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(54) Title: DIG-305 INSECTICIDAL CRY TOXINS

(57) Abstract: DIG-305 insecticidal protein toxins, polynucleotides encoding such toxins, and transgenic plants that produce such toxins are disclosed. Also disclosed are methods for using such toxins to control insect pests in plants, especial crop plants. Methods for preparing transgenic plants expressing the protein toxins and methods for detecting the claimed toxins and polynucleotides in transgenic plants are disclosed.



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DIG-305 INSECTICIDAL CRY TOXINS

FIELD OF THE DISCLOSURE

[0001] The present invention relates generally to the field of molecular biology as applied to agricultural sciences. Methods of making and using the claimed nucleic and amino acid sequences in the development of t incorporated protectants in transgenic plant cells and plants are disclosed herein.

BACKGROUND OF THE DISCLOSURE

[0002] *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. Some Cry toxins have been shown to have activity against nematodes. An extensive list of delta endotoxins is maintained and regularly updated at the *Bacillus thuringiensis* Toxin Nomenclature web site maintained by Neil Crickmore. (See Crickmore et al. 1998, page 808).

[0003] Coleopterans are a significant group of agricultural pests that cause extensive damage to crops each year. Examples of coleopteran pests include corn rootworm, alfalfa weevil, boll weevil, and Japanese beetle. The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is one of the most devastating corn rootworm species in North America and is a particular concern in corn-growing areas of the Midwestern United States. The northern corn rootworm (NCR), *Diabrotica barberi* Smith and Lawrence, is a closely-related species that co-inhabits much of the same range as WCR. There are several other related subspecies of *Diabrotica* that are significant pests in North America: the Mexican corn rootworm (MCR), *D. virgifera zea* Krysan and Smith; the southern corn rootworm (SCR), *D. undecimpunctata howardi* Barber; *D. balteata* LeConte; *D. undecimpunctata tenella*; and *D. u. undecimpunctata* Mannerheim. The United States Department of Agriculture currently estimates that corn rootworms cause \$1 billion in lost revenue each year, including \$800 million in yield loss and \$200 million in treatment costs. Cry toxins, including members of the Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C (Frankenhuyzen, 2009) families have insecticidal activity against coleopteran insects.

[0004] Although production of the currently-deployed Cry proteins in transgenic plants can provide robust protection against the aforementioned pests, thereby protecting grain yield, adult pests have emerged in artificial infestation trials, indicating less than complete larval insect control. Additionally, development of resistant insect populations threatens the

long-term durability of Cry proteins in insect pest control. Lepidopteran insects resistant to Cry proteins have developed in the field for *Plutella xylostella* (Tabashnik, 1994), *Trichoplusia ni* (Janmaat and Myers, 2003, 2005), and *Helicoverpa zea* (Tabashnik *et al.*, 2008). Coleopteran insects likewise have developed resistance in the field to Cry proteins (Gassman *et al.* PLoS ONE July 2011 | Volume 6 | Issue 7 | e22629). 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.

[0005] There is interest in the development of new Cry proteins that provide additional tools for management of coleopteran insect pests. Cry proteins with different modes of action produced in combination in transgenic plants would prevent the development of insect resistance and protect the long term utility of *B.t.* technology for insect pest control.

BRIEF SUMMARY OF THE DISCLOSURE

[0006] The present invention is based on the discovery of insecticidal toxins based on the Cry protein toxin designated herein as DIG-305, including variants of DIG-305, 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 express the toxins. The predicted amino acid sequence of native DIG-305 toxin in SEQ ID NO:2 indicates that DIG-305 is best classified to the Cry32 family.

[0007] As described in Example 1, a nucleic acid encoding the DIG-305 protein was discovered and isolated from a *B.t.* strain internally designated by Dow AgroSciences LLC as PS246F10 also known as DBt11519. 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 nucleic acid sequence encoding DIG-305 toxin is given in SEQ ID NO:1. A BLAST search using the insecticidal protein sequence as a query found that DIG-305 toxin protein has 89% sequence identity to the closest insecticidal toxin Cry32Ca1 (BAB78602) and 91% sequence identity to the closest publically disclosed sequence (AGU13873, US20140096281). Thus, DIG-305 represents a new subclass within the Cry32 family of proteins.

[0008] The DIG-305 toxins can be used alone or in combination with other Cry toxins, such as Cry34Ab1/Cry35Ab1 (DAS-59122-7), Cry3Bb1 (MON88017), Cry3A (MIR604), chimeric Cry3A/Cry1Ab (eCry3.1Ab, FR8A, Event 5307, WO 2008/121633 A1), CryET33 and CryET34, Vip1A, CryIIa, CryET84, CryET80, CryET76, CryET71, CryET69, CryET75, CryET39, CryET79, TIC809, TIC810, and CryET74 to control the development of resistant Coleopteran insect populations. Further, DIG-305 toxins can be used alone or in combination with other Cry toxins that control the development of other pest populations, such as, for example, Cry1F, Cry1Ab, Vip Cry2A, Cry1Da, CryIIa, and Cry1Ac to control the development of lepidopteran resistant insect populations.

[0009] DIG-305 insecticidal toxins may also be used in combination with RNAi methodologies for control of other insect pests. For example, DIG-305 insecticidal toxins can be used in transgenic plants in combination with a dsRNA for suppression of an essential gene in WCR or another insect pest (Baum *et. al.*, 2007). Such target genes include, for example, vacuolar ATPase, ARF-1, Act42A, CHD3, EF-1 α , ROP, RNAPII, and TFIIB in corn rootworm. An example of a suitable target gene is vacuolar ATPase, as disclosed in WO2007035650.

[0010] In one embodiment, the invention provides an isolated, treated, or formulated DIG-305 insecticidal toxin polypeptide comprising a core toxin segment selected from the group consisting of (a) the amino acid sequence of residues from approximately 2 to approximately 685 of SEQ ID NO:2; (b) an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues from approximately 2 to approximately 685 of SEQ ID NO:2; and (c) an amino acid sequence of residues from approximately 2 to approximately 685 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 of SEQ ID NO:2; or an insecticidal active fragment of either (a), (b), or (c).

[0011] In certain embodiments the DIG-305 insecticidal toxin polypeptide comprises (a') the amino acid sequence of residues from approximately 2 to 685 of SEQ ID NO:2; (b') an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues from approximately 2 to 685 of SEQ ID NO:2; and (c') an amino acid sequence of residues from approximately 2 to 685 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 of SEQ ID NO:2; or an insecticidal active fragment of either (a'), (b') or (c'). In further embodiments, the DIG-305 insecticidal toxin polypeptide of (a), (b), (c), (a'), (b') or

(c') can be linked to a C-terminal protoxin, e.g., the C-terminal protoxin of cry1Ab or cry1Ac/cry1Ab chimeric toxin.

[0012] In another embodiment the invention provides an isolated, treated, or formulated DIG-305 insecticidal toxin polypeptide comprising a DIG-305 core toxin segment selected from the group consisting of a (a) polypeptide comprising the amino acid sequence of residues 1 to 1241 of SEQ ID NO:2; (b) polypeptide comprising an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 1 to 1241 of SEQ ID NO:2; and (c) polypeptide comprising an amino acid sequence of residues 1 to 1241 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 of SEQ ID NO:2; or an insecticidal active fragment of either (a), (b), or (c).

[0013] In another embodiment the invention provides a plant comprising a DIG-305 insecticidal toxin disclosed herein. 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-305 insecticidal toxin disclosed herein. In another embodiment the invention provides an isolated nucleic acid that encodes a DIG-305 insecticidal toxin disclosed herein. In another embodiment the invention provides a DNA construct comprising a nucleotide sequence that encodes a DIG-305 insecticidal toxin operably linked to a heterologous 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.

[0014] By “isolated” applicants mean that the nucleotide or polypeptide molecules have been removed from their native environment and have been placed in a different environment by the hand of man. Thus, isolated nucleotide and polypeptide molecules include DNA or protein molecules that have been purified, concentrated, or otherwise rendered substantially free of *Bacillus thuringiensis* cellular material. Embodiments of isolated DIG-305 insecticidal polypeptide or nucleotide molecules can have less than about 30%, less than about 20%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3% or less than about 2%, or less than about 1% (by dry weight) of contaminating protein (e.g., from *Bacillus thuringiensis*). When the isolated DIG-305 insecticidal polypeptide or nucleotide embodiments is recombinantly produced, then the culture medium material,

chemical precursors, and/or or non-DIG-305 insecticidal polypeptide or nucleotide represent less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 4%, less than about 3% or less than about 2%, or less than about 1% (by dry weight) of the isolated DIG-305 insecticidal polypeptide or nucleotide.

BRIEF DESCRIPTION OF THE SEQUENCES

- [0015] SEQ ID NO:1 is a DNA sequence encoding a DIG-305 toxin; 3726 nt.
[0016] SEQ ID NO:2 is a deduced DIG-305 protein sequence; 1241 aa.
[0017] SEQ ID NO:3 is a maize-optimized DNA sequence encoding full length DIG-305; 3723 nt.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0018] DIG-305 insecticidal toxins : In addition to the full length DIG-305 toxin of SEQ ID NO:2, the invention encompasses insecticidal active variants thereof. By the term "variant", applicants intend to include fragments, certain deletion and insertion mutants, and certain fusion or chimeric proteins. DIG-305 includes three-domains generally associated with a Cry toxin. As a preface to describing variants of the DIG-305 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-305 protein toxin in particular.

[0019] 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 1241 amino

acid sequence of the partial DIG-305 polypeptide, of which the N-terminal 685 amino acids comprise a DIG-305 core toxin segment.

[0020] 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).

[0021] Domain I is a bundle of seven alpha helices where helix five 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-305 protein comprises amino acid residues approximately 1-320 of SEQ ID NO:2.

[0022] 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 (CPB) 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-305 protein comprises amino acid residues approximately 320-525 of SEQ ID NO:2.

[0023] 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.

[0024] 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, WO1991001087, WO1995006730, US Patent No. 5736131, US Patent No. 6204246, US Patent No. 6780408, WO1998022595, US Patent Application No. 20090143298, and US Patent No. 7618942). Domain III of the DIG-305 protein comprises amino acid residues approximately 526-685 of SEQ ID NO:2.

[0025] In lepidopteran insects it has been reported that Cry1A toxins bind certain classes of receptor proteins including cadherins, aminopeptidases and alkaline phosphatases, others remain to be identified (Honée *et al.*, 1991; Pigott and Ellar, 2007). In coleopteran insects, two receptors have been identified for Cry3Aa; in Colorado potato beetle an ADAM metalloprotease (Biochemical and Biophysical Research Communications 362 (2007) 437–

442), in *Tenebrio* a cadherin has been identified (THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 27, pp. 18401–18410, July 3, 2009). Given the diversity of *Bacillus thuringiensis* toxins and pests it is anticipated that additional receptors will be identified that will include additional classes of proteins and membrane surface substituents.

[0026] 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 brush border membrane vesicles (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 of Cry1Ab; 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.

[0027] Amino terminal deletion variants of DIG-305 In one of its aspects, the invention provides DIG-305 variants in which all or part of one or more α -helices are deleted to improve insecticidal activity and avoid development of resistance by insects. These modifications are made to provide DIG-305 variants with improved attributes, such as improved target pest spectrum, potency, and insect resistance management. In some embodiments of the subject invention, the subject modifications may affect the efficiency of protoxin activation and pore formation, leading to insect intoxication. More specifically, to provide DIG-305 variants with improved attributes, step-wise deletions are described that remove part of the DNA sequence encoding the N-terminus. Such deletions remove all of α -helix 1 and all or part of α -helix 2 in Domain I, while maintaining the structural integrity of the 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 I for more efficient pore formation. More specifically, the subject invention provides improved DIG-305 proteins designed to have N-terminal deletions in regions with putative secondary structure homology to α -helices 1 and 2 in Domain I of Cry1 proteins.

[0028] 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 express 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 may be that proteolytic cleavage by plant proteases near the start of α -helix 3 of subject *B.t.* insecticidal proteins 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 certain examples of N-terminal deletion variants that begin with one of the destabilizing amino acids, a codon that specifies a G (glycine) amino acid can be added between the translational initiation methionine and the destabilizing amino acid.

[0029] 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 CPB, 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 CPB. 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.

[0030] 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 affect 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 such as *Ostrinia nubilalis*, *Diatraea grandiosella*, *Helicoverpa zea*, *Agrotis ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Diatraea saccharalis*, *Loxagrotis albicosta*, Coleopteran pests such as western corn rootworm, southern corn rootworm, northern corn rootworm (*i.e.* *Diabrotica* spp.), and other target pests.

[0031] Serine proteases of the same family 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.

[0032] A preferred location for the introduction of such protease cleavage sites is within the "spacer" region between α -helix2B and α -helix3. A second preferred location for the introduction of protease cleavage sites is within the spacer region between α -helix3 and α -helix4. Modified DIG-305 insecticidal toxin proteins are generated either by gene deletion

or by introduction of protease cleavage sites to provide improved activity on insect pests including but not limited corn rootworm, alfalfa weevil, boll weevil, Japanese beetle, and the like.

[0033] 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-305 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.

[0034] Domains of the DIG-305 toxin The separate domains of the DIG-305 toxin, (and variants that are 90%, 91%, 92%, 93, 94%, 95%, 96%, 97%, 98%, or 99% 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-305 protein comprises approximately amino acid residues 1 to 320 of SEQ ID NO:2. Domain II of the DIG-305 protein comprises approximately amino acid residues 321 to 525 of SEQ ID NO:2. Domain III of the DIG-305 protein comprises approximately amino acid residues 526 to 685 of SEQ ID NO:2. Domain swapping or shuffling is another 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 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).

[0035] 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 -helices 4 and 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 helices proposed to contact the membrane surface like the ribs of an umbrella (Bravo *et al.*, 2007; Gazit *et al.*, 1998).

[0036] DIG-305 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 insecticidal active variants of the core toxin (approximately amino acids 1 to 685 of SEQ ID NO:2), in which up to 10, up to 15, or up to 20 amino acid additions, deletions, or substitutions have been made.

[0037] The invention includes DIG-305 insecticidal toxin variants having a core toxin segment that is 90%, 91%, 92%, 93, 94%, 95%, 96%, 97%, 98%, or 99% identical to amino acids 1 to 685 of SEQ ID NO:2. 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 1 provides a listing of examples of amino acids belonging to each class.

Table 1.

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

Beta-branched Side Chains	Thr, Val, Ile
Aromatic Side Chains	Tyr, Phe, Trp, His

[0038] 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, that is, retaining pesticidal activity.

[0039] 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, for example, US Patent No. 7058515; Larson *et al.* (2002); Stemmer (1994a, 1994b, 1995) and Cramer *et al.* (1996a, 1996b, 1997). US Patent No US 8,513,492 B2

[0040] Nucleic Acids Isolated nucleic acids encoding DIG-305 insecticidal toxins are one aspect of the present invention. This includes nucleic acids encoding SEQ ID NO:2 and complements thereof, as well as other nucleic acids that encode insecticidal variants of SEQ ID NO:2. The term “isolated” is defined herein above. 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.

[0041] Gene synthesis Genes encoding the DIG-305 insecticidal toxins 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 gene synthesis on demand. Full-length genes 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, terminal gene deletions can be made by PCR amplification using site-specific terminal oligonucleotides.

[0042] Nucleic acids encoding DIG-305 insecticidal toxins can be made for example, by synthetic construction by methods currently practiced by any of several commercial suppliers. (*e.g.* US Patent No. 7482119). These genes, 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.

[0043] Given the amino acid sequence for a DIG-305 insecticidal toxin, a coding sequence can be designed by reverse translating the coding sequence using synonymous codons preferred by the intended host, and then refining the sequence using alternative synonymous codons to remove sequences that might cause problems in transcription, translation, or mRNA stability. Further, synonymous codons may be employed to introduce stop codons in the non-DIG-305 reading frames (*i.e.* reading frames 2, 3, 4, 5 and 6) to eliminate spurious long open reading frames.

[0044] Quantifying Polypeptide or Nucleic Acid Sequence Identity The percent identity of two amino acid sequences or of two nucleic acid sequences is determined by first aligning the sequences 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.

[0045] 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 Altschul *et al.* (1990), and 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.

[0046] 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.

[0047] 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.

[0048] 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.

[0049] 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 nws gapdna. 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.

[0050] 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.

[0051] 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).

[0052] Alignment may also be performed manually by inspection.

[0053] 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 recombinant host cell. The treated cell, which comprises a treated toxin polypeptide of the invention that retains the insecticidal activity, can be applied to the environment of the target pest to control for the pest.

[0054] Where the *B.t.* toxin gene is introduced via a suitable DNA construct, *e.g.*, a 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.

[0055] 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*. 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*. Of further interest are fungi, particularly yeast, *e.g.* genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*, and of particular interest are 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.

[0056] Isolated toxin polypeptides and compositions of the invention. The DIG-305 insecticidal toxin polypeptides of the invention can be treated or prepared, for example, to make a formulated pesticide composition. Examples of formulated pesticide compositions include protein composition, sprayable protein composition, a bait matrix, or in other delivery systems. In one example, *B.t.* cells or recombinant host cells expressing a DIG-305 insecticidal toxin of the invention can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle, the *B.t.* spores or other recombinant host cells and/or toxin crystals from the fermentation broth can be isolated by methods known in the art. *B.t.* spores or recombinant host cells also can be treated prior to being applied or formulated for application to plants. For example, isolated *B.t.* spores and/or

toxin crystals can be chemically treated to prolong insecticidal activity and thereby include a treated polypeptide of the invention. Methods of growing *B.t.* toxin polypeptides in recombinant hosts and then treating the *B.t.* to prolong pesticidal activity are known and have been published. See, e.g., U.S. Patent Nos. 4,695,462, and 4,695,455 and Gaertner et al., 1993.

[0057] The isolated or treated DIG-305 insecticidal toxin of the invention can be formulated into compositions of finely-divided particulate solids granules, pellets, wettable powders, dusts, aqueous suspensions or dispersions, emulsions, spray, liquid concentrate, or other insecticide formulations. These insecticide formulations are made by combining a DIG-305 insecticide polypeptide herein with adjuvants, diluents, surfactants, dispersants, inert carriers and other components to facilitate handling and application to control one or more target pests. Such formulation ingredients are known in the art, as are methods of application and methods of determining levels of the *B.t.* spores and/or isolated DIG-305 polypeptide crystals that provide desired insecticidal activity.

[0058] Methods for controlling insect pests. When an insect comes into contact with an effective amount of DIG-305 toxin disclosed herein, which is delivered via an insecticide composition (e.g., a formulated protein composition (s), sprayable protein composition(s), a bait matrix, transgenic plant expression, or another 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.

[0059] The subject protein toxins can be “applied” or provided to contact the target insects in a variety of ways. For example, the DIG-305 insecticidal toxin of the invention can be applied after being formulated with adjuvants, diluents, carriers, etc. to provide compositions in the form of finely-divided particulate solids, granules, pellets, wettable powders, dusts, aqueous suspensions or dispersions, and emulsions. Alternatively, the DIG-305 insecticidal polypeptide can be delivered by 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.

[0060] Transgenic plants. The DIG-305 insecticidal toxin disclosed herein can be used to protect practically any type of plant from damage by an insect pest. Examples of such plants include potato, eggplant, tomato, pepper, tobacco, and other plants in the nightshade family. Other 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.

[0061] A preferred embodiment of the subject invention is the transformation of plants with genes encoding the DIG-305 insecticidal toxin, 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, European Patent No. EP131624B1, European Patent No. EP159418B1, European Patent No. EP176112B1, US Patent No. 5149645, EP120516B1, US Patent No. 5464763, US Patent No. 4693976, European Patent No. EP116718B1, European Patent No. EP290799B1, European Patent No. EP320500B1, European Patent No. EP604662B1, US Patent No. 7060876, US Patent No. 6037526, US Patent No. 6376234, European Patent No. EP292435B1, US Patent No. 5231019, US Patent No. 5463174, US Patent No. 4762785, US Patent No. 5608142, 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 WO1987006614, US Patent No. 5472869, US Patent No. 5384253, WO199209696, US Patent No. 6074877,

WO1993021335, and US Patent No. 5679558. 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 type I and type II, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques within the skill of an artisan.

[0062] Genes encoding DIG-305 insecticidal 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 *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.

[0063] The use of T-DNA-containing vectors for the transformation of plant cells has been intensively researched and sufficiently described in European Patent No. EP120516B1; Lee and Gelvin (2008), Fraley *et al.* (1986), and An *et al.* (1985), and is well established in the field.

[0064] 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 phosphinothricin Bialaphos, Kanamycin, Neomycin, G418, Bleomycin, Hygromycin, or a gene which codes for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorosulfuron, bromoxynil, dalapon and the like. Of further interest are genes conferring tolerance to herbicides such as haloxyfop, quizalofop,

diclofop, and the like, as exemplified by AAD genes (US Patent Application No. 20090093366). 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.

[0065] 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.

[0066] 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. For example, the DIG-305 insecticidal toxin of the invention can be optimized for expression in a dicot such as potato, eggplant, tomato, pepper, tobacco, and another plant in the nightshade family. The DIG-305 insecticidal toxin of the invention can also be optimized for expression in other dicots, or in monocots such as *Zea mays* (corn). 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).

[0067] 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, promoters of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the 35S and 19S promoters of cauliflower mosaic virus (CaMV), and the like may be used. Plant-derived 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, 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)), 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.

[0068] 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.

[0069] The subject invention includes plant cells that are not totipotent (non-totipotent), plant cells that are not propagative material (for example, leaf cells in some embodiments; seed cells are excluded from some embodiments) and are incapable of differentiating into whole plants. The subject invention includes plant cells that have uses other than for regenerating into a whole plant. For example, said plant cells can be used to produce a protein (such as a DIG-305 protein of the subject invention). Thus, plant cells of the subject invention include those that have uses other than totipotency (that is, some cells of subject invention are not regenerable into a whole plant). However, some embodiments do include seed cells and plant cells that can be regenerated into a whole plant.

[0070] 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 WO2007053482), or phenoxy acids herbicides and aryloxyphenoxypropionates herbicides (see US Patent Application No. 20090093366). 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 or co-transformation). 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.

[0071] Target Pests. The DIG-305 insecticidal toxins of the invention are particularly suitable for use in control of insects pests. Coleopterans are one important group of agricultural, horticultural, and household pests which cause a very large amount of damage each year. This large insect order encompasses foliar- and root-feeding larvae and adults, including members of, for example, the insect families-Chrysomelidae, Coccinellidae, Curculionidae, Dermestidae, Elateridae, Scarabaeidae, Scolytidae, and Tenebrionidae. Included within these families are leaf beetles and leaf miners in the family Chrysomelidae, potato beetles (*e.g.* Colorado potato beetle (*Leptinotarsa decemlineata* Say), grape colaspis (*Colaspis brunnea* Fabricius), cereal leaf beetle (*Oulema melanopus* Linnaeus), sunflower beetle (*Zygogramma exclamationis* Fabricius), and beetles in the family Coccinellidae (*e.g.* Mexican bean beetle (*Epilachna varivestis* Mulsant)). Further examples are chafers and other beetles in the family Scarabaeidae (*e.g.* Japanese beetle (*Popillia japonica* Newman),

northern masked chafer (white grub, *Cyclocephala borealis* Arrow), southern masked chafer (white grub, *Cyclocephala immaculata* Olivier), European chafer (*Rhizotrogus majalis* Razoumowsky), white grub (*Phyllophaga crinita* Burmeister), carrot beetle (*Ligyrus gibbosus* De Geer), and chafers of the genera *Holotrichia* spp and *Melolontha* spp.). Further examples of coleopteran insects are weevils (e.g. boll weevil (*Anthonomus grandis* Boheman), rice water weevil (*Lissorhoptrus oryzophilus* Kuschel), granary weevil (*Sitophilus granarius* Linnaeus), rice weevil (*Sitophilus oryzae* Linnaeus), and clover leaf weevil (*Hypera punctata* Fabricius)). Also included are maize billbug (*Sphenophorus maidis* Chittenden), flea beetles (e.g. corn flea beetle (*Chaetocnema pulicaria* Melsheimer), and crucifer flea beetle (*Phyllotreta cruciferae* Goeze)), spotted cucumber beetle (*Diabrotica undecimpunctata*), and rootworms, (e.g. western corn rootworm (*Diabrotica virgifera virgifera* LeConte), northern corn rootworm (*Diabrotica barberi* Smith & Lawrence), the Mexican corn rootworm ((MCR) *D. virgifera zeae* Krysan and Smith), *D. balteata* LeConte, *D. undecimpunctata tenella*, *D. u. undecimpunctata* Mannerheim, and southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber)). Further examples of coleopteran pests are beetles of the family Rutelinae (shining leaf chafers) such as the genus *Anomala* (including *A. marginata*, *A. lucicola*, *A. obliqua* and *A. orientalis*). Additional coleopteran insects are carpet beetles from the family Dermestidae, wireworms from the family Elateridae (e.g. *Melanotus* spp., *Conoderus* spp., *Limonius* spp., *Agriotes* spp., *Ctenicera* spp., *Aeolus* spp.)), bark beetles from the family Scolytidae, and beetles from the family Tenebrionidae (e.g. *Eleodes* spp). Any genus listed above (and others), generally, can also be targeted as a part of the subject invention by insecticidal compositions including DIG-305 insecticidal polypeptide alone or in combination with another insecticidal agent. Any additional insects in any of these genera (as targets) are also included within the scope of this invention.

[0072] Use of DIG-305 insecticidal toxins to control coleopteran pests of crop plants is 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 western corn rootworm (*Diabrotica virgifera virgifera* LeConte), northern corn rootworm (*Diabrotica barberi* Smith & Lawrence), and southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber), and grubs such as the larvae of *Cyclocephala borealis* (northern masked chafer), *Cyclocephala immaculata* (southern masked chafer), and *Popillia japonica* (Japanese beetle).

[0073] Lepidopterans are another important group of agricultural, horticultural, and household pests which cause a very large amount of damage each year. The invention provides use of DIG-305 toxins in combination with other insecticides to control insect pests within this order by is within the scope of this invention. This insect order encompasses foliar- and root-feeding larvae and adults, including members of, for example, the insect families Arctiidae, Gelechiidae, Geometridae, Lasiocampidae, Lymantriidae, Noctuidae, Pyralidae, Sesiidae, Sphingidae, Tineidae, and Tortricidae. Lepidopteran insect pests include, but are not limited to: *Achoroia 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 gemmatilis*, *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 feneralis*, *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*, *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*, *Manduca quinquemaculata*, *Manduca sexta*, *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*, *Sabulodes aegrotata*, *Schizura concinna*, *Sitotroga cerealella*, *Spilonta ocellana*, *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), *Thaurnstopoea pityocampa*, *Ensola bisselliella*, *Trichoplusia ni*, (cabbage looper), *Udea rubigalis*, *Xylomyges curiails*, and *Yponomeuta padella*.

[0074] Use of the DIG-305 insecticidal toxins to control parasitic nematodes including, but not limited to, root knot nematode (*Meloidogyne incognita*) and soybean cyst nematode (*Heterodera glycines*) is also contemplated.

[0075] 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-305 toxins using standard ELISA methods.

[0076] Detection using probes. 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 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.

[0077] Hybridization. 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.

[0078] 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.

[0079] 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 .

[0080] 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):

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

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 (w/v), and L is the length of the hybrid in base pairs.

[0081] 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 $[\text{Na}^+]$ 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 (w/v), and L is the length of the hybrid in base pairs

[0082] 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).

[0083] 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 ^{32}P , ^{33}P , ^{14}C , 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 °C to 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 NaHPO_4 , 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)).

[0084] 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).

[0085] For oligonucleotide probes, hybridization may be carried out overnight at 10 °C to 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).

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

[0086] 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).

[0087] 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.

[0088] 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.

[0089] By the use of the term “genetic material” herein, it is meant to include all genes, nucleic acid, DNA and RNA. The term “dsRNA” refers to double-stranded RNA. 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.

[0090] 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.

[0091] Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

[0092] 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-305 toxin

[0093] Unless otherwise indicated, molecular biological and biochemical manipulations described in this and subsequent Examples were performed by standard methodologies as disclosed in, for example, Ausubel *et al.* (1995), and Sambrook *et al.* (1989), and updates

thereof. Nucleic acid encoding the insecticidal Cry protein designated herein as DIG-305 was isolated from *B.t.* strain PS18A also known as DBt10340. Degenerate Forward and Reverse primers for Polymerase Chain Reactions (PCR) were designed and used to amplify a DNA fragment with homology to Cry32 from genomic DNA library. The determined sequence of the amplified fragment was used for genome walking to obtain the complete open reading frame of DIG-305. SEQ ID NO:1 is the 3726 bp nucleotide sequence encoding the full length DIG-305 protein. SEQ ID NO:2 is the 1241 amino acid sequence of the full length DIG-305 protein deduced from SEQ ID NO:1.

EXAMPLE 2

DIG-305 chimeric toxin in bacterial hosts

[0094] Standard cloning methods are used in the construction of *Pseudomonas fluorescens* (Pf) expression plasmids engineered to produce DIG-305 chimera toxin consisting of the DIG-305 core toxin encoding sequence (encoding amino acids 1-685) and the Cry1Ab protoxin encoding segment as described above, each encoded by the maize-optimized coding sequences. Restriction endonucleases are obtained from New England BioLabs (NEB; Ipswich, MA) and T4 DNA Ligase (Invitrogen) are used for DNA ligation. Plasmid preparations are performed using the NucleoSpin® Plasmid Kit (Macherey-Nagel Inc, Bethlehem, PA) following the instructions of the supplier. DNA fragments are purified using the QIAquick Gel Extraction kit (Qiagen) after agarose Tris-acetate gel electrophoresis. The linearized vector is phosphatased with NEB Antarctic Phosphatase to enhance formation of recombinant molecules.

[0095] The basic cloning strategy entailed subcloning a DNA fragment having the DIG-305 Cry1Ab chimera coding sequence (CDS) into pDOW1169 at, for example, *SpeI* and *Sall* restriction sites, whereby the DIG-305 chimera CDS 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 Patent No. 7618799). The expression plasmids are transformed by electroporation into DC454 (a near wild-type *P. fluorescens* strain having mutations *ΔpyrF* and *lsc::lacIQI*), 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 transformation and selection methods are generally described available in Squires *et al.*

(2004), US Patent Application No. 20060008877, US Patent No. 7681799, and US Patent Application No. 20080058262, incorporated herein by reference. Recombinant colonies are identified by restriction digestion of miniprep plasmid DNA.

[0096] Production of DIG-305 chimera for characterization and insect bioassay is accomplished by shake-flask-grown *P. fluorescens* strains harboring expression constructs. Seed cultures grown in M9 medium supplemented with glucose and trace elements are used to inoculate defined minimal medium. Expression of the DIG-305 chimera coding sequences are induced by addition of isopropyl- β -D-1-thiogalactopyranoside (IPTG) after an initial incubation of 24 hours at 30 °C with shaking. Cultures are sampled at the time of induction and at various times post-induction. Cell density is 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 No. 20060008877 in cells from *P. fluorescens* fermentations that produced insoluble *B.t.* insecticidal protein inclusion bodies (IB). Briefly, cells are lysed, pellet and supernatant fractions are prepared by centrifugation, The pellet is resuspended and repeatedly washed by resuspension in lysis buffer until the supernatant becomes colorless and the IB pellet becomes firm and off-white in color. The final pellet is washed, resuspended in sterile-filtered distilled water containing 2 mM EDTA, and stored at -80°C. The supernatant fraction is enriched for the recombinant protein by column chromatography.

[0097] Preparations are analyzed by SDS-PAGE. Quantification of target bands is done by comparing densitometric values for the bands against Bovine Serum Albumin (BSA) samples run on the same gel to generate a standard curve. The sample buffer is then changed to 10 mM CAPS (3-(cyclohexamino)1-propanesulfonic acid) pH10, using disposable PD-10 columns (GE Healthcare, Piscataway, NJ).

[0098] The concentrated extract is analyzed and quantified by SDS-PAGE relative to background-subtracted BSA standards to generate a standard curve to calculate the concentration of DIG-305 chimera.

EXAMPLE 3

Design of a plant-optimized coding sequence for the DIG-305 *B.t.* insecticidal toxin

[0099] One skilled in the art of plant molecular biology will understand that multiple DNA sequences may be designed to encode a single amino acid sequence. A common means of increasing the expression of a coding region for a protein of interest is to tailor the coding region in such a manner that its codon composition resembles the overall codon

composition of the host in which the gene is destined to be expressed. Guidance regarding the design and production of synthetic genes can be found in, for example, WO1997013402, US Patent No. 6166302, and US Patent No. 5380831.

[00100] A DNA sequence having a maize codon bias is designed and synthesized to produce a DIG-305 insecticidal protein in transgenic monocot plants. A codon usage table for maize (*Zea mays* L.) was calculated from hundreds of protein coding sequences obtained from sequences deposited in GenBank (www.ncbi.nlm.nih.gov). A rescaled maize codon set was calculated after omitting any synonymous codon used less than about 10% of total codon uses for that amino acid.

[00101] To derive a maize-codon-optimized DNA sequence encoding the DIG-305 protein core toxin of SEQ ID NO:3, or insecticidal fragments thereof, or DIG-305 chimera toxins, are the subject of codon substitutions to the experimentally determined (native) DIG-305 DNA sequence (SEQ ID NO:1) encoding the toxin were made such that the resulting DNA sequence had the overall codon composition of the maize-optimized codon bias table. Further refinements of the sequences 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 mRNA 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 maize-biased Rescaled codon composition. A maize-optimized DNA sequence encoding DIG-305 core toxin is disclosed as SEQ ID NO:3.

EXAMPLE 4

Construction of expression plasmid encoding the DIG-305 toxin in bacterial hosts

[00102] Standard cloning methods were used in the construction of *Pseudomonas fluorescens* (Pf) expression plasmids engineered to produce DIG-305 encoded by the maize-optimized coding sequences. 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 NucleoSpin® Plasmid Kit (Macherey-Nagel Inc, Bethlehem, PA) following the instructions of the supplier. DNA fragments were purified using the QIAquick Gel Extraction kit (Qiagen) after agarose Tris-acetate gel electrophoresis. The linearized vector was phosphatased with NEB Antarctic Phosphatase to enhance formation of recombinant molecules.

[00103] A DNA fragment having the DIG-305 coding sequence (CDS), as provided by SEQ ID NO:3, was subcloned into pDOW1169 at, for example, *SpeI* and *Sall* restriction sites, whereby the DIG-305 CDS was 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 Patent No. 7618799). The expression plasmid (pDAB107162, containing the DIG-305 coding sequence) was transformed by electroporation into DC454 (a near wild-type *P. fluorescens* strain having mutations $\Delta pyrF$ and *lsc::lacIQI*), or derivatives thereof, recovered in SOC-Soy hydrolysate medium, and plated on selective medium (M9 glucose agar lacking uracil, Sambrook *et al.*, *supra*). The transformation and selection methods are generally described available in Squires *et al.* (2004), US Patent Application No. 20060008877, US Patent No. 7681799, and US Patent Application No. 20080058262, incorporated herein by reference. Recombinant colonies were identified by restriction digestion of miniprep plasmid DNA. The resulting expression strain is known as DPF21990 in the Dow AgroSciences Recombinant Culture Collection.

EXAMPLE 5

Preparation of DIG-305 Protein Samples

[00104] Production of DIG-305 for characterization and insect bioassay was accomplished by expression of DIG-305 in shake-flask-grown *P. fluorescens* strain DPF21990 which harbors expression plasmid pDAB107162. Seed cultures grown in M9 medium supplemented with glucose and trace elements were used to inoculate defined minimal medium with 5% glycerol (Teknova Cat. # 3D7426, Hollister, CA). Expression of the DIG-305 coding region was induced by addition of isopropyl- β -D-1-thiogalactopyranoside (IPTG) after an initial incubation of 24 hours at 30 °C 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 No. 20060008877. Briefly, cells are lysed, IB pellet is collected by centrifugation, IB is resuspended and repeatedly washed by resuspension in lysis buffer until the supernatant becomes colorless and the IB pellet

becomes firm and off-white in color. The final pellet is washed, resuspended in sterile-filtered distilled water containing 2 mM EDTA, and stored at -80°C.

[00105] IB preparations are analyzed by SDS-PAGE. Quantification of target bands is done by comparing densitometric values for the bands against Bovine Serum Albumin (BSA) samples run on the same gel to generate a standard curve. Target protein is subsequently extracted from the inclusion body using sodium carbonate buffer and gently rocking on a platform at 4 °C overnight. Solubilized DIG-305 is centrifuged and the resulting supernatant is concentrated. The sample buffer is then changed to 10 mM CAPS (3-(cyclohexamino)1-propanesulfonic acid) pH10, using disposable PD-10 columns (GE Healthcare, Piscataway, NJ).

[00106] The concentrated extract is analyzed and quantified by SDS-PAGE relative to background-subtracted BSA standards to generate a standard curve to calculate the concentration of DIG-305.

EXAMPLE 6

Insect activity of DIG-305 insecticidal toxin

[00107] DIG-305 was tested and found to have insecticidal activity on larvae of the coleopteran insect, the Western corn rootworm (*Diabrotica virgifera virgifera*).

[00108] A solution containing purified proteins (either solubilized or as inclusion bodies; Table 2) were tested for insecticidal activity in bioassays conducted with Western corn rootworm (*Diabrotica virgifera virgifera*) larvae. Insect eggs were received from CROP CHARACTERISTICS, INC. (Farmington, MN).

[00109] *D. virgifera virgifera* bioassays were conducted in 128-well bioassay trays and a Dow AgroSciences LLC proprietary rootworm diet was used and 80 to 100 µl of aliquot solution was used to treat the diet surface. The treated trays were air dried, and one individual larva was deposited on the treated diet surface. 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 h light:dark photoperiod) for 5 days. The total number of insects exposed to each protein sample, the number of dead insects, and the weight of surviving insects were recorded in all bioassays. Trypsin activated Cry3Aa was used as a positive control. Negative controls included water; untreated; Cry1Fa; 20 mM NaCitrate, p.H. 3.5; and 10 mM CAPS, pH 10.

[00110] Percent mortality and percent growth inhibition were calculated for each treatment. Growth inhibition (GI) is 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). Bioassay results are summarized in Table 2 below. Replicated bioassays demonstrated that ingestion of DIG-305 preparations caused mortality and growth inhibition of Western corn rootworm (Table 2).

Table 2

Treatment	Dose (ug/cm2)	N	Average % Mortality	St.Dev. (%)	Average GI	St.Dev.
DIG-305	300	4	90.0	12.25	0.964	0.042
Cry3Aa (Positive Control)	350	2	80.6	0.85	0.902	0.111
Water	0	2	7.7	12.87	NA	NA
Buffer 10mM CAPS pH10 (Negative Control)	0	2	7.3	10.11	NA	NA
20 mM NaCitrate, p.H. 3.5 (Negative Control)	0	2	15.0	7.07	NA	NA
Cry1Fa (Negative Control)	350	2	33.3	17.68	0.372	0.196
Untreated	0	2	47.4	6.22	NA	NA

DIG-305 protein from inclusion bodies solubilized (10 mM CAPS pH10) and tested against Western corn rootworm.

EXAMPLE 7

Agrobacterium transformation

[00111] Standard cloning methods are used in the construction of binary plant transformation and expression plasmid. 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.

[00112] DNA comprising a nucleotide sequence that encodes a DIG-305 insecticidal toxin is synthesized by a commercial vendor (*e.g.* DNA2.0, Menlo Park, CA) and supplied as cloned fragments in a plasmid vector. Other DNA sequences encoding other DIG-305 toxins are obtained by standard molecular biology manipulation of constructs containing appropriate nucleotide sequences. The DNA fragments encoding the modified DIG-305 fragments are joined to other DIG-305 insecticidal toxin coding region fragments or other *B.t.* (Cry) coding region fragments at appropriate restriction sites to obtain a coding region encoding the desired full-length DIG-305 toxin protein.

[00113] Full length or modified coding sequences (CDS) for DIG-305 insecticidal toxin is subcloned into a plant expression plasmid at *NcoI* and *SacI* restriction sites. The resulting plant expression cassettes containing the appropriate Cry 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. The binary plant transformation vector includes a bacterial selectable marker gene that confers resistance to the antibiotic spectinomycin when the plasmid is present in *E. coli* and *Agrobacterium* cells. The binary vector plasmid also includes a plant-expressible selectable marker gene that is functional in the desired host plants, namely, the aminoglycoside phosphotransferase gene of transposon Tn5 (*aphII*) which encodes resistance to the antibiotics Kanamycin, Neomycin and G418.

[00114] 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 °C 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 °C. Well separated single colonies are selected and streaked onto fresh YEP + agar plates with spectinomycin and streptomycin, and incubated at 28 °C for 1-3 days.

[00115] The presence of the DIG-305 insecticidal toxin 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 manufacturer'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 °C for 3 minutes; Step 2) 94 °C for 45 seconds; Step 3) 55 °C for 30 seconds; Step 4) 72 °C for 1 minute per kb of expected product length; Step 5) 29 times to Step 2; Step 6) 72 °C for 10 minutes. The reaction is maintained at 4 °C 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.

[00116] Another binary plant transformation vector containing the DIG-305 insecticidal toxin 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.

EXAMPLE 8

Production of DIG-305 insecticidal toxins in dicot plants

[00117] 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 °C 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 °C 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 °C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds are harvested.

[00118] *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 °C 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™ growth chamber (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 °C) 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.

[00119] 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™ growth chamber under the above-mentioned growth conditions.

[00120] 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.

[00121] Insect Bioassays of transgenic *Arabidopsis* Transgenic *Arabidopsis* lines expressing DIG-305 insecticidal toxin 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 9

Agrobacterium transformation for generation of superbinary vectors

[00122] The *Agrobacterium* superbinary system is conveniently used for transformation of monocot plant hosts. Methodologies for constructing and validating superbinary vectors are well established. See, for example, European Patent No. EP604662B1 and US Patent No. 7060876. 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.

EXAMPLE 10

Production of DIG-305 insecticidal toxins in monocot plants

[00123] *Agrobacterium*-Mediated Transformation of Maize Seeds from a Hi-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.

[00124] 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 *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 °C 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 is vortexed until a uniform suspension is achieved, and the concentration is adjusted to a final density of 200 Klett units, using a Klett-Summerson colorimeter with a purple filter, or an equivalent optical density measured at 600 nm (OD₆₀₀). 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 or equivalent OD₆₀₀, 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 °C under dark conditions.

[00125] 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 are obtained. Recovered isolates are bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

[00126] 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.

[00127] 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 are transferred to glass culture tubes containing SHGA medium (Schenk and Hildebrandt (1972) salts and vitamins); 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 11

Bioassay of transgenic maize

[00128] Bioactivity of the DIG-305 insecticidal toxins 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-305 insecticidal 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-305 insecticidal toxin and the extracted proteins incorporated into artificial diet bioassays 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 a DIG-305 insecticidal toxin, or to other control samples.

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WE CLAIM:

1. An isolated, treated, or formulated DIG-305 insecticidal toxin polypeptide comprising a core toxin segment that includes an amino acid sequence selected from the group consisting of:
 - (a) residues 2 to 685 of SEQ ID NO:2;
 - (b) a sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO:2; and
 - (c) residues 2 to 685 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 insecticidal active fragment thereof.
2. The isolated, treated, or formulated polypeptide of claim 1 comprising an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:5.
3. The isolated, treated, or formulated polypeptide of claim 1, wherein the core toxin segment is linked to a C-terminal protoxin portion of a Cry toxin other than DIG-305.
4. The isolated, treated, or formulated polypeptide of claim 2, wherein the polypeptide is a chimeric protein and the C-terminal protoxin portion comprises the C-terminal protoxin portion of cry1Ab or a cry1Ac/cry1Ab chimeric toxin.
5. The isolated, treated, or formulated polypeptide of claim 4, wherein the C-terminal protoxin portion comprises the C-terminal protoxin portion of Cry1Ab.
6. The isolated, treated, or formulated polypeptide of claim 4, wherein the C-terminal protoxin portion comprises the C-terminal protoxin portion of cry1Ac/cry1Ab chimeric toxin.
7. A method for controlling a pest population comprising contacting said population with a pesticidally effective amount of the polypeptide of any one of claims 1-5.
8. The polypeptide of any one of claims 1-5 having activity against a coleopteran pest.
9. The polypeptide of any one of claims 1-5 having activity against corn rootworm.
10. A composition comprising the polypeptide of any one of claims 1-5.
11. The composition of claim 9, wherein the composition is a sprayable protein composition, encapsulated protein composition, or bait matrix that comprises a formulated DIG-305 insecticidal toxin.
12. A nucleic acid construct, wherein the construct comprises a heterologous nucleic acid sequence that is recombinantly linked to a sequence encoding a DIG-305 insecticidal toxin

comprising a core toxin segment that includes an amino acid sequence selected from the group consisting of:

- (a) residues 2 to 685 of SEQ ID NO:2;
- (b) a sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO:2; and
- (c) residues 2 to 685 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 insecticidal active fragment thereof.

- 13. The nucleic acid construct of claim 11, wherein the heterologous nucleic acid sequence is a promoter sequence capable of driving expression in a plant.
- 14. The nucleic acid construct of claim 0, wherein the sequence encoding the polypeptide is codon-optimized for expression in a plant.
- 15. The nucleic acid construct of claim 13, wherein the promoter is capable of driving expression in corn and the sequence encoding the polypeptide is codon optimized for expression in corn.
- 16. The nucleic acid construct of any one of claims 11 to 14, wherein the sequence encoding the polypeptide comprises SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:7.
- 17. The nucleic acid construct of claim 15, wherein the construct is a vector and the vector comprises SEQ ID NO:3.
- 18. The nucleic acid construct of claim 13, wherein the promoter is capable of driving expression in potato and the sequence encoding the polypeptide is codon optimized for expression in potato.
- 19. A transgenic plant comprising the nucleic acid construct of any one of claims 11 to 17 stably incorporated into its genome.
- 20. A method for protecting a plant from a pest comprising introducing into said plant the construct of any one of claims 11 to 18.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/66189

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/87; C12N 5/04; A61K 38/00; C07H 21/04; A01H 5/00 (2016.01)

CPC - C12N 15/8286; C12N 15/8216; C07K 14/325

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)

IPC(8) - C12N 15/87; C12N 5/04; A61K 38/00; C07H 21/04; A01H 5/00 (2016.01)

CPC - C12N 15/8286; C12N 15/8216; C07K 14/325; USPC - 800/279; 435/419; 514/4.5; 536/23.71; 800/302

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

IPC(8) - C12N 15/87; C12N 5/04; A61K 38/00; C07H 21/04; A01H 5/00 (2016.01) - see keyword below

CPC - C12N 15/8286; C12N 15/8216; C07K 14/325; USPC - 800/279; 435/419; 514/4.5; 536/23.71; 800/302

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: DIG-305, Cry32Ca, PS246F10, DBt1519, isolated, treated, formulated, purified, toxin, insecticidal, pesticidal, core toxin, polypeptide, delta-endotoxin, coleopteran, corn rootworm, spray, encapsulated, bait matrix, chimeric, C-terminal, carboxyl, terminal, protoxin, Cry, Cry1, Cry1Ab, Cry1Ac

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 2014/0096281 A1 (Sampson) 03 April 2014 (03.04.2014), para [0005], [0007], [0010], [0012], [0013], [0015], [0022], [0037], [0040], [0041], [0047], [0049], [0083], [0086], [0087], [0089], [0093], and SEQ ID NO: 4	1, 7-11/(1) ----- 3, 7-11/(3) ----- 2, 4-6, 7-11/(2, 4-5)
Y --- A	US 2013/0247254 A1 (LIRA et al.) 19 September 2013 (19.09.2013), para [0009], [0020], [0029], [0052], [0061], [0090], and [0104]	3, 7-11/(3) ----- 2, 4-6, 7-11/(2, 4-5)



Further documents are listed in the continuation of Box C.



* 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 application or patent 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

12 February 2016

Date of mailing of the international search report

01 APR 2016

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 19-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-11, directed to an isolated, treated, or formulated DIG-305 insecticidal toxin polypeptide comprising a core toxin segment. [Please Note: The core toxin segment will be searched to the extent that the core toxin segment encompasses (b) a sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO: 2, which includes (a) and (c). The limitation "an insecticidal active fragment thereof" is searched based on the search of the full length of the amino acid sequence of residues 2 to 685 of SEQ ID NO: 2, because unless the applicants provide specific active region(s) for search, it is impossible to search for active fragments thereof].

*****Continued in the extra sheet*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/66189

Continuation of:
Box No III (unity of invention is lacking)

Group II, claims 12-18, directed to a nucleic acid construct, wherein the construct comprises a heterologous nucleic acid sequence that is recombinantly linked to a sequence encoding a DIG-305 insecticidal toxin comprising a core toxin segment. [Please Note: The core toxin segment will be searched to the extent that the core toxin segment encompasses (b) a sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO: 2, which includes (a) and (c). The limitation "an insecticidal active fragment thereof" is searched based on the search of the full length of the amino acid sequence of residues 2 to 685 of SEQ ID NO: 2, because unless the applicants provide specific active region(s) for search, it is impossible to search for active fragments thereof].

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Group I includes the special technical feature of an isolated, treated, or formulated insecticidal toxin polypeptide, not required by Group II.

Group II includes the special technical feature of a nucleic acid construct, wherein the construct comprises a heterologous nucleic acid sequence that is recombinantly linked to a sequence, not required by Group I.

Common Technical Features

The inventions of Groups I-II share the technical feature of a DIG-305 insecticidal toxin polypeptide comprising a core toxin segment that includes an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO:2, or an insecticidal active fragment thereof.

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2014/0096281 A1 to Sampson as follows:

Sampson discloses an insecticidal toxin polypeptide comprising a core toxin segment that includes an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO:2 (para [0040] - 'By "pesticidal protein" is intended a protein having the amino acid sequence set forth in SEQ ID NO:4', wherein SEQ ID NO: 4 shares 91.2% of Query Match and 91.0% of the Best Local Similarity to the claimed SEQ ID NO: 2, and comprising a region between amino acid residues 2- 685, that shares 92.9% of Query Match and 92.7% of the Best Local Similarity to the amino acid sequence of residues 2 to 685 of the claimed SEQ ID NO:2; para [0022] - 'proteolytic cleavage fragment ... SEQ ID NO:4... the fragments encompassed herein result from the removal of the C-terminal crystallization domain'; para [0089] - '"Pest" includes ... insects, ... Insect pests include insects selected from the orders Coleoptera'; para [0093] - 'Insect pests ... include: ... corn rootworm'; Specification: para [0071] - 'The DIG-305 insecticidal toxins ... use in control of insects pests. Coleopteran'; para [0072] - 'Use of DIG-305 insecticidal toxins to control coleopteran pests of crop plants ... rootworms'), and --an insecticidal active fragment thereof (para [0041] - 'Fragments ... "biologically active portions" include polypeptide fragments comprising amino acid sequences sufficiently identical to the amino acid sequence set forth in SEQ ID NO:4... that exhibit pesticidal activity. A biologically active portion of a pesticidal protein can be a polypeptide that is... 50, 100, ... or more amino acids in length', wherein SEQ ID NO: 4 comprising a region between amino acid residues 70-134, that is 100% identical to the amino acid residues 70-134 of the claimed SEQ ID NO: 2; and a region between amino acid residues 2-186, that is 97.8% identical to the amino acid residues 2-187 of the claimed SEQ ID NO: 2; Specification: para [0010] - 'DIG-305 insecticidal toxin polypeptide comprising a core toxin segment... (b) an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues from approximately 2 to approximately 685 of SEQ ID NO:2; ... or an insecticidal active fragment of either (a), (b), or (c)').

Although Sampson does not specifically teach wherein the insecticidal toxin is a DIG-305 insecticidal toxin, this limitation is determined by the amino acid sequence of the insecticidal toxin polypeptide disclosed by Sampson, because the DIG-305 insecticidal toxin polypeptide is determined by an insecticidal toxin polypeptide comprising a core toxin segment that includes an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues 2 to 685 of SEQ ID NO:2, which includes the insecticidal toxin polypeptide disclosed by Sampson (para [0040] - 'By "pesticidal protein" is intended a protein having the amino acid sequence set forth in SEQ ID NO:4', wherein SEQ ID NO: 4 comprising a region between amino acid residues 2- 685, that shares 92.9% of Query Match and 92.7% of the Best Local Similarity to the amino acid sequence of residues 2 to 685 of the claimed SEQ ID NO:2; para [0022] - 'proteolytic cleavage fragment ... SEQ ID NO:4... the fragments encompassed herein result from the removal of the C-terminal crystallization domain'; Specification: para [0007] - 'A BLAST search using the insecticidal protein sequence as a query found that DIG-305 toxin protein has 89% ...insecticidal toxin Cry32Cal (BAB78602) and 91% sequence identity to ... sequence (AGU13873, US20140096281). Thus, DIG-305 represents a new subclass within the Cry32 family of proteins', wherein '91% sequence identity to the closest publicly disclosed sequence ... US20140096281' is the sequence disclosed by Sampson; para [0010] - 'DIG-305 insecticidal toxin polypeptide comprising a core toxin segment... (b) an amino acid sequence having at least 90% sequence identity to the amino acid sequence of residues from approximately 2 to approximately 685 of SEQ ID NO:2; ... or an insecticidal active fragment of either (a), (b), or (c)').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 19-20 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.

*****Continued in the next extra sheet*****

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/66189

Continuation of:

The previous extra sheet - Box No III (unity of invention is lacking)

Note:

I) Claim 2 is directed to an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:5. However, there is no SEQ ID NO: 5 in the Sequence Listing. Claim 2 is limited to search for SEQ ID NO: 2 only.

II) Claim 11 is objected to as lacking a proper antecedent basis for the "The composition" limitation. It is assumed that claim 11 depends from claim 10. For the purposes of this ISR, claim 11 is construed as follows:

11. The composition of claim 10, wherein the composition is a spray able protein composition, encapsulated protein composition, or bait matrix that comprises a formulated DIG-305 insecticidal toxin.

III) Claims 13-14 are objected to as lacking a proper antecedent basis for the "The nucleic acid construct" limitation. It is assumed that claims 13-14 depend from claim 12. For the purposes of this ISR, claims 13-14 are construed as follows:

13. The nucleic acid construct of claim 12, wherein the heterologous nucleic acid sequence is a promoter sequence capable of driving expression in a plant.

14. The nucleic acid construct of claim 12, wherein the sequence encoding the polypeptide is codon-optimized for expression in a plant.

IV) Claim 16 is objected to as partially lacking a proper antecedent basis for the "The nucleic acid construct" limitation. For the purposes of this ISR, it is assumed that claim 16 depends from any one of claims 12 to 14.

V) Claim 16 is directed to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:7. However, there is no SEQ ID NO: 4 or SEQ ID NO:7 in the Sequence Listing. Claim 16 is limited to search for SEQ ID NO: 1 or SEQ ID NO:3 only.