isolated polypeptide of claim 1 comprising a core toxin segment selected from the group consisting of
 - (a) a polypeptide comprising the amino acid sequence of residues 1 to 643 of SEQ ID NO: 2;
 - (b) a polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 1 to 643 of SEQ ID NO:2;

(c) a polypeptide comprising an amino acid sequence of residues 1 to 643 of SEQ ID NO: 2 with up to 20 amino acid substitutions, deletions, or modifications that do not adversely affect expression or activity of the toxin encoded by SEQ ID NO: 2;

or an insecticidally active fragment thereof.

4. A plant comprising the polypeptide of claim 1.
5. A method for controlling a pest population comprising contacting said population with a pesticidally effective amount of the polypeptide of claim 1.
7. An isolated nucleic acid that encodes a polypeptide of claim 1.
8. The isolated nucleic acid of claim 7 of SEQ ID NO: 1 or SEQ ID NO:3.
9. A polypeptide of claim 1 of SEQ ID NO: 2 or SEQ ID NO:5.
10. A DNA construct comprising the nucleotide sequence of claim 1 operably linked to a promoter that is not derived from *Bacillus thuringiensis* and is capable of driving expression in a plant.
11. A transgenic plant that comprises the DNA construct of claim 10 stably incorporated into its genome.
12. A method for protecting a plant from a pest comprising introducing into said plant the construct of claim 10.
13. The polypeptide of claim 1 having activity against European corn borer.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/028381

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/325 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUO WHITE-SHANG ET AL: "Cloning of two new cry genes from <i>Bacillus thuringiensis</i> subsp. <i>wuhanensis</i> strain" CURRENT MICROBIOLOGY, vol. 40, no. 4, April 2000 (2000-04), pages 227-232, XP002589373 ISSN: 0343-8651 approx. 90% identity to aa 113-643, 73-643 and 1-643 of SEQ ID NO:2; the whole document ----- -/--	1-13

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

7 July 2010

Date of mailing of the international search report

22/07/2010

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/028381

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 1 October 2002 (2002-10-01), "SubName: Full=CryIBII;" XP002590686 retrieved from EBI accession no. UNIPROT:Q8KNY2 Database accession no. Q8KNY2 compound -----	1-13
A	WO 99/24581 A2 (MYCOGEN CORP [US]) 20 May 1999 (1999-05-20) 85.5% sequence identity to SEQ ID NO:5 -----	1-13
A	WO 2004/020636 A1 (MONSANTO TECHNOLOGY LLC [US]; BOGDANOVA NATALIA N [US]; ROMANO CHARLES) 11 March 2004 (2004-03-11) 85% sequence identity to SEQ ID NO:1 and 71% identity to SEQ ID NO:3 -----	1-13
A	WO 2008/145406 A1 (BAYER BIOSCIENCE NV [BE]; GOSSELE VERONIQUE [BE]; SAEY BERNADETTE [BE]) 4 December 2008 (2008-12-04) -----	1-13
A	US 2009/023653 A1 (MALVAR THOMAS [US] ET AL) 22 January 2009 (2009-01-22) -----	1-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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