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(54) **Title**: INSECTICIDAL CRY TOXINS

(57) **Abstract**: Insecticidal toxins derived from *Bacillus thuringiensis*, polynucleotides encoding such toxins, use of such toxins to control Coleopteran plant pests, and transgenic plants that produce, and are protected, by these toxins are described.

INSECTICIDAL CRY TOXINS

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional application No. 62/319,428, filed April 7, 2016, entitled "INSECTICIDAL CRY TOXINS", the disclosure of which is being incorporated by reference.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named "78559-US-NP-20170328-Sequence-Listing_ST25", created on March 7, 2017, and having a size of 106 kilobytes, and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification, and is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0003] The present invention relates generally to the field of molecular biology as applied to agricultural sciences. More particularly, certain embodiments concern methods for using DNA segments as diagnostic probes and templates for protein production, and the use of proteins, fusion protein carriers and peptides for insect control and in various immunological and diagnostic applications. Also disclosed are methods of making and using nucleic acid segments in the development of plant incorporated protectants in transgenic plant cells containing the DNA segments disclosed herein.

BACKGROUND

[0004] *Bacillus thuringiensis* is a Gram-positive bacterium that produces delta-endotoxins known as crystal proteins which are specifically toxic to certain orders and species of insects. Many different strains of *B. thuringiensis* have been shown to produce insecticidal crystal proteins. Compositions including *B. thuringiensis* strains which produce insecticidal proteins have been commercially available and used as environmentally acceptable insecticides.

[0005] The majority of insecticidal *B. thuringiensis* strains are active against insect of the order Lepidoptera, i.e., caterpillar insects. Other *B. thuringiensis* strains are active against

insects of the order Diptera, i.e., flies and mosquitoes, or against both lepidopteran and dipteran insects. In recent years, a few *B. thuringiensis* strains have been reported as producing crystal proteins that are toxic to insects of the order Coleoptera, i.e., beetles, such as corn rootworms. Such currently deployed toxic proteins include Cry3Bbl, a modified Cry3A, eCry3.1Ab, and a binary toxin Cry34Abl/Cry35Abl (requiring two different proteins for toxic activity). These proteins are effective for controlling *Diabrotica* species that infest corn roots, whether deployed singly, or in various combinations to decrease the likelihood of the development of resistance. Even though these proteins have been successfully deployed as insect control agents in transgenic crop plants, resistance to their effects can develop.

[0006] The classification of these crystal proteins was previously based on their target insect types. However, ongoing discovery of crystal proteins with very different amino acid sequences and insecticidal activities necessitated the development of a new classification system. The currently accepted nomenclature groups crystal proteins based on their amino acid sequences only. (Crickmore, N. et al. Microbiol. and Mol. Bio. Rev. (1998) Vol. 62: 807-813; http://www.btnomenclature.info/).

[0007] Resistance to a deployed toxin, whether chemistry or protein, is more likely to develop in a number of situations which enhance resistance development. Generally, the development of resistance is directly dependent on the length of time that a toxin is deployed into the environment. Resistance development is also more likely to increase in situations in which the dose of the toxin is insufficient to ensure mortality to the pest consuming a single bite of tissue containing the toxin. Accordingly, it is crucial to deliver a lethal dose of toxin with each bite, otherwise development of resistance to a particular toxin is more likely to occur. Repetitive use of the same toxin within a common geographic region on or in multiple species of plants which are susceptible to the same or similar pests within a common geographic region is more likely to cause rapid development of resistance to the toxin, particularly in climates in which there are multiple generations of a particular target pest within a single growing season. For all the forgoing reasons, dependence on a limited number of toxic proteins or toxic chemistries can result in the development of resistance to these pest control agents.

[0008] The western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) is a major corn insect pest throughout the United States Corn Belt. The options available for WCR management are limited due to the insect's propensity to adapt to both chemical pesticides and

transgenic corn hybrids expressing *B.t.* Cry proteins, as well as cultural measures such as crop rotation with soybean. In recent years, WCR at specific geographic areas has developed significant resistance to Cry3Bb, likely due to continuously planting Cry3Bb transgenic corn. It has been shown that WCR selected with Cry3Bb is cross resistant to mCry3Aa (Gassmann et al., 2014; Gassmann et al., 2011). Although there has been no report of resistance issue with Cry34/35Ab1 maize traits, Cry34/35 trait is under increasing selection pressure due to larger planting area, e.g. the commercialization of SmartStax, and the possibility of Cry34/35Ab1 selection on top of Cry3-resistant WCR population. The corn rootworm trait market needs novel insecticidal genes with a new mode of action (MOA) for sustainable corn rootworm control (Narva et al., 2013). Other proteins disclosed in the art that are asserted to exhibit toxic effects to corn rootworms include patatin, TICl00/101 binary toxin, ET33/34 binary toxin, TIC863, ET80/76 binary toxin, ET70, Cry3Bb (U.S. Patent No. 6,501,009), CrylC variants, Cry3A variants, Cry3, Cry3B, Cry34/35, 5307, Axmil84, Axmi205, AxmiRl, TIC901, TIC1201, TIC407, TIC417, TIC431, TIC807, TIC853, TIC3131, eHIPs (U.S. Patent Application Publication No. 2010/0017914), and ω-Hex atoxin-Hv la (U.S. Patent Application Publication US2014-0366227 Al).

[0009] Despite the discovery of many selective protein toxins from *B. thuringiensis*, there remains a critical need to discover new, effective pest control agents that provide economic benefits to farmers especially against *Diabrotica virgifera virgifera* (Western Corn Rootworm (WCR)), and are environmentally acceptable. Particularly needed are agents targeted to control a wide spectrum of economically important insect pests that effectively control insect populations that are, or could become, resistant to existing insect control agents and those with equal to or increased potency compared to currently deployed insecticidal IRDIG protein toxins.

SUMMARY OF THE INVENTION

[0010] The present invention is based on the discovery of novel protein toxins having insecticidal activity against WCR. These insecticidal proteins and the genes have little homology to all the known proteins and genes of the prior art and demonstrate surprising insecticidal activity against insects including but not limited to the order Coleoptera. Based on the amino acid sequence of these native insecticidal toxins, they are not related at the primary sequence level to any known family *B. thuringiensis* proteins.

[0011] The present invention provides novel *B. thuringiensis* insecticidal IRDIG protein toxins and the genes encoding them. The invention also includes homologs, N-terminal deletions, derivatives, analogs, and mutant forms of these insecticidal toxins, plant codon optimized nucleic acid sequences encoding the claimed toxins, methods for making, using the toxins and antibodies that selectively bind these toxins.

[0012] The present invention also concerns DNA segments, which can be isolated from virtually any source, that are free from total genomic DNA and that encode the whole or a portion of the novel peptides disclosed herein. The insecticidal IRDIG protein gene encodes the insecticidal IRDIG protein having an amino acid sequence and size as referenced in Table 1.

Table 1

Name	Gene SEQ ID NO	Protein SEQ ID NO	Predicted Protein Size (kDa)
IRDIG28688.1	1	2	21.8
IRDIG28686.1	3	4	21.6
IRDIG28684.1	5	6	21.5
IRDIG28682.1	7	8	21.6
IRDIG28680.1	9	10	12.3
IRDIG28674.1	11	12	20.9
IRDIG28672.1	13	14	21.1
IRDIG27642.1	15	16	21.1
IRDIG28678.2	17	18	21.4
IRDIG28678.1	19	20	21.4
IRDIG31125.1	21	22	22.0
IRDIG28696.1	23	24	13.5
IRDIG29781.1	25	26	14.3
IRDIG29779.1	27	28	20.7
IRDIG30844.1	29	30	21.8
IRDIG30850.1	31	32	21.1

IRDIG30852.1	33	34	21.8
IRDIG30854.1	35	36	21.5
IRDIG30856.1	37	38	13.5
IRDIG30858.1	39	40	18.4
IRDIG30862.1	41	42	10.7
IRDIG30860.1	43	44	10.7
IRDIG30848.1	45	46	21.4
IRDIG30858.1	119	120	12.8
IRDIG28676.1	121	122	23.7
IRDIG28692.1	123	124	22.1
IRDIG28694.1	125	126	13.6

[0013] In particular embodiments, the invention concerns isolated DNA segments, recombinant vectors incorporating DNA sequences that encode the claimed toxins, and functional genetic inserts found in the genomes of plants that result from transforming plants with such recombinant vectors. More preferably, the DNA segments comprise a nucleic acid sequence that encodes a protein or peptide toxin that includes within its amino acid sequence an at least ten amino acid contiguous sequence of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122.

[0014] Similarly, a DNA segment comprising an isolated or purified protein-encoding gene refers to a DNA segment which may include in addition to peptide encoding sequences, certain other elements such as, regulatory sequences, isolated substantially away from other naturally occurring genes or protein-encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein-, polypeptide- or peptide-encoding unit. As will be understood by those in the art, this functional term includes not only genomic sequences, including extrachromosomal DNA sequences, but also operon sequences and engineered gene segments that express, or may be adapted to express, functional proteins, polypeptides or peptides.

BRIEF DESCRIPTION OF THE SEQUENCES

[0015] SEQ ID NO:1 is a *B. thuringiensis* DNA sequence encoding IRDIG28688.1 toxin; 570 nt.

[0016] SEQ ID NO:2 is the *B. thuringiensis* IRDIG28688.1protein sequence encoded by SEQ ID NO:1, 189 aa.

[0017] SEQ ID NO:3 is a *B. thuringiensis* DNA sequence encoding IRDIG28686.1; 564 nt.

[0018] SEQ ID NO:4 is a *B. thuringiensis* protein sequence from IRDIG28686.1; 187 aa.

[0019] SEQ ID NO:5 is a *B. thuringiensis* DNA sequence from IRDIG28684.1; 564 nt.

[0020] SEQ ID NO:6 is a *B. thuringiensis* protein sequence from IRDIG28684.1; 187 aa.

[0021] SEQ ID NO:7 is a *B. thuringiensis* DNA sequence from IRDIG28682.1; 564 nt.

[0022] SEQ ID NO:8 is a *B. thuringiensis* protein sequence from IRDIG28682.1; 187 aa.

[0023] SEQ ID NO:9 is a *B. thuringiensis* DNA sequence from IRDIG28680.1; 333 nt.

[0024] SEQ ID NO:10 is a *B. thuringiensis* protein sequence from IRDIG28680.1; 110 aa.

[0025] SEQ ID NO:11 is a *B. thuringiensis* DNA sequence from IRDIG28674.1; 555 nt.

[0026] SEQ ID NO:12 is a *B. thuringiensis* protein sequence from IRDIG28674.1; 184 aa.

[0027] SEQ ID NO:13 is a *B. thuringiensis* DNA sequence from IRDIG28672.1; 555 nt.

[0028] SEQ ID NO:14 is a *B. thuringiensis* protein sequence from IRDIG28672.1; 184 aa.

[0029] SEQ ID NO:15 is a *B. thuringiensis* DNA sequence from IRDIG27642; 555 nt.

[0030] SEQ ID NO:16 is a *B. thuringiensis* protein sequence from IRDIG27642; 184 aa.

[0031] SEQ ID NO:17 is a *B. thuringiensis* DNA sequence from IRDIG28678.2; 558 nt.

[0032] SEQ ID NO:18 is a *B. thuringiensis* protein sequence from IRDIG28678.2; 185 aa.

[0033] SEQ ID NO:19 is a *B. thuringiensis* DNA sequence from IRDIG28678.1; 558 nt.

[0034] SEQ ID NO:20 is a *B. thuringiensis* protein sequence from IRDIG28678.1; 185 aa.

[0035] SEQ ID NO:21 is a *B. thuringiensis* DNA sequence from IRDIG31125.1; 576 nt.

[0036] SEQ ID NO:22 is a *B. thuringiensis* protein sequence from IRDIG31125.1; 191 aa

[0037] SEQ ID NO:23 is a *B. thuringiensis* DNA sequence from IRDIG28696.1; 366 nt.

[0038] SEQ ID NO:24 is a *B. thuringiensis* protein sequence from IRDIG28696.1; 121 aa.

[0039] SEQ ID NO:25 is a *B. thuringiensis* DNA sequence from IRDIG29781.1; 387 nt.

[0040] SEQ ID NO:26 is a *B. thuringiensis* protein sequence from IRDIG29781.1; 128 aa.

[0041] SEQ ID NO:27 is a *B. thuringiensis* DNA sequence from IRDIG29779.1; 537 nt.

[0042] SEQ ID NO:28 is a *B. thuringiensis* protein sequence from IRDIG29779.1; 178 aa.

[0043] SEQ ID NO:29 is a *B. thuringiensis* DNA sequence from IRDIG 30844.1; 570 nt.

[0044] SEQ ID NO:30 is a *B. thuringiensis* protein sequence from IRDIG30844.1; 189 aa.

[0045] SEQ ID NO:31 is a *B. thuringiensis* DNA sequence from IRDIG30850.1; 639 nt.

[0046] SEQ ID NO:32 is a *B. thuringiensis* protein sequence from IRDIG30850.1; 212 aa.

[0047] SEQ ID NO:33 is a *B. thuringiensis* DNA sequence from IRDIG30852.1; 570 nt.

[0048] SEQ ID NO:34 is a *B. thuringiensis* protein sequence from IRDIG30852.1; 189 aa.

[0049] SEQ ID NO:35 is a *B. thuringiensis* DNA sequence from IRDIG30854.1; 564 nt.

[0050] SEQ ID NO:36 is a *B. thuringiensis* protein sequence from IRDIG30854.1; 187 aa.

[0051] SEQ ID NO:37 is a *B. thuringiensis* DNA sequence from IRDIG30856.1; 366 nt.

[0052] SEQ ID NO:38 is a *B. thuringiensis* protein sequence from IRDIG30856.1; 121 aa.

[0053] SEQ ID NO:39 is a *B. thuringiensis* DNA sequence from IRDIG30858.1; 492 nt.

[0054] SEQ ID NO:40 is a *B. thuringiensis* protein sequence from IRDIG30858.1; 163 aa.

[0055] SEQ ID NO:41 is a *B. thuringiensis* DNA sequence from IRDIG30862.1; 285 nt.

[0056] SEQ ID NO:42 is a *B. thuringiensis* protein sequence from IRDIG30862.1; 94 aa.

[0057] SEQ ID NO:43 is a *B. thuringiensis* DNA sequence from IRDIG30860.1; 285 nt.

[0058] SEQ ID NO:44 is a *B. thuringiensis* protein sequence from IRDIG30860.1; 94 aa.

[0059] SEQ ID NO:45 is a *B. thuringiensis* DNA sequence from IRDIG30848.1; 561 nt.

[0060] SEQ ID NO:46 is a *B. thuringiensis* protein sequence from IRDIG30848.1; 186 aa.

[0061] SEQ ID NOs:47-68 primers used to amplify portions of insecticidal IRDIG proteins listed above.

[0062] SEQ ID NO:69 IRDIG27642.2 is a codon-optimized DNA sequence encoding IRDIG27642 toxin using the ZmHGC strategy; 555 nt

[0063] SEQ ID NO:70 IRDIG27642.3 is a codon-optimized DNA sequence encoding IRDIG27642 toxin using the Zm Highest GC strategy; 555 nt

[0064] SEQ ID NO:71 is a *B. thuringiensis* protein sequence from IRDIG27642.2 and IRDIG27642.3; 184 aa.

[0065] SEQ ID NO:72 IRDIG27642.4 TraP8 fused to IRDIG27642 Zm Highest GC; 753

[0066] SEQ ID NO:73 is a *B. thuringiensis* protein sequence from IRDIG27642.4 and IRDIG27642.6; 250 aa.

[0067] SEQ ID NO:74 IRDIG27642.5 TraP4 fused to IRDIG27642 ZmHGC; 768

[0068] SEQ ID NO:75 is a *B. thuringiensis* protein sequence from IRDIG27642.5; 255 aa

[0069] SEQ ID NO:76 IRDIG27642.6 TraP8 fused to IRDIG27642 ZmHGC; 753

[0070] SEQ ID NO:77 IRDIG27642-F1BamHI is the forward primer sequence for PCR and for expression after the PCR product was cloned into BamI/KpnI cut pDAB101622 in Bt 4Q7

[0071] SEQ ID NO:78 IRDIG27642-R1KpnI is the reverse primer sequence for PCR and for expression after the PCR product was cloned into BamI/KpnI cut pDAB101622 in Bt 4Q7

[0072] SEQ ID NO:79 IRDIG27642-00733-F2SpeI is the forward primer sequence for PCR and for expression after the PCR product was cloned into SpeI/XhoI cut pDOW1169 in Pf

[0073] SEQ ID NO:80 IRDIG27642-00733-R2XhoI is the reverse primer for PCR and for expression after the PCR product was cloned into SpeI/XhoI cut pDOW1169 in Pf

[0074] SEQ ID NO:81 is a truncated *B. thuringiensis* DNA sequence from IRDIG27642.1

[0075] SEQ ID NO:82 is a truncated *B. thuringiensis* protein sequence from IRDIG27642.1

[0076] SEQ ID NO:83 is a *B. thuringiensis* DNA sequence from IRDIG28672.1

[0077] SEQ ID NO:84 is a truncated *B. thuringiensis* protein sequence from IRDIG28672.1

[0078] SEQ ID NO:85 is a *B. thuringiensis* DNA sequence from IRDIG28674.1

[0079] SEQ ID NO:86 is a truncated *B. thuringiensis* protein sequence from IRDIG28674.1

[0080] SEQ ID NO:87 is a *B. thuringiensis* DNA sequence from IRDIG28678.1

[0081] SEQ ID NO:88 is a truncated *B. thuringiensis* protein sequence from IRDIG28678.1

[0082] SEQ ID NO:89 is a *B. thuringiensis* DNA sequence from IRDIG31125.1

[0083] SEQ ID NO:90 is a truncated *B. thuringiensis* protein sequence from IRDIG31125.1

[0084] SEQ ID NO:91 is a *B. thuringiensis* DNA sequence from IRDIG28680.1

[0085] SEQ ID NO:92 is a truncated *B. thuringiensis* protein sequence from IRDIG28680.1

[0086] SEQ ID NO:93 is a *B. thuringiensis* DNA sequence from IRDIG28682.1

[0087] SEQ ID NO:94 is a truncated *B. thuringiensis* protein sequence from IRDIG28682.1

[0088] SEQ ID NO:95 is a *B. thuringiensis* DNA sequence from IRDIG28684.1

[0089] SEQ ID NO:96 is a truncated *B. thuringiensis* protein sequence from IRDIG28684.1

[0090] SEQ ID NO:97 is a *B. thuringiensis* DNA sequence from IRDIG28686.1

[0091] SEQ ID NO:98 is a truncated *B. thuringiensis* protein sequence from IRDIG28686.1

[0092] SEQ ID NO:99 is a *B. thuringiensis* DNA sequence from IRDIG28688.1

[0093] SEQ ID NO:100 is a truncated *B. thuringiensis* protein sequence from IRDIG28688.1

[0094] SEQ ID NO:101 is a *B. thuringiensis* DNA sequence from IRDIG28696.1

[0095] SEQ ID NO:102 is a truncated *B. thuringiensis* protein sequence from IRDIG28696.1

[0096] SEQ ID NO:103 is a *B. thuringiensis* DNA sequence from IRDIG29779.1

[0097] SEQ ID NO:104 is a truncated *B. thuringiensis* protein sequence from IRDIG29779.1

[0098] SEQ ID NO:105 is a *B. thuringiensis* DNA sequence from IRDIG29781.1

[0099] SEQ ID NO:106 is a truncated *B. thuringiensis* protein sequence from IRDIG29781.1

[00100] SEQ ID NO:107 is a *B. thuringiensis* DNA sequence from IRDIG30844.1

[00101] SEQ ID NO:108 is a truncated *B. thuringiensis* protein sequence from IRDIG30844.1

[00102] SEQ ID NO:109 is a *B. thuringiensis* DNA sequence from IRDIG30848.1

[00103] SEQ ID NO:110 is a truncated *B. thuringiensis* protein sequence from IRDIG30848.1

[00104] SEQ ID NO:111 is a *B. thuringiensis* DNA sequence from IRDIG30850.1

[00105] SEQ ID NO:112 is a truncated *B. thuringiensis* protein sequence from IRDIG30850.1

[00106] SEQ ID NO:113 is a *B. thuringiensis* DNA sequence from IRDIG30852.1

[00107] SEQ ID NO:114 is a truncated *B. thuringiensis* protein sequence from IRDIG30852.1

[00108] SEQ ID NO:115 is a *B. thuringiensis* DNA sequence from IRDIG30854.1

[00109] SEQ ID NO:116 is a truncated *B. thuringiensis* protein sequence from IRDIG30854.1

[00110] SEQ ID NO:117 is a *B. thuringiensis* DNA sequence from IRDIG30856.1

[00111] SEQ ID NO:118 is a truncated *B. thuringiensis* protein sequence from IRDIG30856.1

[00112] SEQ ID NO:119 is a *B. thuringiensis* DNA sequence from IRDIG30858.1

[00113] SEQ ID NO:120 is a truncated *B. thuringiensis* protein sequence from IRDIG30858.1

[00114] SEQ ID NO:121 is a *B. thuringiensis* DNA sequence from IRDIG28676.1; 624 nt.

[00115] SEQ ID NO:122 is a *B. thuringiensis* protein sequence from IRDIG28676.1; 207 aa.

[00116] SEQ ID NO:123 is a *B. thuringiensis* DNA sequence from IRDIG28692.1; 579 nt.

[00117] SEQ ID NO:124 is a *B. thuringiensis* protein sequence from IRDIG28692.1; 192 aa

[00118] SEQ ID NO:125 is a *B. thuringiensis* DNA sequence from IRDIG28694.1; 366 nt.

[00119] SEQ ID NO:126 is a *B. thuringiensis* protein sequence from IRDIG28694.1; 121 aa.

[00120] SEQ ID NOs:127-132 primers used to amplify portions of insecticidal IRDIG proteins.

DETAILED DESCRIPTION OF THE INVENTION

[00121] The following words and phrases have the meanings set forth below. Unless specifically indicated, the terms "a", "an", and "the" signify "at least one" as used herein.

[00122] An "insecticidal IRDIG protein" is defined as SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 protein toxins have at least 70% sequence identity with any of the foregoing including derivatives, analogs, and mutant forms. A more preferred group of insecticidal IRDIG proteins consists of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, protein toxins have at least 80% sequence identity with any of the foregoing sequences. Another preferred group of insecticidal IRDIG proteins consists of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, protein toxins have at least 90% sequence identity with any of the foregoing sequences. Another preferred group of insecticidal IRDIG proteins consists of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, protein toxins have at least 95% sequence identity with any of the foregoing sequences. Another preferred group of insecticidal IRDIG proteins consists of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, protein toxins have at least 99% sequence identity with any of the foregoing sequences. The most preferred group of insecticidal IRDIG proteins toxins consists of SEQ ID

NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[00123] A "formulated insecticidal IRDIG protein" means purified or isolated insecticidal IRDIG protein that has been expressed or placed into a synthetic composition suitable for agricultural application, including but not limited to transgenic plants, sprayable liquid formulations, powdered solid formulations, or granular formulations.

[00124] "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a protein or peptide refers to a DNA segment that contains protein coding sequences yet is isolated away from, or purified free from, total genomic DNA of the species from which the DNA segment is obtained, which in the instant case is the genome of the Gram-positive bacterial genus, *Bacillus*, and in particular, the species known as *B. thuringiensis*. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, viruses, and the like.

[00125] "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding a bacterial insecticidal IRDIG protein, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or operon coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes, recombinant genes, synthetic linkers, or coding regions later added to the segment by the hand of man.

[00126] "A sequence essentially as set forth in SEQ ID NO:2" means that the sequence substantially corresponds to a portion of the sequence of SEQ ID NO:2 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of any of these sequences. The term "biologically functional equivalent" is well understood in the art. Accordingly, sequences that have between about 70% and about 80%, or more preferably between about 81% and about 90%, or even more preferably between about 91% and about 99% amino acid sequence identity or functional equivalence to the amino acids of SEQ ID NO:2 will be sequences that are "essentially as set forth in SEQ ID NO:2."

[00127] "Expression" means the combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

[00128] "Genetic material" means 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.

[00129] "Promoter" means a recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

[00130] "Nucleic acid construct" is an artificially created genetic sequence comprising a structural gene such as an IRDIG gene sequence and heterologous regulatory elements such as promoter, terminators, enhancers, or other genetic elements designed to cause the transcription or translation of the structural gene in an appropriate host.

[00131] "Regeneration" means the process of growing a plant from a plant cell (e.g., plant protoplast or explant).

[00132] "Structural gene" means a gene that is expressed to produce a polypeptide.

[00133] "Transformation" means process of introducing an exogenous DNA sequence (e.g., a vector, a recombinant DNA molecule) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

[00134] "Transformed cell" means a cell whose DNA has been altered by the introduction of an exogenous DNA molecule into that cell.

[00135] "Transgenic cell" means any cell derived or regenerated from a transformed cell or derived from a transgenic cell. Exemplary transgenic cells include plant calli derived from a transformed plant cell and particular cells such as leaf, root, stem, e.g., somatic cells, or reproductive (germ) cells obtained from a transgenic plant.

[00136] "Transgenic plant" means a plant or progeny thereof derived from a transformed plant cell or protoplast, wherein the plant DNA contains an introduced exogenous DNA molecule not originally present in a native, non-transgenic plant of the same strain. The terms

"transgenic plant" and "transformed plant" have sometimes been used in the art as synonymous terms to define a plant whose DNA contains an exogenous DNA molecule. However, it is thought more scientifically correct to refer to a regenerated plant or callus obtained from a transformed plant cell or protoplast as being a transgenic plant, and that usage will be followed herein.

[00137] "Vector" means a DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

[00138] It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5′ or 3′ sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5′ or 3′ portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

[00139] The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding regions, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch encoding the whole or a portion of the peptide sequence disclosed in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, or that are identical to or complementary to DNA sequences which encode the peptide disclosed in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, and particularly the DNA segment disclosed in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39,41, 43, 45, 69,

70, 72, 74, 76, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125. For example, DNA sequences such as about 14 nucleotides, and that are up to about 10,000, about 5,000, about 3,000, about 2,000, about 1,000, about 500, about 200, about 100, about 50, and about 14 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

[00140] It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; and up to and including sequences of about 10,000 nucleotides and the like.

[00141] It will also be understood that this invention is not limited to the particular nucleic acid sequences which encode peptides of the present invention, or which encode the amino acid sequence of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, including the DNA sequence which is particularly disclosed in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39,41, 43, 45, 69, 70, 72, 74, 76, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125. Recombinant vectors and isolated DNA segments may therefore variously include the peptide-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include these peptide-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

[00142] The DNA segments of the present invention encompass biologically-functional, equivalent peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally-equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the molecular level.

[00143] Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a gene encoding peptides of the present invention, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

[00144] In addition to their use in directing the expression of insecticidal IRDIG proteins or peptides of the present invention, the nucleic acid sequences contemplated herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous DNA segment of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39,41, 43, 45, 69, 70, 72, 74, 76, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125, will find particular utility. Longer contiguous identical or complementary sequences, e.g., those of about 20, 30, 40, 50, 100, 200, 500, 1000, 2000, 5000, 10000 etc. (including all intermediate lengths and up to and including full-length sequences) will also be of use in certain embodiments.

[00145] The ability of such nucleic acid probes to specifically hybridize to protein-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

[00146] Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to the DNA sequence of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39,41, 43, 45, 69, 70, 72, 74, 76, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, and 125, are particularly contemplated as hybridization probes for use in, e.g., Southern and Northern blotting. Smaller

fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 or 200 nucleotides, but larger contiguous complementary stretches may be used, according to the length complementary sequences one wishes to detect.

[00147] The use of a hybridization probe of about 14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 14 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

[00148] Of course, fragments may also be obtained by other techniques such as, e.g., by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Pat. Nos. 4,683,195 and 4,683,202 (each incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

[00149] Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. 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. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating protein-encoding DNA segments. Detection of DNA segments via hybridization is well-known to those of skill in the art, and the teachings of U.S. Pat. Nos. 4,965,188 and 5,176,995 (each incorporated herein by reference) are exemplary of the methods of hybridization analyses.

[00150] 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.

[00151] 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:

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

[00152] 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.

[00153] Alternatively, the T_m is described by the following formula (Beltz *et al.,* 1983).

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

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

[00154] 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 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). Also see Sambrook *et al.* (1989).

[00155] Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate protein-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20° C. to about 55° C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

[00156] In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known that can be employed to provide a means visible to

the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

[00157] In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

[00158] The invention also discloses and claims a composition comprising an insecticidal IRDIG protein. The composition may comprises bacterial host cells which express an insecticidal IRDIG protein, in the soluble fraction, inclusion bodies or crystals containing the insecticidal IRDIG protein, culture supernatant, disrupted cells, cell extracts, lysates, homogenates, and the like. The compositions may be in aqueous form, or alternatively, in dry, semi-wet, or similar forms such as cell paste, cell pellets, or alternatively freeze dried, powdered, lyophilized, evaporated, or otherwise similarly prepared in dry form. Such means for preparing insecticidal IRDIG proteins are well-known to those of skill in the art of bacterial protein isolation and purification. In certain embodiments, the proteins may be purified, concentrated, admixed with other reagents, or processed to a desired final form. Preferably, the composition will comprise from about 1% to about 90% by weight of the protein, and more preferably from about 5%, to about 50% by weight.

[00159] In a preferred embodiment, the protein compositions of the invention may be prepared by a process which comprises the steps of culturing a *Bacillus thuringiensis* cell which expresses an insecticidal IRDIG protein under conditions effective to produce such a protein, and then obtaining the protein from the cell. The obtaining of such a protein may further include purifying, concentrating, processing, or mixing the protein with one or more reagents. Preferably, the insecticidal IRDIG protein toxin is obtained in an amount of from between about 1% to about 90% by weight and more preferably from about 5% to about 50% by weight.

[00160] The invention also relates to a method of preparing an insecticidal IRDIG protein composition. Such a method generally involves the steps of culturing a *Bacillus thuringiensis* cell which expresses an insecticidal IRDIG protein toxin under conditions effective to produce the protein, and then obtaining the protein so produced. In a preferred embodiment the *Bacillus thuringiensis* cell is any *Bacillus thuringiensis* cell which contains an insecticidal IRDIG protein gene segment. Alternatively, the recombinant plasmid vectors of the invention may be used to transform other suitable bacterial or eukaryotic cells to produce the protein of the invention. Prokaryotic host cells including Gram-negative cells such as *E. coli, Pseudomonas fluorescens* and related *Enterobacteraceae*, or Gram-positive cells such as *Bacillus* spp. (including *B. megaterium, B. subtilis*, and *B. thuringiensis*) and the like are all contemplated to be useful in the preparation of the insecticidal IRDIG proteins of the invention. Particularly preferred are the commonly used *E. coli* and *Pseudomonas fluorescens* expression strains.

[00161] In such embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a DNA segment encoding a protein or peptide in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or plant cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology).

[00162] In connection with expression embodiments to prepare recombinant proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire peptide sequence being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of peptides or

epitopic core regions, such as may be used to generate anti-protein antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 8 to about 50 amino acids in length, or more preferably, from about 8 to about 30 amino acids in length, or even more preferably, from about 8 to about 20 amino acids in length are contemplated to be particularly useful. Such peptide epitopes may be amino acid sequences which comprise contiguous amino acid sequences from SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, or 126.

[00163] In yet another aspect, the present invention provides methods for producing a transgenic cell, and in particular a plant cell which expresses a nucleic acid segment encoding the novel insecticidal IRDIG proteins of the present invention. The process of producing transgenic cells is well-known in the art. In general, the method comprises transforming a suitable host cell with a DNA segment which contains a promoter operatively linked to a coding region that encodes an insecticidal IRDIG protein toxin. Such a coding region is generally operatively linked to a transcription-terminating region, whereby the promoter is capable of driving the transcription of the coding region in the cell, and hence providing the cell the ability to produce the recombinant protein *in vivo*. Alternatively, in instances where it is desirable to control, regulate, or decrease the amount of a particular recombinant protein expressed in a particular transgenic cell, the invention also provides for the expression of protein antisense mRNA. The use of antisense mRNA as a means of controlling or decreasing the amount of a given protein of interest in a cell is well-known in the art.

[00164] In a preferred embodiment, the invention encompasses a plant cell which has been transformed with a nucleic acid segment of the invention, and which expresses a gene or gene segment encoding one or more of the novel polypeptide compositions disclosed herein. As used herein, the term "transgenic plant cell" is intended to refer to a plant cell that has incorporated DNA sequences, including but not limited to genes which are perhaps not normally present, DNA sequences not normally transcribed into RNA or translated into a protein ("expressed"), or any other genes or DNA sequences which one desires to introduce into the non-transformed plant, such as genes which may normally be present in the non-transformed plant but which one desires to either genetically engineer or to have altered expression.

[00165] It is contemplated that in some instances the genome of a transgenic plant of the present invention will have been augmented through the stable introduction of an insecticidal IRDIG protein toxin-expressing transgene. In some instances, more than one transgene may be incorporated into the genome of the transformed host plant cell. Such is the case when more than one protein-encoding DNA segment is incorporated into the genome of such a plant. In certain situations, it may be desirable to have one, two, three, four, or even more *B. thuringiensis* crystal proteins or other insecticidal IRDIG proteins or nucleic acids incorporated and stably expressed in the transformed transgenic plant. In preferred embodiments, the introduction of the transgene into the genome of the plant cell results in a stable integration wherein the offspring of such plants also contain a copy of the transgene in their genome. The inheritability of this genetic element by the progeny of the plant into which the gene was originally introduced is a preferred aspect of this invention.

[00166] A gene encoding an insecticidal IRDIG protein of the invention may be stably introduced into the genome of a plant cell using well-known methods which is then regenerated into a whole fertile plant. Such plants are highly desirable to growers to protect against crop damage caused by Coleoptera, especially WCR, feeding.

[00167] Means for transforming a plant cell and the preparation of a transgenic cell line are well-known in the art (as exemplified in U.S. Pat. Nos. 5,550,318; 5,508,468; 5,482,852; 5,384,253; 5,276,269; and 5,225,341, all specifically incorporated herein by reference), and are briefly discussed herein. Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes), and DNA segments for use in transforming such cells will, of course, generally comprise either the operons, genes, or gene-derived sequences of the present invention, either native, or synthetically-derived, and particularly those encoding the disclosed proteins. These DNA or nucleic acid constructs can further include structures such as promoters, enhancers, polylinkers, or even gene sequences which have positively- or negatively-regulating activity upon the particular genes of interest as desired. The DNA segment or gene may encode either a native or modified protein, which will be expressed in the resultant recombinant cells, and/or which will impart an improved phenotype to the regenerated plant.

[00168] Such transgenic plants may be desirable for increasing the insecticidal resistance of a monocotyledonous or dicotyledonous plant, by incorporating into such a plant, a transgenic DNA segment encoding an insecticidal IRDIG protein which is toxic to insects. Particularly

preferred plants include corn, wheat, soybeans, cotton, turf grasses, ornamental plants, fruit trees, shrubs, vegetables, grains, legumes, and the like, or any plant into which introduction of a insecticidal IRDIG protein transgene is desired.

[00169] In a related aspect, the present invention also encompasses a seed produced by the transformed plant, a progeny from such seed, and a seed produced by the progeny of the original transgenic plant, produced in accordance with the above process. Such progeny and seeds will have a protein encoding transgene stably incorporated into its genome, and such progeny plants will inherit the traits afforded by the introduction of a stable transgene in Mendelian fashion. All such transgenic plants having incorporated into their genome transgenic DNA segments encoding an insecticidal IRDIG protein toxin are aspects of this invention.

[00170] In particular embodiments, the inventors contemplate the use of antibodies, either monoclonal or polyclonal which bind to the proteins disclosed herein. Means for preparing and characterizing antibodies are well known in the art.

[00171] The present invention also provides compositions, methods and kits for screening samples suspected of containing an insecticidal IRDIG protein toxin or a gene encoding such a toxin. Such screening may be performed on samples such as transformed host cells, transgenic plants, progeny or seed thereof, or laboratory samples suspected of containing or producing such a polypeptide or nucleic acid segment. A kit can contain a novel nucleic acid segment or an antibody of the present invention. The kit can contain reagents for detecting an interaction between a sample and a nucleic acid or an antibody of the present invention. The provided reagent can be radio-, fluorescently- or enzymatically-labeled. The kit can contain a known radiolabeled agent capable of binding or interacting with a nucleic acid or antibody of the present invention.

[00172] The reagent of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. Preferably, when the reagent is provided in a liquid solution, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatograph media, a test plate having a plurality of wells, or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent that may be provided.

[00173] In still further embodiments, the present invention concerns immunodetection methods and associated kits. It is proposed that the proteins or peptides of the present invention

may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect proteins or protein-related epitope-containing peptides. In general, these methods will include first obtaining a sample suspected of containing such a protein, peptide or antibody, contacting the sample with an antibody or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of an immunocomplex, and then detecting the presence of the immunocomplex.

[00174] In general, the detection of immunocomplex formation is quite well known in the art and may be achieved through the application of numerous approaches. For example, the present invention contemplates the application of ELISA, RIA, immunoblot (e.g., dot blot), indirect immunofluorescence techniques and the like. Generally, immunocomplex formation will be detected through the use of a label, such as a radiolabel or an enzyme tag (such as alkaline phosphatase, horseradish peroxidase, or the like). Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

[00175] For assaying purposes, it is proposed that virtually any sample suspected of comprising either a protein or peptide or a protein-related peptide or antibody sought to be detected, as the case may be, may be employed. It is contemplated that such embodiments may have application in the titering of antigen or antibody samples, in the selection of hybridomas, and the like. In related embodiments, the present invention contemplates the preparation of kits that may be employed to detect the presence of proteins or related peptides and/or antibodies in a sample. Samples may include cells, cell supernatants, cell suspensions, cell extracts, enzyme fractions, protein extracts, or other cell-free compositions suspected of containing proteins or peptides. Generally speaking, kits in accordance with the present invention will include a suitable protein, peptide or an antibody directed against such a protein or peptide, together with an immunodetection reagent and a means for containing the antibody or antigen and reagent. The immunodetection reagent will typically comprise a label associated with the antibody or antigen, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first antibody or antigen or a biotin or avidin (or streptavidin) ligand having an associated label. Of course, as noted above, a number of

exemplary labels are known in the art and all such labels may be employed in connection with the present invention.

[00176] The container will generally include a vial into which the antibody, antigen or detection reagent may be placed, and preferably suitably aliquoted. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

[00177] ELISAs and Immunoprecipitation

[00178] ELISAs may be used in conjunction with the invention. In an ELISA assay, proteins or peptides incorporating protein antigen sequences are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of milk powder. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

[00179] After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/TWEEN® surface active agent (ICI Americas, Inc., Wilmington, Del.). These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hours, at temperatures preferably on the order of about 25 °C to about 27 °C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/TWEEN® surface active agent, or borate buffer.

[00180] Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting to a second antibody having specificity for the first. To provide a detecting means, the second antibody will preferably have an associated enzyme

that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS/TWEEN®) surface active agent.

[00181] After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2′-azino-di-(3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

[00182] The anti-protein antibodies of the present invention are particularly useful for the isolation of other protein antigens by immunoprecipitation. Immunoprecipitation involves the separation of the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Non-ionic salts are preferred, since other agents such as bile salts, precipitate at acid pH or in the presence of bivalent cations.

[00183] In an alternative embodiment the antibodies of the present invention are useful for the close juxtaposition of two antigens. This is particularly useful for increasing the localized concentration of antigens, e.g. enzyme-substrate pairs.

[00184] The compositions of the present invention will find great use in immunoblot or western blot analysis. The anti-peptide antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immuno-precipitation, followed by gel electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. This is especially useful when the antigens studied are immunoglobulins (precluding the use of immunoglobulins binding bacterial cell wall components), the antigens studied cross-react with the detecting agent, or they migrate at the same relative molecular weight as a cross-reacting signal.

[00185] Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

[00186] The present invention is also directed to protein or peptide compositions, free from total cells and other peptides, which comprise a purified protein or peptide which incorporates an epitope that is immunologically cross-reactive with one or more anti-protein antibodies. In particular, the invention concerns epitopic core sequences derived from insecticidal IRDIG proteins or peptides.

[00187] As used herein, the term "incorporating an epitope(s) that is immunologically cross-reactive with one or more anti-protein antibodies" is intended to refer to a peptide or protein antigen which includes a primary, secondary, or tertiary structure similar to an epitope located within a protein or polypeptide. The level of similarity will generally be to such a degree that monoclonal or polyclonal antibodies directed against the protein or polypeptide will also bind to, react with, or otherwise recognize, the cross-reactive peptide or protein antigen. Various immunoassay methods may be employed in conjunction with such antibodies, such as, for example, Western blotting, ELISA, RIA, and the like, all of which are known to those of skill in the art.

[00188] The identification of Cry immunodominant epitopes, or their functional equivalents, is a relatively straightforward matter. For example, one may employ the methods of Hopp, as taught in U.S. Pat. No. 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. The methods described in several other papers, and software programs based thereon, can also be used to identify epitopic core sequences (see, e.g., U.S. Pat. No. 4,554,101). The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into peptides, either through the application of peptide synthesis or recombinant technology.

[00189] Preferred peptides for use in accordance with the present invention will generally be on the order of about 8 to about 20 amino acids in length, and more preferably about 8 to about 15 amino acids in length. It is proposed that shorter antigenic protein-derived peptides will provide advantages in certain circumstances, for example, in the preparation of immunologic detection assays. Exemplary advantages include the ease of preparation and purification, the

relatively low cost and improved reproducibility of production, and advantageous biodistribution.

[00190] It is proposed that particular advantages of the present invention may be realized through the preparation of synthetic peptides which include modified or extended epitopic/immunogenic core sequences which result in a "universal" epitopic peptide directed to proteins, and in particular insecticidal and insecticidal-related sequences. These epitopic core sequences are identified herein in particular aspects as hydrophilic regions of the particular polypeptide antigen. It is proposed that these regions represent those which are most likely to promote T-cell or B-cell stimulation, and, hence, elicit specific antibody production.

[00191] An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is "complementary" to, and therefore will bind, antigen binding sites on the protein-directed antibodies disclosed herein. Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with antibodies directed against the peptide compositions of the present invention. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired protein antigen with the corresponding protein-directed antisera.

[00192] In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would generally be on the order of about 8 amino acids in length, with sequences on the order of 10 to 20 being more preferred. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

[00193] The identification of epitopic core sequences is known to those of skill in the art, for example, as described in U.S. Pat. No. 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. Moreover, numerous computer programs are available for use in predicting antigenic portions of proteins. Computerized peptide sequence analysis programs (e.g.,

DNAStar® software, DNAStar, Inc., Madison, Wis.) may also be useful in designing synthetic peptides in accordance with the present disclosure.

[00194] Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (e.g., through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

[00195] In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, e.g., up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of about 7.0 to about 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at about 4 °C, or more preferably, frozen. Of course, where the peptides are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, e.g., in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

[00196] The inventors contemplate that the protein compositions disclosed herein will find particular utility as insecticides for topical or systemic application to field crops, grasses, fruits and vegetables, and ornamental plants. In a preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension of bacterial cells which expresses a novel protein disclosed herein. Preferably the cells are *B. thuringiensis,* however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a protein is contemplated to be useful, including but not limited to *B. megaterium, B. subtilis, E. coli*, or *Pseudomonas* spp.

[00197] In another important embodiment, the bioinsecticide composition comprises a water dispersible granule. This granule comprises bacterial cells which expresses a novel protein disclosed herein. Preferred bacterial cells are *B. thuringiensis* cells, however, bacteria such as *B.*

megaterium, B. subtilis, E. coli, or *Pseudomonas* spp. cells transformed with a DNA segment disclosed herein and expressing the protein are also contemplated to be useful.

[00198] In a third important embodiment, the bioinsecticide composition comprises a wettable powder, dust, pellet, or collodial concentrate. This powder comprises bacterial cells which expresses a novel protein disclosed herein. Preferred bacterial cells are *B. thuringiensis* cells, however, bacteria such as *B. megaterium, B. subtilis, E. coli*, or *Pseudomonas* spp. cells transformed with a DNA segment disclosed herein and expressing the protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained release, or other time-dependent manner.

[00199] In a fourth important embodiment, the bioinsecticide composition comprises an aqueous suspension of bacterial cells such as those described above which express the protein. Such aqueous suspensions may be provided as a concentrated stock solution which is diluted prior to application, or alternatively, as a diluted solution ready-to-apply.

[00200] For these methods involving application of bacterial cells, the cellular host containing the protein gene(s) may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B. thuringiensis* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

[00201] When the insecticidal compositions comprise intact *B. thuringiensis* cells expressing the protein of interest, such bacteria may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

[00202] Alternatively, the novel insecticidal IRDIG proteins or insecticidal IRDIG protein-derived toxins may be prepared by native or recombinant bacterial expression systems *in vitro*

and isolated for subsequent field application. Such protein may be either in crude cell lysates, suspensions, colloids, etc., or alternatively may be purified, refined, buffered, and/or further processed, before formulating in an active biocidal formulation. Likewise, under certain circumstances, it may be desirable to isolate crystals and/or spores from bacterial cultures expressing the protein and apply solutions, suspensions, or collodial preparations of such crystals and/or spores as the active bioinsecticidal composition.

[00203] Regardless of the method of application, the amount of the active component(s) are applied at an insecticidally-effective amount, which will vary depending on such factors as, for example, the specific insects to be controlled, the specific plant or crop to be treated, the environmental conditions, and the method, rate, and quantity of application of the insecticidally-active composition.

[00204] The insecticide compositions described may be made by formulating the bacterial cell, crystal and/or spore suspension, or isolated protein component with the desired agriculturally-acceptable carrier. The compositions may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, desiccated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, e.g., inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well known to those skilled in insecticide formulation. The formulations may be mixed with one or more solid or liquid adjuvants and prepared by various means, e.g., by homogeneously mixing, blending and/or grinding the insecticidal composition with suitable adjuvants using conventional formulation techniques.

[00205] The insecticidal compositions of this invention are applied to the environment of the target insect, typically onto the foliage and in the rhizosphere (the soil surrounding plant roots) of the plant or crop to be protected, by conventional methods, preferably by spraying. The strength and duration of insecticidal application will be set with regard to conditions specific to the particular pest(s), crop(s) to be treated and particular environmental conditions. The proportional ratio of active ingredient to carrier will naturally depend on the chemical nature,

solubility, and stability of the insecticidal composition, as well as the particular formulation contemplated.

[00206] Other application techniques, e.g., dusting, sprinkling, soaking, soil injection, seed coating, seedling coating, spraying, aerating, misting, atomizing, and the like, are also feasible and may be required under certain circumstances such as e.g., insects that cause root or stalk infestation, or for application to delicate vegetation or ornamental plants. These application procedures are also well-known to those of skill in the art.

[00207] The insecticidal composition of the invention may be employed in the method of the invention singly or in combination with other compounds, including and not limited to other pesticides. The method of the invention may also be used in conjunction with other treatments such as surfactants, detergents, polymers or time-release formulations. The insecticidal compositions of the present invention may be formulated for either systemic or topical use.

[00208] The concentration of insecticidal composition which is used for environmental, systemic, or soil application will vary widely depending upon the nature of the particular formulation, means of application, environmental conditions, and degree of biocidal activity. Typically, the bioinsecticidal composition will be present in the applied formulation at a concentration of at least about 1% by weight and may be up to and including about 99% by weight. Dry formulations of the compositions may be from about 1% to about 99% or more by weight of the composition, while liquid formulations may generally comprise from about 1% to about 99% or more of the active ingredient by weight.

[00209] The insecticidal formulation may be administered to a particular plant or target area in one or more applications as needed, with a typical field application rate per hectare ranging on the order of from about 50 g to about 500 g of active ingredient, or of from about 500 g to about 1000 g, or of from about 1000 g to about 5000 g or more of active ingredient.

[00210] Modification and changes may be made in the primary structure of the toxins of the present invention to produce derivatives, analogs and mutants and DNA segments which encode them and still obtain a functional insecticidal molecule that encodes a protein or peptide with desirable characteristics. In particular embodiments of the invention, mutated proteins are contemplated to be useful for increasing the insecticidal activity of the protein, and consequently increasing the insecticidal activity or expression of the recombinant transgene in a

plant cell. The amino acid changes may be achieved by changing the codons of the nucleic sequence, according to the codons given in Table 2.

Table 2

Amino Acids	Abbreviation	Abbreviation	Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUA GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

[00211] For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a

protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

[00212] In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art. It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

[00213] Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

[00214] It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

[00215] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

[00216] As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3);

valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (–3.4).

[00217] It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

[00218] As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

[00219] In another aspect, DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of the selected polynucleotides disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of a selected protein gene sequence, e.g., a sequence such as that shown in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39,41, 43, 45, 69, 70, 72, 74, 76, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, and 121. The ability of such nucleic acid probes to specifically hybridize to a protein-encoding gene sequence lends them particular utility in a variety of embodiments. Most importantly, the probes may be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

[00220] In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined segment of a protein gene from *B. thuringiensis* using PCR™ technology. Segments of related protein genes from other species may also be amplified by PCR™ using such primers.

[00221] The present invention contemplates an expression vector comprising a polynucleotide of the present invention. Thus, in one embodiment an expression vector is an isolated and purified DNA molecule comprising a promoter operatively linked to a coding

region that encodes a polypeptide of the present invention, which coding region is operatively linked to a transcription-terminating region, whereby the promoter drives the transcription of the coding region.

[00222] As used herein, the term "operatively linked" means that a promoter is connected to a coding region in such a way that the transcription of that coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region are well known in the art.

[00223] In a preferred embodiment, the recombinant expression of DNAs encoding the proteins of the present invention is preferable in a *Bacillus* host cell. Preferred host cells include *B. thuringiensis, B. megaterium, B. subtilis*, and related bacilli, with *B. thuringiensis* host cells being highly preferred. Promoters that function in bacteria are well-known in the art. An exemplary and preferred promoter for the *Bacillus* crystal proteins include any of the known crystal protein gene promoters, including the insecticidal IRDIG protein gene promoter, and promoters specific for *B. thuringiensis* sigma factors, such as σ^E and σ^K. Alternatively, mutagenized or recombinant crystal protein-encoding gene promoters may be engineered by the hand of man and used to promote expression of the novel gene segments disclosed herein.

[00224] In an alternate embodiment, the recombinant expression of DNAs encoding the proteins of the present invention is performed using a transformed Gram-negative bacterium such as an *E. coli* or *Pseudomonas* spp. host cell. Promoters which function in high-level expression of target polypeptides in *E. coli* and other Gram-negative host cells are also well-known in the art.

[00225] Where an expression vector of the present invention is to be used to transform a plant, a promoter is selected that has the ability to drive expression in plants. Promoters that function in plants are also well known in the art. Useful in expressing the polypeptide in plants are promoters that are inducible, viral, synthetic, constitutive, and temporally regulated, spatially regulated, and spatio-temporally regulated.

[00226] A promoter is also selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Structural genes can be driven by a variety of promoters in plant tissues. Promoters can be near-constitutive, such as the CaMV 35S promoter, or tissue-specific or developmentally specific promoters affecting dicots or monocots.

[00227] 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 seeds (e.g., zein, oleosin, lectin, 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.

[00228] Exemplary tissue-specific promoters are corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, pea small subunit RuBP Carboxylase, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, CaMV 35s transcript and Potato patatin. Preferred promoters are the cauliflower mosaic virus (CaMV 35S) promoter and the S-E9 small subunit RuBP carboxylase promoter.

[00229] 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.

[00230] An expression vector containing a coding region that encodes a polypeptide of interest is engineered to be under control of the lectin promoter and that vector is introduced into plants using, for example, a protoplast transformation method. The expression of the polypeptide is directed specifically to the seeds of the transgenic plant.

[00231] A transgenic plant of the present invention produced from a plant cell transformed with a tissue specific promoter can be crossed with a second transgenic plant developed from a plant cell transformed with a different tissue specific promoter to produce a hybrid transgenic plant that shows the effects of transformation in more than one specific tissue.

[00232] The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depends directly on the functional properties desired, e.g., the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the expression of the polypeptide coding region to which it is operatively linked.

[00233] Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. However, several other plant integrating vector systems are known to function in plants including pCaMVCN transfer control vector. Plasmid pCaMVCN (available from Pharmacia, Piscataway, N.J.) includes the cauliflower mosaic virus CaMV 35S promoter.

[00234] In preferred embodiments, the vector used to express the polypeptide includes a selection marker that is effective in a plant cell, preferably a drug resistance selection marker. One preferred drug resistance marker is the gene whose expression results in kanamycin resistance; i.e., the chimeric gene containing the nopaline synthase promoter, Tn5 neomycin phosphotransferase II (nptII) and nopaline synthase 3′ nontranslated region.

[00235] RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences

are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

[00236] Means for preparing expression vectors are well known in the art. Expression (transformation vectors) used to transform plants and methods of making those vectors are described in U.S. Pat. Nos. 4,971,908, 4,940,835, 4,769,061 and 4,757,011, the disclosures of which are incorporated herein by reference. Those vectors can be modified to include a coding sequence in accordance with the present invention.

[00237] A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

[00238] A coding region that encodes a polypeptide having the ability to confer insecticidal activity to a cell is preferably an insecticidal IRDIG protein toxin-encoding gene.

[00239] A bacterium, a yeast cell, plant cell, or a plant transformed with an expression vector of the present invention is also contemplated. A transgenic bacterium, yeast cell, plant cell, or plant derived from such a transformed or transgenic cell is also contemplated. Means for transforming bacteria and yeast cells are well known in the art. Typically, means of transformation are similar to those well-known means used to transform other bacteria or yeast such as *E. coli* or *Saccharomyces cerevisiae.*

[00240] Methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is well known which methods are useful for a particular plant strain.

[00241] There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as by *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts, by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation

with silicon carbide fibers, by acceleration of DNA coated particles, etc. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

[00242] Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods; (2) physical methods such as microinjection, electroporation and the gene gun; (3) viral vectors; and (4) receptor-mediated mechanisms.

[00243] More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced insecticidal activity relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

[00244] It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a polypeptide of interest. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

[00245] Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments.

[00246] Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (WO 1997/013843 A1).

[00247] To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected. In addition, "particle gun" or high-velocity microprojectile technology can be utilized.

[00248] Using that latter technology, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles. The metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants.

[00249] By transforming a suitable host cell, such as a plant cell, with a recombinant insecticidal IRDIG protein encoding gene-containing segment, the expression of the encoded protein (*i.e.*, a bacterial protein or polypeptide having insecticidal activity against coleopterans) can result in the formation of insect-resistant plants.

[00250] By way of example, one may utilize an expression vector containing a coding region for a *B. thuringiensis* protein and an appropriate selectable marker to transform a suspension of embryonic plant cells, such as wheat or corn cells using a method such as particle bombardment to deliver the DNA coated on microprojectiles into the recipient cells. Transgenic plants are then regenerated from transformed embryonic calli that express the insecticidal IRDIG proteins.

[00251] The formation of transgenic plants may also be accomplished using other methods of cell transformation which are known in the art such as *Agrobacterium*-mediated DNA transfer. Alternatively, DNA can be introduced into plants by direct DNA transfer into pollen, by injection of the DNA into reproductive organs of a plant, or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos.

[00252] The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art. This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

[00253] The development or regeneration of plants containing the foreign, exogenous gene that encodes a polypeptide of interest introduced by *Agrobacterium* from leaf explants can be achieved by methods well known in the art. In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described.

[00254] This procedure typically produces shoots within two to four months and those shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Shoots that rooted in the presence of the selective

agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant strain employed, such variations being well known in the art.

[00255] Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, as discussed before. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

[00256] A transgenic plant of this invention thus has an increased amount of a coding region (e.g., an insecticidal gene) that encodes the polypeptide of interest. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for, by way of example, increased insecticidal capacity against insects, preferably in the field, under a range of environmental conditions. The inventors contemplate that the present invention will find particular utility in the creation of transgenic plants of commercial interest including corn and various turf grasses, wheat, corn, rice, barley, oats, a variety of ornamental plants and vegetables, as well as a number of nut- and fruit-bearing trees and plants.

[00257] 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.

[00258] 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.

[00259] 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 the genes encoding insecticidal IRDIG proteins

[00260] Nucleic acids encoding the insecticidal IRDIG proteins were isolated from various *B.t.* strains. Forward and reverse primers for Polymerase Chain Reaction (PCR) were designed and used to amplify nucleotide sequences encoding the full-length insecticidal IRDIG proteins (Table 3). The amplified fragments were subcloned into a protein expression vector backbone. BLAST searches of these gene sequences against NCBI, Pfam and GenomeQuest databases did not return any significant hits. These sequence search results indicate that these genes are novel among all known protein sequences.

[00261] Standard cloning methods were used in the construction of *Pseudomonas fluorescens* (*Pf*) expression plasmids engineered to produce full-length insecticidal IRDIG protein toxins encoded by native and plant-optimized coding regions (described below). Restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA) and T4 DNA Ligase (NEB; Ipswich, MA) was used for DNA ligation. DNA fragments were purified using a QIAquick® Gel Extraction Kit (Qiagen, Venio, Limburg) after agarose Tris-acetate gel electrophoresis. Plasmid preparations were performed using the NucleoSpin® Plasmid Kit (Macherey-Nagel Inc, Bethlehem, PA) following the instructions of the suppliers for low-copy plasmid purification or the Qiagen Plasmid Plus Midi kit® (Qiagen, Hilden, Germany).

[00262] All insecticidal IRDIG proteins were analyzed phylogenetically and non-redundant representative proteins were selected for WCR activity validation. The open reading frame (ORF) sequences of unique representative insecticidal IRDIG proteins were identified. These ORFs were amplified through PCR using the genomic DNAs isolated from the wild-type *B.t.* strains containing the target insecticidal IRDIG proteins as a template and using the primers listed in Table 3.

Table 3

Primers used for PCR to amplify the open reading frames of insecticidal IRDIG proteins for cloning and expression in either *Bt* or *Pf*

IRDIG #	Primer ID	Sequence	SEQ ID NO

28674	28674F	GTGTACTAGTATGGATAATCATTTTTTAGATTTAATCTCAAAAG	47
	28674R	TCTCCTCGAGTTAAGCTCTACCATCATAATGTAAATCATATTTTTGAC	48
28672	28672F	GTGTACTAGTATGAATAACCAGTTATTAGATTTACTGTCAAAAACTC	49
	28672R	TCTCCTCGAGTTAAGCTCTGCCATCATAATGTAAATCATATTTTTGAC	50
28680	28680F	GTGTACTAGTATGGCAACAGTTAGCGGAAAAATAATAATAAATAC	51
	28680R	TCTCCTCGAGTTAATTACTACTGTCATATTTTAAAATATATTTTTGTCCAAGC	52
28682	28682F	GTGTACTAGTATGAATAATACATTATTGGAATTACTTTCAAAAATAAAAAAAGAATTCTTTGG	53
	28682R	TCTCCTCGAGTTAAGCTCTACCTTTGTATTGTAATGTATATCTTTCTC	54
28684	28684F	GTGTACTAGTATGAATAATACATTATTGGAATTACTTTCAAAAATAAAAAAAGAATTCTTTGG	55
	28684R	TCTCCTCGAGTTAAGCTCTACCTTTGTATTGTAATGTATATCTTTCTCC	56
28686	28686F	GTGTACTAGTATGAATAACACATTATTGGAATTACTTTCAAAAATAAAAAAAGAATTCTTTGG	57
	28686R	TCTCCTCGAGTTAAGCCCTACCTTTGTATTGTAATGTATATCTTTCTCC	58
28688	28688F	GTGTACTAGTATGTATATGAATAATACATTATTGGAATTACTTTCAAAAATAAAAAAAGAATTCTTTGG	59
	28688R	TCTCCTCGAGTTAAGCTCTACCTTTGTATTGTAATGTATATCTTTCTCC	60
28696	28696F	GTGTACTAGTATGAAGAAAAGATTTTCTGCCGTATCG	61
	28696R	TCTCCTCGAGTTAACGCGGGCTTAATGTATAGGTTT	62
29779	29779FSpeI	GTGTACTAGTATGGATAATAATTTATTATATTTAATTTCAAAAATCCAAACC	63
	29779RXhoI	TCTCCTCGAGTTATTGTTTAGTTAAAGTATAATTTTCTTTAGATACCTTAAC	64
29781	29781FXbaI	GTGTTCTAGAATGAAAGAAGGAGTGTTTAAATTGAAAAAAGG	65
	29781RXhoI	TCTCCTCGAGTTAGCGTGGAGCTAATGTATAGGTTTCAC	66
28678	28678FXbaI	GTGTTCTAGAATGGATAATCATTTATTAGAGTTACTTTTAAAAGTTCAAAAC	67
	28678RPstI	GTCACTGCAGTTAAGCTCTACCATCATATTTTAAAGTATATCTTTCTCC	68

EXAMPLE 2

Design of a plant codon-optimized insecticidal toxin gene

[00263] 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.

[00264] A DNA sequence having a maize codon bias was designed and synthesized to produce an insecticidal IRDIG 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.

[00265] 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. The maize-optimized DNA sequences encoding insecticidal toxins are disclosed as SEQ ID NOs:69, 70, 72, 74, and 76.

[00266] The foregoing provides several embodiments of the isolated polynucleotide(s) according to the invention, including polynucleotides that are codon-optimized for expression of insecticidal toxin polypeptides of the invention.

EXAMPLE 3

Construction of expression plasmid encoding insecticidal IRDIG protein toxins in bacterial hosts

[00267] Standard cloning methods were used in the construction of *Pseudomonas fluorescens* (*Pf*) expression plasmids engineered to produce the insecticidal IRDIG protein toxins encoded by either the native or 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 treated with Antarctic Phosphatase (NEB) to enhance formation of recombinant molecules.

[00268] The resulting PCR products lacking the native regulatory element sequences were cloned into pDAB122775 for expression in *B.t.* host 4Q7 and into pDOW1169 for expression in a *Pf* host, respectively. The cloning sites used for both *B.t.* and *Pf* systems were XbaI/XhoI or SpeI/XhoI. *B.t.* expression vector pDAB122775 includes a Cry1Ac crystal protein gene promoter (expressed during *B.t.* cell sporulation), ribosomal binding site (RBS) and the Cry1Ac terminator, while in pDOW1169 these target gene expressions were driven by Ptac promoter and IPTG induction. pDOW1169 is a low 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). If not expressed in either *B.t.* or *Pf*, they were cloned into an *E. coli* expression vector such as pET280(Kan) at SpeI/XhoI. Constructs were generated using standard molecular cloning procedures that are well known in the art.

[00269] The expression plasmids (pDAB121093, 127479, 127480, 127481, 127482, 127484, 127485, 127486, 127487, 127488, 127489, 127490, 127491) were transformed by electroporation into DC454 (a near wild-type *P. fluorescens* strain having mutations *ΔpyrF* 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*).

[00270] Protein expression experiments for these insecticidal IRDIG proteins were performed first in 4Q7 *B.t.* host and then in DPf10 *Pf* host. Briefly, recombinant *B.t.* cultures were grown in 50 ml of Dow AgroSciences Proprietary medium broth that promotes *B.t.* bacterial sporulation, in a 250-ml baffled flask at 28 °C/180-200 rpm for 24-32 hours. The mixture of the crystals and endospores was harvested by centrifugation at 6,000 g at 4 °C for 15 min and followed by washing in 10 ml of 1M NaCl, 0.1% Triton X-100 solution and then in 35 ml of ionized water. The final pellet was suspended in 2 ml deionized water for WCR feeding assays. The *B.t.* 4Q7 transformed with empty vector pDAB122775 was included as a negative control.

[00271] The transformation and selection methods are generally described available in 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.

EXAMPLE 4

Preparation of insecticidal IRDIG protein samples

[00272] Production of insecticidal IRDIG proteins for characterization and insect bioassay was accomplished by shake-flask-grown *P. fluorescens* strain harboring expression construct strains DPf46314, 48284, 48285, 48286, 48287, 48289, 48290, 48291, 48292, 48293, 48294, 48295, 48296. Stored glycerol stocks of the strain were used to inoculate defined production medium with 9.5% glycerol (Teknova Catalog No. 3D7426, Hollister, CA). Expression of the insecticidal gene 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 (OD_{600}). Other culture media suitable for growth of *Pseudomonas fluorescens* may also be utilized, for example, as described in US Patent Application No. 20060008877. The post- and pre-induction samples were analyzed for target protein expression in both cellular soluble and insoluble fractions following BugBuster® lysis and extraction procedures. To estimate expression levels, densitometry analysis was performed using a GE Image Scanner III (GE Healthcare). Protein bands were detected and quantified using ImageQuant TL software (GE Healthcare) and BSA as a standard. The insecticidal IRDIG protein accumulated in the insoluble fraction of lysed cells as inclusion bodies (IB). The cells were flash frozen in liquid nitrogen and stored at -80 C.

[00273] Inclusion body (IB) preparation for insecticidal IRDIG proteins. *Pf* derived cell pastes expressing full length insecticidal IRDIG proteins were transferred from -80 °C storage to room temperature. Approximately 10g of each was taken out and resuspended in cold lysis buffer (40 mL of 50 mM Tris, 200 mM NaCl, 10% glycerol, 0.5% Triton X-100, 20 mM EDTA, 1 mM DTT, pH 7.5) at 20% w/v. The resuspended pellet was incubated at room temperature while rocking with 0.4 mg/ml lysozyme for 20 minutes. This was followed by adding 0.1 mg/mL DNase with 0.1 M $MgCl_2$ and further incubation at 30 °C in a water bath for 20 minutes. The sample was sonicated using a Branson sonifier for 1 minute, duty cycle-60, output control 4 followed by centrifugation at 16,000 rpm for 30 minutes in a JA-17 rotor. The pellets were resuspended 2 additional times in 20% w/v cold lysis buffer with metal beads. The final two washes were carried out using the lysis buffer in the absence of triton-x-100, the supernatants were colorless and the IB pellets became firm and off-white in color. The inclusion

bodies were resuspended in sterile-filtered distilled water containing 10 mM EDTA, pH 8.0, aliquoted into 1.5 mL and frozen at -80 °C until needed.

[00274] *Inclusion body solubilization.* Inclusion bodies were thawed at room temperature in a water bath. The inclusion bodies were brought up to 10 mL in 0.1 M CAPS pH 11 followed by sonication for 1 min at output control 4, duty cycle 40%. The solubilized protein was centrifuged for 20 min at 16,000 rpm in a JA17 rotor. The samples were concentrated by 15 mL Amicon 3K MWCO to give a final volume of about 2 mL and buffer exchanged once by adding 18 mL 10 mM CAPS, pH 11 and concentrating them down to 2 mL. This was followed by desalting on PD10 columns that had been previously equilibrated using 10 mM CAPS pH 11.

[00275] Insecticidal IRDIG protein purified from the IB preparations was analyzed by SDS-PAGE. Molecular weight was determined from amino acid sequence. Quantification of target bands was done by comparing densitometric values for the bands against Bovine Serum Albumin (BSA) samples run on the same gel to generate a standard curve.

[00276] The foregoing provides isolated polynucleotides, including nucleic acid constructs, and isolated insecticidal polypeptides according to the invention.

EXAMPLE 5

Insecticidal activity of proteins

[00277] Insecticidal IRDIG proteins were tested and found to have insecticidal activity on larvae of the coleopteran insect, the western corn rootworm (*Diabrotica virgifera virgifera* LeConte).

[00278] Test insects were first instar (<24 hr after eclosion) western corn rootworm (WCR), *Diabrotica virgifera virgifera*. Non-diapausing *Diabrotica virgifera virgifera* eggs (Crop Characteristics, Inc., Farmington, MN) were incubated for 10 days at 28 °C and 60% RH. Black head eggs were surface sterilized with 10% formalin following the method by Pleau et al. (2002). Lepidopteran test insects comprised fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), corn earworm (CEW), *Heliothis zea* (Boddie), European corn borer (ECB), *Ostrinia nubilalis* (Hübner), and soybean looper (SBL), *Chrysodeixis includens*.

[00279] Proteins were bioassayed using a 48-well WCR bioassay format. In this assay, non-diapausing WCR eggs (Crop Characteristics Inc., Farmington, MN) were incubated at 28 °C in soil for 10 days. These eggs were washed from the soil with water, surface sterilized with 10%

formaldehyde and triple rinsed with sterile water. These eggs were hatched and fed with a Dow AgroSciences proprietary WCR diet. An overlay diet bioassay was conducted in 48-well titer plates with each well containing 0.75 ml of the artificial WCR diet. Each test aliquot was pipetted at 40 uL/well onto diet surface (0.95 cm^2) of 8 wells and dried under room temperature in a laminar flow. The treated diet surface of each well was infested with two *D. virgifera* neonates (24-48 hr old) and test insects were enclosed in the bioassay arena with Breathe Easy® gas permeable sealing membrane for micro titer plates (USA Scientific, Orlando, FL). Negative controls were 20 mM sodium citrate buffer, pH 3.5; 10 mM CAPS buffer, pH 11; the positive control was 100 ug/cm^2 Cry34/35Ab1 in sodium citrate buffer.

[00280] Bioassay trays were held under controlled environmental conditions (28 °C, 60% relative humidity, 16:8 h light/dark) 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 insect bioassays. Larvae which weighed 0.1 mg were considered moribund insects and were included in the percent practical mortality computation. Growth inhibition was calculated as follows:

[00281] GI = [1 – (TWIT/TNIT)/(TWIBC/TNIBC)]

[00282] 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). Bioassays were conducted under randomized complete block design and replicated at least 4 times, with 16 *D. virgifera virgifera* larvae per replicate. Data were analyzed with ANOVA and mean separation using Tukey HSD (Pr>0.05). When dose response analyses were performed, the growth inhibition concentration-response curves were determined using a nonlinear logistic 3-parameter model, and the effective concentrations required to cause 50% growth inhibition (GI_{50}) was estimated. These analyses were performed using JMP Pro, version 9.0.3, software (SAS Institute Inc., Cary, NC). Probit analyses of the pooled practical mortality data were conducted using POLO-PC (LeOra Software) to estimate the 50% lethal concentration (LC_{50}) of the concentration-response curves.

[00283] Insecticidal IRDIG proteins were significantly efficacious compared to negative controls. Efficacy of IRDIG28686, 28684 and 28682 at the tested concentrations were comparable to the positive control Cry34/35Ab1 (Table 5).

[00284] Table 5 shows the mean percent practical mortality and mean percent growth inhibition of WCR when exposed to various concentrations of insecticidal IRDIG proteins.

Table 5

Sample name	Rep	Conc. (µg/cm2)	% Practical mortality			% Growth inhibition		
			Mean	Mean Std Error	Tukey HSD (p>0.05)	Mean	Mean Std Error	Tukey HSD (p>0.05)
Cry34/35Ab1	18	100	82.83	4.05	A	98.69	3.55	A
IRDIG28684	6	23	35.75	7.02	BC	79.15	6.15	AB
IRDIG28686	6	42	50.27	7.02	B	79.12	6.15	AB
IRDIG28682	4	42	26.78	8.60	BCD	69.45	7.53	ABC
IRDIG28688	6	42	49.67	7.02	B	69.27	6.15	B
IRDIG28674	4	25	11.78	8.60	BCD	61.38	7.53	BCD
IRDIG28680	4	42	21.11	8.60	BCD	56.51	7.53	BCD
IRDIG28672	4	22	3.13	8.60	CD	34.20	7.53	CDE
IRDIG27642	8	42	9.54	6.08	CD	30.59	5.32	DE
10 mM CAPS pH 11	18	0	3.57	4.05	D	0.00	3.55	F
20 mM NaCitrate pH 3.5	18	0	5.64	4.05	D	0.00	3.55	F

[a] Means followed by the same letter within each column are not significantly different according to Tukey HSD (p>0.05).

[00285] Table 6 shows the mean percent practical mortality and mean percent growth inhibition of WCR when exposed to 100 µg/cm^2 concentrations of insecticidal IRDIG proteins

Table 6

Sample	Rep	Conc (µg/cm2)	% Practical mortality			% Growth inhibition		
			Mean	Mean Std Error	Tukey HSD (p>0.05)[a]	Mean	Mean Std Error	Tukey HSD (p>0.05)[a]
Cry34/35Ab1	4	100	100.0	4.6	A	100.0	3.6	A
IRDIG28682	4	100	81.3	6.5	AB	94.4	5.1	A
IRDIG28686	4	100	81.0	6.5	AB	93.7	5.1	A
IRDIG28684	4	100	70.3	6.5	B	88.2	5.1	AB
IRDIG28688	4	100	75.2	6.5	AB	84.4	5.1	ABC

IRDIG27642	4	100	38.0	6.5	C	68.4	5.1	BCD
IRDIG28674	4	100	32.1	6.5	CD	66.3	5.1	BCDE
IRDIG28672	4	100	28.9	6.5	CD	63.0	5.1	CDE
IRDIG28692	4	100	28.4	6.5	CD	62.5	5.1	CDE
IRDIG28694	4	100	23.6	6.5	CD	45.5	5.1	DE
IRDIG28676	4	100	18.8	6.5	CD	41.9	5.1	E
Na-citrate 20 mM pH 3.5	4	0	3.3	6.5	D	0.0	5.1	F
10 mM CAPS pH 11	4	0	3.1	6.5	D	0.0	5.1	F

[a] Means followed by the same letter within each column are not significantly different according to Tukey HSD (p>0.05).

[00286] IRDIG28682, IRDIG28686, IRDIG28688 showed the highest percent mortality and percent growth inhibition and were not significantly different from the positive control of Cry34/35. IRDIG28684 and IRDIG28642 showed significant mortality and growth inhibition compared to the controls. IRDIG28674, IRDIG28672, IRDIG28692, IRDIG28694, IRDIG28676 showed significant growth inhibition in comparison to the respective controls.

[00287] IRDIG27642 and IRDIG28686 were tested in a range of concentrations ranging from 2.6 to 168 µg/cm^2 and the WCR activity provided sufficient data for dose response analyses (Tables 6 and 7). There was a significant difference in percent practical mortality of IRDIG28686 at doses of 21, 42, 84, and 168 µg/cm^2. Significant growth inhibition was determined for IRDIG27642 at doses of 10.5, 42, 84, 168 µg/cm^2 and for IRDIG28686 at 21, 42, 84, and 168 µg/cm^2.

[00288] Table 7 shows the dose response of insecticidal IRDIG proteins against WCR in a 48 well bioassay format.

Table 7

Sample name	Conc. (ug/cm2)	Rep	% Practical mortality			% Growth inhibition		
			Mean	Mean Std Error	Tukey HSD (p>0.05)[a]	Mean	Mean Std Error	Tukey HSD (p>0.05)[a]
10 mM CAPS buffer pH 11	0	8	2.5	3.1	E	2.2	6.0	FG
20 mM Sodium citrate buffer pH3.5	0	8	6.9	3.1	DE	0.0	6.0	G
Cry34/35Ab1	100	8	98.3	3.1	A	99.8	6.0	A
IRDIG27642	2.625	4	0.0	4.4	E	9.6	8.4	EFG

IRDIG27642	5.25	4	7.2	4.4	DE	31.2	8.4	CDEFG
IRDIG27642	10.5	4	10.1	4.4	DE	40.3	8.4	CDE
IRDIG27642	21	4	7.5	4.4	DE	38.3	8.4	CDEF
IRDIG27642	42	4	9.2	4.4	DE	53.0	8.4	BCD
IRDIG27642	84	4	7.6	4.4	DE	41.2	8.4	CDE
IRDIG27642	168	4	21.8	4.4	CDE	57.6	8.4	BCD
IRDIG28686	2.625	2	3.2	6.2	DE	4.9	11.9	DEFG
IRDIG28686	5.25	4	10.1	4.4	DE	24.9	8.4	CDEFG
IRDIG28686	10.5	4	17.2	4.4	DE	34.0	8.4	CDEFG
IRDIG28686	21	4	25.4	4.4	CD	50.8	8.4	BCDE
IRDIG28686	42	4	42.8	4.4	BC	65.5	8.4	ABC
IRDIG28686	84	4	48.9	4.4	B	61.6	8.4	BC
IRDIG28686	168	4	65.3	4.4	B	86.2	8.4	AB

[a] Means followed by the same letter within each column are not significantly different according to Tukey HSD (p>0.05).

[00289] Table 8 shows the LC_{50} and GI_{50} of IRDIG27642 and IRDIG28686 insecticidal IRDIG proteins in 48-well bioassay format.

Table 8

Protein	LC_{50}, µg/cm^2 (95% CI*)	GI_{50}, µg/cm^2 (95% CI*)
IRDIG28686	78.4 (58.0-116.6)	22.6 (11.4-44.7)
IRDIG27642	>168	74.2 (25.2-218.1)

*CI= Confidence interval

[00290] Table 9 shows percent practical mortality and percent growth inhibition of WCR when exposed to 33µg/cm^2 concentrations of IRDIG proteins.

Table 9

		% Practical mortality			% Growth inhibition		
Sample	Conc (ug/cm^2)	Mean	SE	Tukey HSD (Pr>0.05)	Mean	SE	Tukey HSD (Pr>0.05)
Cry34/35Ab1	100	100.0	5.3	A	100.0	6.4	A
IRDIG28686	33	61.8	7.5	B	79.9	9.1	AB
IRDIG28682	33	57.6	7.5	BC	78.8	9.1	AB
IRDIG28684	33	53.0	7.5	BCD	76.4	9.1	AB
IRDIG28688	33	45.8	7.5	BCDE	62.4	9.1	ABC
IRDIG28674	33	25.0	7.5	BCDEF	57.3	9.1	BC
IRDIG27642	33	23.7	7.5	CDEF	55.8	9.1	BC
IRDIG28672	33	12.8	7.5	EF	30.9	9.1	CD
IRDIG28692	33	19.3	7.5	DEF	29.9	9.1	CD

IRDIG28694	33	23.5	7.5	CDEF	29.5	9.1	CD
IRDIG28676	33	7.8	7.5	F	26.8	9.1	CD
Na-citrate 20 mM pH 3.5	0	3.3	7.5	F	0.0	9.1	D
10 mM CAPS pH 11	0	3.1	7.5	F	0.0	9.1	D

[00291] IRDIG28686, IRDIG28682, IRDIG 28684, and IRDIG 28688 exhibited similar growth inhibition as the positive control. IRDIG28674and IRDIG28646 showed a significant difference in growth inhibition when compared to controls. Percent mortality for IRDIG28686, IRDIG28682, IRDIG 28684, and IRDIG 28688 were significantly different than the controls.

[00292] Table 10 shows percent practical mortality and percent growth inhibition of WCR when exposed to 11 $\mu g/cm^2$ concentrations of IRDIG proteins.

Table 10

		% Practical mortality			% Growth inhibition		
Sample	Conc (ug/cm^2)	Mean	SE	Tukey HSD (Pr>0.05)	Mean	SE	Tukey HSD (Pr>0.05)
Cry34/35Ab1	100	100.0	4.2	A	100.0	8.2	A
IRDIG28686	11	53.2	6.0	B	72.9	11.6	AB
IRDIG28684	11	27.1	6.0	BCD	59.6	11.6	ABC
IRDIG28682	11	33.6	6.0	BC	51.2	11.6	ABCD
IRDIG28676	11	20.3	6.0	CD	43.3	11.6	BCD
IRDIG28688	11	11.3	6.0	CD	34.2	11.6	BCD
IRDIG27642	11	12.5	5.3	CD	33.0	10.4	BCD
IRDIG28692	11	9.4	6.0	CD	29.0	11.6	BCD
IRDIG28672	11	8.6	6.0	CD	28.1	11.6	BCD
IRDIG28674	11	11.2	6.0	CD	21.3	11.6	BCD
IRDIG28694	11	11.0	6.0	CD	13.9	11.6	CD
Na-citrate 20 mM pH 3.5	0	3.3	6.0	D	0.0	11.6	D
10 mM CAPS pH 11	0	3.1	6.0	D	0.0	11.6	D

[00293] Percent practical mortality was significantly higher for IRDIG28686 and IRDIG28682 compared to the controls. Percent growth inhibition was significantly higher for IRDIG28686 and IRDIG 28684.

[00294] For Lepidoptera, bioassays were conducted in 128-well bioassay trays (C-D International, Pitman, NJ). A 40uL aliquot of protein sample was delivered onto the surface of

multispecies lepidopteran diet (Southland Products, Lake Village, AR) in each well. The treated trays were air dried, and one individual larva (24 to 48 h after eclosion) was deposited on the treated diet surface. Each sample was tested with eight larvae per replication. There were two to three replications performed. The infested wells were then sealed with adhesive sheets of clear plastic vented to allow gas exchange (C-D International, Pitman, NJ). Negative controls were untreated diet surface, water, 10 mM CAPS buffer, pH 10.5 and 9 ug/cm^2 BSA in CAPS buffer, the positive control was 0.03 ug/cm^2 Cry1A in CAPS buffer and 0.12 μg/cm^2 Cry1Fa in CAPS buffer.

[00295] Enriched insecticidal IRDIG proteins from inclusion bodies was tested on lepidopteran insects, methods similar to those described above were followed for *Helicoverpa zea* (corn earworm (CEW)), *Ostrinia nubilalis* (European corn borer (ECB)), *Spodoptera frugiperda* (fall armyworm (FAW)), and *Chrysodeixis includens* (soybean looper (SBL)). IRDIG27642 insecticidal toxin activity was not significantly different in mortality or growth inhibition from the negative controls.

[00296] The foregoing describes a method of applying an isolated insecticidal polypeptide and controlling a coleopteran pest population in accordance with the invention.

EXAMPLE 6

Insecticidal activity of proteins on Cry3Bb-resistant WCR

[00297] The insecticidal IRDIG proteins are bioassayed with larvae generated from a selected and a non-selected Cry3Bb WCR strains, as well as a non-diapausing WCR control line. The non-diapausing WCR control eggs (Crop Characteristics Inc., Farmington, MN), Cry3Bb selected WCR eggs (Meihls et al., 2008 and 2012) and Cry3Bb non-selected WCR eggs are processed similarly as described above in preparation for a 48-well WCR bioassay format. The Cry3Bb non-selected eggs are an unexposed lab population, originating from South Dakota.

[00298] Percent practical mortality (dead plus moribund insects) and growth inhibition are calculated. Control mortality should not exceed 20%. Bioassays are conducted under completely randomized design and replicated 3-4 times, with 16 *D. virgifera virgifera* larvae per replicate. Percent practical mortality and growth inhibition are analyzed with a one-way analysis of variance (ANOVA) and mean separations by using the Tukey-Kramer HSD test ($P>0.05$).

EXAMPLE 7

Protein processing by WCR midgut fluid and Corn Root Juice

[00299] Midgut fluid collection. Approximately 150 third instar western corn rootworm (WCR) larvae were ordered from Crop Characteristics. The insects were shipped with corn roots. Under a light microscope, using a scalpel, both the posterior and anterior ends of the larvae were removed. Using forceps, the gut was pulled out and stored in the buffer (0.15 M NaCl filtered and sterile buffer containing 8.5% sucrose) and kept on ice.

[00300] Procedure for protein digestion by WCR midgut fluid. Western corn rootworm (WCR) active protein (IRDIG) stability was analyzed in the presence of 12µg WCR gut juice to determine potential cleavage (activation or inactivation) sites. Proteins expressed and purified from *Pseudomonas* were incubated with WCR gut juice or extract for 20 hr at 30 °C. WCR concentrations and pH of 7.5 (50 mM Tris pH 7.5, 0.15 M KCl, 0.015 M $CaCl_2$ final concentration from 10X stock) were chosen based on protease activity testing.

[00301] Reactions were stopped with the addition of proteinase inhibitors. Thirty µl of reaction was then mixed 10 µl of LDS buffer (10 mM TCEP) and loaded onto a 4-12% PAGE gel using MES running buffer. Results indicate a significant amount of processing of WCR actives by WCR and maize root extract (MRE). Identical gels were blotted to allow identification of cleavage motifs by Edman N-terminal sequencing.

[00302] Procedure for protein digestion by corn root juice

[00303] 100 ug/cm^2 of Insecticidal IRDIG27642 protein is treated by WCR midgut fluid and corn root juice are tested against the WCR following the bioassay method of 48-well format, described in Example 5, Insecticidal activity of proteins.

EXAMPLE 8

Agrobacterium transformation

[00304] Standard cloning methods were used in the construction of binary plant transformation and expression plasmid. Restriction endonucleases and T4 DNA Ligase were obtained from NEB. Plasmid preparations were 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 were purified using the QIAquick PCR Purification Kit or the QIAEX II Gel Extraction Kit (both from Qiagen) after gel isolation.

[00305] DNA comprising a nucleotide sequence that encodes an insecticidal IRDIG protein was synthesized by a commercial vendor (e.g., DNA2.0, Menlo Park, CA) and was supplied as cloned fragments in plasmid vectors. Other DNA sequences encoding other insecticidal IRDIG proteins were obtained by standard molecular biology manipulation of constructs containing appropriate nucleotide sequences.

[00306] Full-length or modified coding sequences (CDS) for insecticidal IRDIG proteins were subcloned into a plant expression plasmid at appropriate sites. The resulting plant expression cassettes containing the appropriate 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) were 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 included 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 included 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.

[00307] Electro-competent cells of *Agrobacterium tumefaciens* strain Z707S (a streptomycin-resistant derivative of Z707) were 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) was added to the cuvette and the cell-YEP suspension was transferred to a 15 mL culture tube for incubation at 28 °C in a water bath with constant agitation for 4 hours. The cells were plated on YEP plus agar (25 gm/L) with spectinomycin (200 µg/mL) and streptomycin (250 µg/mL) and the plates were incubated for 2-4 days at 28 °C. Well separated single colonies were selected and streaked onto fresh YEP + agar plates with spectinomycin and streptomycin, and incubated at 28 °C for 1-3 days.

[00308] The presence of the insecticidal toxin gene insert in the binary plant transformation vector was confirmed 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 was extracted using Qiagen Spin Mini Preps, performed per manufacturer's instructions. Plasmid DNA from the binary vector used in the *Agrobacterium* electroporation transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Invitrogen per manufacturer's instructions at 0.5X concentrations. PCR reactions were 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 was maintained at 4 °C after cycling. The amplification products were analyzed by agarose gel electrophoresis (e.g., 0.7 % to 1% agarose, w/v) and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[00309] Another binary plant transformation vector containing the insecticidal toxin gene insert was confirmed 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.

[00310] The foregoing discloses nucleic acid constructs comprising a polynucleotide that encodes an insecticidal toxin polypeptide in accordance with the invention.

EXAMPLE 9

Production of insecticidal toxins in dicot plants

[00311] *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.

[00312] *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 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 µmol/m^2sec 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.

[00313] 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 *bar* gene, 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.

[00314] 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.

[00315] Insect Bioassays of transgenic *Arabidopsis* Transgenic *Arabidopsis* lines expressing 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.

[00316] The foregoing provides methods for making and using transgenic plants comprising insecticidal toxin polypeptides according to the invention.

EXAMPLE 10

Production of insecticidal IRDIG proteins in monocot plants

[00317] *Agrobacterium*-Mediated Transformation of Maize. Transgenic maize cells, tissues, and plants that produce one or more insecticidal IRDIG proteins through expression of a chimeric gene stably-integrated into the plant genome are produced following *Agrobacterium*-mediated transformation. Maize transformation methods employing binary transformation vectors are known in the art, as described, for example, in U.S. Patent No. 8,304,604, which is herein incorporated by reference in its entirety. Transformed tissues are selected by their ability to grow on Haloxyfop-containing medium and are screened for protein production, as appropriate. Portions of such transformed tissue cultures are presented to insect larvae for bioassay, essentially as described in EXAMPLE 5.

[00318] *Agrobacterium* Culture Initiation. Glycerol stocks of the project vectors in the host *Agrobacterium tumefaciens* strain DAt13192 (RecA minus ternary strain) are obtained from the DAS Recombinant Culture Collection (RCC). *Agrobacterium* cultures are streaked from glycerol stocks onto AB minimal medium and incubated at 20 °C in the dark for 3 days. *Agrobacterium* cultures are then streaked onto a plate of YEP medium and incubated at 20 °C in the dark for 1 day.

[00319] On the day of an experiment, a mixture of Inoculation medium and acetosyringone is prepared in a volume appropriate to the number of constructs in the experiment. Inoculation medium is pipetted into a sterile, disposable, 250 ml flask. A 1 M stock solution of acetosyringone in 100% dimethyl sulfoxide is added to the flask containing inoculation medium in a volume appropriate to make a final acetosyringone concentration of 200 µM.

[00320] For each construct, 1-2 loops of *Agrobacterium* from the YEP plate are suspended in 15 mL of the inoculation medium/acetosyringone mixture inside a sterile, disposable, 50 mL centrifuge tube and the optical density of the solution at 600 nm ($O.D._{600}$) is measured in a spectrophotometer. The suspension is then diluted down to 0.25-0.35 $O.D._{600}$ using additional Inoculation medium/acetosyringone mixture. The tube of *Agrobacterium* suspension is then placed horizontally on a platform shaker set at about 75 rpm at room temperature for between 1 and 4 hours before use.

[00321] Ear sterilization and embryo isolation. Ears from *Zea mays* cultivar B104 are produced in greenhouse facilities and harvested 10-12 days post pollination. Harvested ears are de-husked and surface-sterilized by immersion in a 20% solution of commercial bleach (Ultra Clorox® Germicidal Bleach, 6.15% sodium hypochlorite) and two drops of soap, for 20 minutes, followed by three rinses in sterile, deionized water inside a laminar flow hood. Immature zygotic embryos (1.8–2.2 mm long) are aseptically excised from each ear and distributed into one or more micro-centrifuge tubes containing 2.0 mL of *Agrobacterium* suspension into which 2 µl of 10% Break-Thru® S233 surfactant has been added.

[00322] *Agrobacterium* co-cultivation. Upon completion of the embryo isolation activity the tube of embryos is closed and placed on a rocker platform for 5 minutes. The contents of the tube are then poured out onto a plate of co-cultivation medium and the liquid *Agrobacterium* suspension is removed with a sterile, disposable, transfer pipette and the embryos are oriented with the scutellum facing up using a microscope. The plate is then closed, sealed with 3M Micropore tape, and placed in an incubator at 25 °C with 24 hours/day light at approximately 60 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR).

[00323] Callus Selection and Regeneration of Transgenic Events. Following the co-cultivation period, embryos are transferred to Resting medium. No more than 36 embryos are moved to each plate. The plates are incubated at 27 °C with 24 hours/day light at approximately 50 µmol m^{-2} s^{-1} PAR for 7-10 days. Callused embryos are then transferred onto Selection I

medium. No more than 18 callused embryos are moved to each plate of Selection I. The plates are incubated at 27 °C with 24 hours/day light at approximately 50 μmol m^{-2} s^{-1} PAR for 7 days. Callused embryos are then transferred to Selection II medium. No more than 12 callused embryos are moved to each plate of Selection II. The plates are incubated at 27 °C with 24 hours/day light at approximately 50 μmol m^{-2} s^{-1} PAR for 14 days.

[00324] At this stage resistant calli are moved to Pre-Regeneration medium. No more than 9 calli are moved to each plate of Pre-Regeneration. The plates are held at 27 °C with 24 hours/day light at approximately 50 μmol m^{-2} s^{-1} PAR for 7 days. Regenerating calli are then transferred to Regeneration medium in Phytatrays™ (Sigma-Aldrich). and incubated at 28 °C with 16 hours light/8 hours dark per day at approximately 150 μmol m^{-2} s^{-1} PAR for 7-14 days or until shoots develop. No more than 5 calli are placed in each Phytatray™. Small shoots with primary roots are then isolated and transferred to Shoot/Root medium. Rooted plantlets about 6 cm or taller are transplanted into soil and moved out to a growth chamber for hardening off.

[00325] Transformed plant shoots selected by their ability to grow on medium containing Haloxyfop are transplanted from Phytatrays™ to small pots filled with growing medium (ProMix BX; Premier Tech Horticulture), covered with cups or HUMI-DOMES (Arco Plastics), and then hardened-off in a Conviron growth chamber (27 °C day/24 °C night, 16-hour photoperiod, 50-70% RH, 200 μmol $m^{-2}s^{-1}$ PAR). In some instances, putative transgenic plantlets are analyzed for transgene relative copy number by quantitative real-time PCR assays using primers designed to detect the herbicide tolerance gene integrated into the maize genome. Further, RNA qPCR assays are used to detect the presence of the linker sequence in expressed dsRNAs of putative transformants. Selected transformed plantlets are then moved into a greenhouse for further growth and testing.

[00326] Transfer and establishment of T_0 plants in the greenhouse for bioassay and seed production. Plants are transplanted from Phytatrays™ to small pots (T. O. Plastics, 3.5" SVD, 700022C) filled with growing media (Premier Tech Horticulture, ProMix BX, 0581 P) and covered with Humidomes to acclimate the plants. They are placed in a Conviron growth chamber (28 °C/24 °C, 16-hour photoperiod, 50-70% RH, 200 μmol m^{-2} s^{-1} PAR) until they reach the V3-V4 stage. This aids in acclimating the plants to soil and harsher temperatures. Plants are then moved to the greenhouse (Light Exposure Type: Photo or Assimilation; High Light Limit: 1200 μmol m^{-2} s^{-1} PAR; 16-hour day length; 27 °C day/24 °C

night) and transplanted from the small pots to TINUS™ 350-4 Rootrainers® (Spencer-Lemaire Industries, Acheson, Alberta, Canada) prior to insect bioassay, at one plant per event per Rootrainer®. About 30 events are tested per construct. Approximately four days after transplanting to Rootrainers®, the V3-V4 stage plants are infested for bioassay, with about ready to hatch 150 western corn rootworm eggs (Crop Characteristics LLC, Farmington, MN) per plant. The bioassay is conducted for 2 weeks in the greenhouse and then, each event is graded following a modified method recommended by Oleson et al. (2005).

[00327] ROOT DAMAGE RATING (modified from Oleson et al, 2005)

0.00 = No damage

0.01 = Only a few minor feedings

0.02 = Feeding scars evident – very light tunneling or channeling & no roots eaten off to within 4 cm of stalk (a root eaten to within 4 cm of the stalk is considered a "pruned root")

0.05 = Severe scarring or when only the tips of several roots are injured on the entire root system

0.10 = One root pruned

0.25 = 2-3 roots pruned or ¼ of roots pruned

0.50 = 4-5 roots pruned, considerable feeding damage on the outer portion of the root system; ½ of node pruned

0.75 = 6+ roots pruned, but with extensive feeding on outer portion of the root system; ¾ of node pruned

1.00 = At least one full node of roots pruned,

[00328] The inbred B104 and 7SH382 negative controls consistently provide 0.5 to 1.0 root ratings (high damage). T_0 events that provide 0.5 unit of root rating or less are saved and transplanted into 5 gallon pots for seed productions.

[00329] Plants of the T_1 generation are obtained by pollinating the silks of T_0 transgenic plants with pollen collected from plants of non-transgenic inbred line B104 or other appropriate pollen donors, and planting the resultant seeds. Reciprocal crosses are performed when possible. Selective T_1 events are tested for root protection against the western corn rootworm following the procedures used in T_0 event insect bioassay.

[00330] The foregoing provides methods for making and regenerating transgenic plants comprising insecticidal toxin polypeptides according to the invention.

[00331] Leaf Sampling for Western blot Analyses. The plants are sampled at the V-3 to V-5 stage. Two 6mm diameter leaf samples are stored in a 96 well cluster tube rack at -80°C until the day of analysis. Two Daisy™ steel BB's and 300 µl of extraction buffer (PBS solution containing 0.05% of Tween 20 and 5ul / ml of Sigma protease inhibitors, catalog number 9599) is added to each tube. The samples are milled in a Kelco bead mill for 3 minutes, on maximum setting. Samples are centrifuged at 3,000 x g for 5 minutes; 100µl of the supernatant is transferred to an empty sample tube. Another 100µl of extraction buffer is added to the plant sample and bead milled 3 additional minutes, centrifuged and 100µl of this extract is combined with the first 100µl. The combined supernatants are mixed and analyzed the same day as the extraction.

[00332] *Western Blot (Qualitative Methods)*: Conventional electrophoresis and blotting methods are used with Invitrogen™ devices and basic reagents. A Dow AgroSciences rabbit anti-IRDIG27642 antibody is the primary antibody for the detection of IRDIG27642 in leaf tissue. All proteins are detected with a CY-3 fluorescence detection system.

EXAMPLE 11

Bioassay of transgenic maize

[00333] Bioactivity of the insecticidal toxins produced in plant cells is demonstrated by conventional bioassay methods (see, for example Huang *et al.*, 2006). In one assay of efficacy, various plant tissues or tissue pieces derived from a plant producing an insecticidal toxin are fed to target insects in a controlled feeding environment. In another bioactivity assay, protein extracts are prepared from various plant tissues derived from the plant producing the insecticidal toxin and the extracted proteins are incorporated into artificial diet bioassays. The results of each feeding assay are compared to similarly conducted bioassays that employ appropriate control tissues from host plants that do not produce an insecticidal toxin, or to other control samples.

EXAMPLE 12

Transgenic *Glycine max* comprising an insecticidal IRDIG protein

[00334] Ten to 20 transgenic T_0 *Glycine max* plants harboring expression vectors for nucleic acids comprising an insecticidal IRDIG protein are generated by *Agrobacterium*-mediated transformation. Mature soybean (*Glycine max)* seeds are sterilized overnight with chlorine gas for sixteen hours. Following sterilization with chlorine gas, the seeds are placed in an open container in a Laminar™ flow hood to dispel the chlorine gas. Next, the sterilized seeds are imbibed with sterile H_2O for sixteen hours in the dark using a black box at 24° C.

[00335] Preparation of split-seed soybeans. The split soybean seed comprising a portion of an embryonic axis protocol required preparation of soybean seed material which is cut longitudinally, using a #10 blade affixed to a scalpel, along the hilum of the seed to separate and remove the seed coat, and to split the seed into two cotyledon sections. Careful attention is made to partially remove the embryonic axis, wherein about 1/2 – 1/3 of the embryo axis remains attached to the nodal end of the cotyledon.

[00336] Inoculation. The split soybean seeds comprising a partial portion of the embryonic axis are then immersed for about 30 minutes in a solution of *Agrobacterium tumefaciens* (e.g., strain EHA 101 or EHA 105) containing binary plasmid comprising an insecticidal IRDIG protein. The *Agrobacterium tumefaciens* solution is diluted to a final concentration of λ=0.6 OD_{650} before immersing the cotyledons comprising the embryo axis.

[00337] Co-cultivation. Following inoculation, the split soybean seed is allowed to co-cultivate with the *Agrobacterium tumefaciens* strain for 5 days on co-cultivation medium (Wang, Kan. *Agrobacterium Protocols*. 2. 1. New Jersey: Humana Press, 2006. Print.) in a Petri dish covered with a piece of filter paper.

[00338] Shoot induction. After 5 days of co-cultivation, the split soybean seeds are washed in liquid Shoot Induction (SI) media consisting of B5 salts, B5 vitamins, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose, 0.6 g/L MES, 1.11 mg/L BAP, 100 mg/L TIMENTIN™, 200 mg/L cefotaxime, and 50 mg/L vancomycin (pH 5.7). The split soybean seeds are then cultured on Shoot Induction I (SI I) medium consisting of B5 salts, B5 vitamins, 7 g/L Noble agar, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose, 0.6 g/L MES, 1.11 mg/L BAP, 50 mg/L TIMENTIN™, 200 mg/L cefotaxime, 50 mg/L vancomycin (pH 5.7), with the flat side of the cotyledon facing up and the nodal end of the cotyledon imbedded into the medium. After 2 weeks of culture, the explants from the transformed split soybean seed are transferred to the

Shoot Induction II (SI II) medium containing SI I medium supplemented with 6 mg/L glufosinate (Liberty®).

[00339] Shoot elongation. After 2 weeks of culture on SI II medium, the cotyledons are removed from the explants and a flush shoot pad containing the embryonic axis are excised by making a cut at the base of the cotyledon. The isolated shoot pad from the cotyledon is transferred to Shoot Elongation (SE) medium. The SE medium consists of MS salts, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose and 0.6 g/L MES, 50 mg/L asparagine, 100 mg/L L-pyroglutamic acid, 0.1 mg/L IAA, 0.5 mg/L GA3, 1 mg/L zeatin riboside, 50 mg/L TIMENTIN™, 200 mg/L cefotaxime, 50 mg/L vancomycin, 6 mg/L glufosinate, 7 g/L Noble agar, (pH 5.7). The cultures are transferred to fresh SE medium every 2 weeks. The cultures are grown in a Conviron™ growth chamber at 24 °C with an 18 h photoperiod at a light intensity of 80-90 $\mu mol/m^2sec$.

[00340] Rooting. Elongated shoots which developed from the cotyledon shoot pad are isolated by cutting the elongated shoot at the base of the cotyledon shoot pad, and dipping the elongated shoot in 1 mg/L IBA (Indole 3-butyric acid) for 1–3 minutes to promote rooting. Next, the elongated shoots are transferred to rooting medium (MS salts, B5 vitamins, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 20 g/L sucrose and 0.59 g/L MES, 50 mg/L asparagine, 100 mg/L L-pyroglutamic acid 7 g/L Noble agar, pH 5.6) in phyta trays.

[00341] Cultivation. Following culture in a Conviron™ growth chamber at 24° C, 18 h photoperiod, for 1-2 weeks, the shoots which have developed roots are transferred to a soil mix in a covered sundae cup and placed in a Conviron™ growth chamber (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu mol/m^2sec$ under constant temperature (22 °C) and humidity (40-50%) for acclimatization of plantlets. The rooted plantlets are acclimated in sundae cups for several weeks before they are transferred to the greenhouse for further acclimatization and establishment of robust transgenic soybean plants.

[00342] Development and morphological characteristics of transgenic lines are compared with nontransformed plants. Plant root, shoot, foliage and reproduction characteristics are compared. There are no observable difference in root length and growth patterns of transgenic and nontransformed plants. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are similar. In general, there are no observable

morphological differences between transgenic lines and those without expression of IRDIG proteins when cultured in vitro and in soil in the glasshouse.

[00343] The foregoing provides methods for making and selecting transgenic dicot plants (soybeans) comprising insecticidal toxin polypeptides according to the invention.

EXAMPLE 13

Transformation of Additional Crop Species

[00344] Cotton is transformed with insecticidal IRDIG proteins (with or without a chloroplast transit peptide) to provide control of insects by utilizing a method known to those of skill in the art, for example, substantially the same techniques previously described in EXAMPLE 14 of U.S. Patent 7,838,733, or Example 12 of PCT International Patent Publication No. WO 2007/053482.

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CLAIMS

What is claimed is:

1. A nucleic acid construct comprising a nucleic acid sequence encoding an insecticidal IRDIG protein.

2. A nucleic acid construct comprising a nucleic acid sequence of Claim 1 chosen from the group consisting of:
    a. insecticidal IRDIG proteins having at least 80% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    b. insecticidal IRDIG proteins having at least 90% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    c. insecticidal IRDIG proteins having at least 95% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    d. insecticidal IRDIG proteins having at least 99% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122, and;
    e. insecticidal IRDIG proteins having 100% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122.

3. A composition comprising a formulated insecticidal IRDIG protein.

4. A composition comprising a formulated insecticidal IRDIG protein in which the insecticidal IRDIG protein is chosen from the group consisting of:
    a. insecticidal IRDIG proteins having at least 80% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    b. insecticidal IRDIG proteins having at least 90% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    c. insecticidal IRDIG proteins having at least 95% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122;
    d. insecticidal IRDIG proteins having at least 99% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122, and;
    e. insecticidal IRDIG proteins having 100% sequence identity with any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122.

5. A plant or plant part comprising a nucleic acid sequence encoding an insecticidal IRDIG protein comprising a nucleic acid sequence of Claim 1.

6. The plant part of claim 5 wherein the plant part is a seed.

7. A plant or plant part comprising a polynucleotide comprising a sequence of Claim 2.

8. The plant part of claim 7 wherein the plant part is a seed.

9. A plant or plant part comprising an insecticidal IRDIG protein comprising a sequence of Claim 3.

10. The plant part of claim 9 wherein the plant part is a seed.

11. A plant or plant part comprising an insecticidal IRDIG protein comprising a sequence of Claim 4.

12. The plant or plant part of Claim 5, wherein the insecticidal IRDIG protein has insecticidal activity against an insect selected from the group consisting of the order Coleoptera and Western Corn Rootworm.

13. A plant or plant part of Claim 7 wherein the polypeptide has insecticidal activity against an insect selected from the group consisting of the order Coleoptera and Western Corn Rootworm.

14. The plant or plant part of Claim 4, wherein the polypeptide has insecticidal activity against an insect selected from the group consisting of the order Coleoptera and Western Corn Rootworm.

15. A method for controlling susceptible insects comprising contacting said insect with an effective amount of an insecticidal IRDIG protein of Claim 3.

16. A method for controlling susceptible insects comprising contacting said insect with an effective amount of an insecticidal IRDIG protein of Claim 4.

17. The method of Claim 15 wherein the susceptible insect is Western Corn Rootworm

18. The method of Claim 15 wherein the susceptible insect is Western Corn Rootworm.

19. A method for producing a coleopteran-tolerant plant comprising breeding a non transgenic plant with a transgenic plant comprising a foreign DNA construct, capable of expressing an insecticidal IRDIG protein, stably incorporated into the genome of the plant and selecting progeny by analyzing for at least a portion of the foreign DNA construct emanating from the transgenic plant.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/025859

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01H 5/00; A01N 63/02; A61K 35/66; A61K 35/742; C07K 14/32; C07K 14/325 (2017.01)
CPC - A01N 63/02; C07K 14/325; C12N 15/8216; C12N 15/8257; C12N 15/8286 (2017.02)

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/93.461; 435/418; 435/252.31; 514/4.5; 800/302 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0203857 A1 (DOW AGROSCIENCES LLC) 23 July 2015 (23.07.2015) entire document	19
A	US 2012/0210462 A1 (BERMUDEZ et al) 16 August 2012 (16.08.2012) entire document	1-19
A	LI et al. "Bacillus thuringiensis Cry34Ab1/Cry35Ab1 Interactions with Western Corn Rootworm Midgut Membrane Binding Sites," PLoS One, 04 January 2013 (04.01.2013), Vol. 8, No. 1, Pgs. 1-9. entire document	1-19
A	US 2011/0183896 A1 (ADANG et al) 28 July 2011 (28.07.2011) entire document	1-19
A	WANGILA et al. "Susceptibility of Nebraska Western Corn Rootworm (Coleoptera: Chrysomelidae) Populations to Bt Corn Events," Journal of Economic Entomology, 04 February 2015 (04.02.2015), Vol.108, Iss. 2, Pgs. 742-751. entire document	1-19
A	US 2012/0222173 A1 (GOERTZ et al) 30 August 2012 (30.08.2012) entire document	1-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier 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	Date of mailing of the international search report
31 July 2017	17 AUG 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/025859

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-20 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/025859

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.:
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:
See extra sheet(s).

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.:
1-19 restricted to an insecticidal IRDIG protein of SEQ ID NO: 2.

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/US2017/025859

Continued from Box No. III Observations where unity of invention is lacking

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 need to be paid.

Group I+: claims 1-19 are drawn to insecticidal IRDIG proteins, nucleic acid constructs, compositions, and methods comprising the same.

The first invention of Group I+ is restricted to an insecticidal IRDIG protein, nucleic acid constructs, compositions, and methods comprising the same, wherein the insecticidal IRDIG protein is selected to be SEQ ID NO:2. It is believed that claims 1-19 read on this first named invention and thus these claims will be searched without fee to the extent that they read on an insecticidal IRDIG protein of SEQ ID NO: 2.

Applicant is invited to elect additional insecticidal IRDIG proteins, each with a specified SEQ ID NO, to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be an insecticidal IRDIG protein, nucleic acid constructs, compositions, and methods comprising the same, wherein the insecticidal IRDIG protein is selected to be SEQ ID NO:4. Additional insecticidal IRDIG proteins will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ 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:

The Groups I+ formulas do not share a significant structural element for controlling susceptible insects, requiring the selection of alternatives for the insecticidal IRDIG proteins, where "the insecticidal IRDIG protein is chosen from the group consisting of: a. insecticidal IRDIG proteins having at least 80% sequence identity with any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122; b. insecticidal IRDIG proteins having at least 90% sequence identity with any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122; c. insecticidal IRDIG proteins having at least 95% sequence identity with any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122; d. insecticidal IRDIG proteins having at least 99% sequence identity with any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122, and; e. insecticidal IRDIG proteins having 100% sequence identity with any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 71, 73, 75, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122".

The Groups I+ share the technical features of a nucleic acid construct comprising a nucleic acid sequence encoding an insecticidal IRDIG protein; a composition comprising a formulated insecticidal IRDIG protein; a composition comprising a formulated insecticidal IRDIG protein; a method for producing a coleopteran-tolerant plant comprising breeding a non-transgenic plant with a transgenic plant comprising a foreign DNA construct, capable of expressing an insecticidal IRDIG protein, stably incorporated into the genome of the plant and selecting progeny by analyzing for at least a portion of the foreign DNA construct emanating from the transgenic plant. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2015/0203857 A1 to Dow Agrosciences LLC discloses a nucleic acid construct ([n]ucleic acid constructs (e.g., vectors) comprising a synthetic polynucleotide encoding a polypeptide of interest may be employed in particular embodiments for transformation of an expression host, Para. [0145]) comprising a nucleic acid sequence encoding an insecticidal IRDIG protein (Each version of the cry1Ab gene was trimmed to yield the corresponding protein consisting of amino acids 1-619...a seventeenth polyadenylation sequence beginning at base 1639 was incorporated into the design. This sequence is referred to herein as "IRDIG, Para. [0202]; Insecticidal proteins, for example, those with activity against Lepidoptera driver pests, are an extremely important tool in crop improvement. The Cry1Ab protein from Bacillus thuringiensis has demonstrated activity against Ostrinia nubilalis (European corn borer (ECB)), Para. [0123]); a composition comprising a formulated insecticidal IRDIG protein (the DNA composition... referred to herein as "IRDIG, Para. [0202]); a composition comprising a formulated insecticidal IRDIG protein (Also included in some embodiments is a composition (e.g., a commodity product) produced from transgenic plants or parts or materials thereof containing a synthetic nucleic acid encoding a polypeptide of interest comprises a coding sequence that is codon-optimized for expression in a heterologous cell, Para. [0019]; Insecticidal proteins, for example, those with activity against Lepidoptera driver pests, are an extremely important tool in crop improvement. The Cry1Ab protein from Bacillus thuringiensis has demonstrated activity against Ostrinia nubilalis (European corn borer (ECB)), Para. [0123]); a method for producing a Lepidopteran-tolerant plant comprising breeding a non-transgenic plant with a transgenic plant comprising a foreign DNA construct (the original variety of interest (recurrent parent) is crossed to a second variety (non-recurrent parent) that carries a gene of interest to be transferred, Para. [0076]), capable of expressing an insecticidal IRDIG protein (Also included in some embodiments is a composition (e.g., a commodity product) produced from transgenic plants or parts or materials thereof containing a synthetic nucleic acid encoding a polypeptide of interest comprises a coding sequence that is codon-optimized for expression in a heterologous cell, Para. [0019]; Insecticidal proteins, for example, those with activity against Lepidoptera driver pests, are an extremely important tool in crop improvement. The Cry1Ab protein from Bacillus thuringiensis has demonstrated activity against Ostrinia nubilalis (European corn borer (ECB)), Para. [0123]), stably incorporated into the genome of the plant (incorporation of the nucleic acid molecule into the cellular genome, Para. [0089]) and selecting progeny by analyzing for at least a portion of the foreign DNA construct emanating from the transgenic plant (The resulting progeny from this cross are then crossed again to the recurrent parent, and the process is repeated until a plant is obtained wherein essentially all of the desired morphological and physiological characteristics of the recurrent plant are recovered in the converted plant, in addition to the transferred gene from the non-recurrent parent, Para. [0076]; specific gene design parameters that generally increase the expression of the polypeptide of interest from the nucleic acid in a host (e.g., a plant cell, plant tissue, and plant), Para. [0010]).

Further, US 2012/0210462 A1to Bermudez et al. discloses a coleopteran-tolerant plant (the host organism is a plant, the synthetic

nucleic acids can be synthesized using plant-preferred codons for improved expression, Para. [0110]; amino acid substitutions can be made to any Cry3 polypeptide, including native (e.g., naturally occurring), mutated, or shuffled sequences. These changes increase the solubility of the Cry3 polypeptide in the rootworm gut. Nucleic acid molecules and nucleotide sequences of the invention can be used to transform any organism to produce the encoded modified Cry3 pesticidal polypeptides, Para. [0062]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.