



- (51) International Patent Classification:
A01H 5/00 (2006.01) *C12N 15/82* (2006.01)
- (21) International Application Number:
PCT/US2012/049491
- (22) International Filing Date:
3 August 2012 (03.08.2012)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/515,553 5 August 2011 (05.08.2011) US
- (71) Applicant (for all designated States except US): **DOW AGROSCIENCES LLC** [US/US]; 9330 Zionsville Road, Indianapolis, IN 46268-1054 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **BURTON, Stephanie, L.** [US/US]; 6820 Bluffgrove Drive, Indianapolis, IN 46278 (US). **MEADE, Thomas** [US/US]; 11471 Huntington Woods, Zionsville, IN 46077 (US). **NARVA, Kenneth** [US/US]; 4372 Creekside Pass, Zionsville, IN 46077 (US). **SHEETS, Joel, J.** [US/US]; 10005 Oak Ridge Drive, Zionsville, IN 46077 (US). **STORER, Nicholas, P.** [US/US]; 10703 Lexington Street, Kensington, MD 20895 (US). **WOOSLEY, Aaron, T.** [US/US]; 8906 Tanner Way, Fishers, IN 46038 (US).
- (74) Agent: **SANDERS, Jay, M.**; Faegre Baker Daniels LLP, 300 North Meridian Street, Suite 2700, Indianapolis, IN 46204 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
- with international search report (Art. 21(3))
 - with sequence listing part of description (Rule 5.2(a))



(54) Title: USE OF DIG3 INSECTICIDAL CRYSTAL PROTEIN IN COMBINATION WITH CRY1AB

(57) Abstract: The subject invention includes methods and plants for controlling European corn borer, said plants comprising a Cry1Ab insecticidal protein and a DIG-3 insecticidal protein to delay or prevent development of resistance by the insect.

USE OF DIG3 INSECTICIDAL CRYSTAL PROTEIN IN COMBINATION WITH CRY1AB

Background of the Invention

[0001] Humans grow corn for food and energy applications. Humans also grow many other crops, including soybeans and cotton. Insects eat and damage plants and thereby undermine these human efforts. Billions of dollars are spent each year to control insect pests and additional billions are lost to the damage they inflict. Synthetic organic chemical insecticides have been the primary tools used to control insect pests but biological insecticides, such as the insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*), have played an important role in some areas. The ability to produce insect-resistant plants through transformation with *Bt* insecticidal protein genes has revolutionized modern agriculture and heightened the importance and value of insecticidal proteins and their genes.

[0002] Several *Bt* proteins have been used to create the insect-resistant transgenic plants that have been successfully registered and commercialized to date. These include Cry1Ab, Cry1Ac, Cry1F and Cry3Bb in corn, Cry1Ac and Cry2Ab in cotton, and Cry3A in potato.

[0003] The commercial products expressing these proteins express a single protein except in cases where the combined insecticidal spectrum of 2 proteins is desired (*e.g.*, Cry1Ab and Cry3Bb in corn combined to provide resistance to lepidopteran pests and rootworm, respectively) or where the independent action of the proteins makes them useful as a tool for delaying the development of resistance in susceptible insect populations (*e.g.*, Cry1Ac and Cry2Ab in cotton combined to provide resistance management for tobacco budworm).

SMART STAX is a commercial product that incorporates several *Cry* proteins. *See also* U.S. Patent Application Publication No. 2008/0311096, which relates in part to Cry1Ab for controlling Cry1F-resistant European corn borer (ECB; *Ostrinia nubilalis* (Hübner)). U.S. Patent Application Publication No. 2010/0269223 relates to DIG-3.

[0004] The rapid and widespread adoption of insect-resistant transgenic plants has given rise to the concern that pest populations will develop resistance to the insecticidal proteins produced by these plants. Several strategies have been suggested for preserving the utility of *Bt*-based insect resistance traits which include deploying proteins at a high dose in

combination with a refuge, and alternation with, or co-deployment of, different toxins (McGaughey *et al.* (1998), “*B.t.* Resistance Management,” *Nature Biotechnol.* 16:144-146).

[0005] The proteins selected for use in an insect resistant management (IRM) stack need to exert their insecticidal effect independently so that resistance developed to one protein does not confer resistance to the second protein (*i.e.*, there is not cross resistance to the proteins). If, for example, a pest population selected for resistance to “Protein A” is sensitive to “Protein B”, one would conclude that there is not cross resistance and that a combination of Protein A and Protein B would be effective in delaying resistance to Protein A alone.

[0006] In the absence of resistant insect populations, assessments can be made based on other characteristics presumed to be related to mechanism of action and cross-resistance potential. The utility of receptor-mediated binding in identifying insecticidal proteins likely to not exhibit cross resistance has been suggested (van Mellaert *et al.* 1999). The key predictor of lack of cross resistance inherent in this approach is that the insecticidal proteins do not compete for receptors in a sensitive insect species.

[0007] In the event that two *Bt* toxins compete for the same receptor in an insect, then if that receptor mutates in that insect so that one of the toxins no longer binds to that receptor and thus is no longer insecticidal against the insect, it might be the case that the insect will also be resistant to the second toxin (which competitively bound to the same receptor). That is, the insect is cross-resistant to both *Bt* toxins. However, if two toxins bind to two different receptors, this could be an indication that the insect would not be simultaneously resistant to those two toxins.

[0008] Additional *Cry* toxins are listed at the website of the official *B.t.* nomenclature committee (Crickmore *et al.*; lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). There are currently nearly 60 main groups of "Cry" toxins (Cry1-Cry59), with additional Cyt toxins and VIP toxins and the like. Many of each numeric group have capital-letter subgroups, and the capital letter subgroups have lower-cased letter sub-subgroups. (Cry1 has A-L, and Cry1A has a-i, for example).

BRIEF SUMMARY OF THE INVENTION

[0009] The subject invention relates in part to the surprising discovery that DIG-3 and Cry1Ab do not compete for binding to sites in European corn borer (ECB; *Ostrinia nubilalis* (Hübner)) gut cell membrane preparations. As one skilled in the art will recognize

with the benefit of this disclosure, plants that produce both of these proteins (including insecticidal portions of the full-length proteins) can be used to delay or prevent the development of resistance to either of these insecticidal proteins alone. Corn is a preferred plant for use according to the subject invention. ECB is the preferred target insect for the subject pair of toxins.

[0010] Thus, the subject invention relates in part to the use of a Cry1Ab protein in combination with a DIG-3 protein. Plants (and acreage planted with such plants) that produce both of these proteins are included within the scope of the subject invention.

[0011] The subject invention also relates in part to triple stacks or "pyramids" of three (or more) toxins, with Cry1Ab and DIG-3 being the base pair. In some preferred pyramid embodiments, the combination of the selected toxins provides three sites of action against ECB. Some preferred "three sites of action" pyramid combinations include the subject base pair of proteins plus Cry1F as the third protein for targeting ECB. (It was known from US 2008 0311096 that Cry1Ab is effective against Cry1Fa-resistant ECB.) This particular triple stack, for example, would, according to the subject invention, advantageously and surprisingly provide three sites of action against ECB. This can help to reduce or eliminate the requirement for refuge acreage.

[0012] Although the subject invention is disclosed herein as a base pair of toxins, Cry1Ab and DIG-3, which, either together as a pair or in a "pyramid" of three or more toxins, provide for insect-resistance against ECB in corn, it should be understood that other combinations with Cry1Ab and DIG-3 can be also used according to the subject invention, preferably in corn.

BRIEF DESCRIPTION OF THE FIGURE

[0013] Figure 1 shows percent specific binding of ^{125}I Cry1Ab (0.5 nM) in BBMVs from *Ostrinia nubilalis* versus competition by unlabeled homologous Cry1Ab (●) and heterologous DIG-3 (■). The displacement curve for homologous competition by Cry1Ab results in a sigmoidal shaped curve showing 50% displacement of the radioligand at about 0.5 nM of Cry1Ab. DIG-3 does not displace any of the binding of ^{125}I Cry1Ab from its binding site at concentrations of 100 nM or lower (200-fold higher than the concentration of ^{125}I Cry1Ab in the assay). Only at 300 nM do we observe about 25% displacement of the binding of ^{125}I Cry1Ab by DIG-3. These results show that DIG-3 does not effectively

compete for the binding of Cry1Ab to receptor sites located in BBMV's from *Ostrinia nubilalis*.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the full-length Cry1Ab exemplified protein. (MR818)

SEQ ID NO:2 is the full-length DIG-3 exemplified protein.

DETAILED DESCRIPTION OF THE INVENTION

[0014] The subject invention relates in part to the surprising discovery that Cry1Ab and DIG-3 do not compete with each other for binding sites in the gut of the European corn borer (ECB; *Ostrinia nubilalis* (Hübner)) or the fall armyworms (FAW; *Spodoptera frugiperda*). Thus, a Cry1Ab protein can be used in combination with a DIG-3 protein, preferably in transgenic corn, to delay or prevent ECB from developing resistance to either of these proteins alone. The subject pair of proteins can be effective at protecting plants (such as maize plants) from damage by Cry-resistant ECB. That is, one use of the subject invention is to protect corn and other economically important plant species from damage and yield loss caused by ECB populations that could develop resistance to Cry1Ab or DIG-3.

[0015] The subject invention thus teaches an insect resistant management (IRM) stack comprising Cry1Ab and DIG-3 to prevent or mitigate the development of resistance by ECB to either or both of these proteins.

[0016] Further, although the subject invention, disclosed herein, teaches an IRM stack comprising Cry1Ab and DIG-3 for preventing resistance by ECB to either or both of these proteins, it is within the scope of the invention disclosed herein that one or both of Cry1Ab and DIG-3 may be adapted, either alone or in combination, to prevent resistance by FAW to either or both of these proteins.

[0017] The present invention provides compositions for controlling lepidopteran pests comprising cells that produce a Cry1Ab core toxin-containing protein and a DIG-3 core toxin-containing protein.

[0018] The invention further comprises a host transformed to produce both a Cry1Ab insecticidal protein and a DIG-3 insecticidal protein, wherein said host is a microorganism

or a plant cell. The subject polynucleotide(s) are preferably in a genetic construct under control of a non-*Bacillus-thuringiensis* promoter(s). The subject polynucleotides can comprise codon usage for enhanced expression in a plant.

[0019] It is additionally intended that the invention provides a method of controlling lepidopteran pests comprising contacting said pests or the environment of said pests with an effective amount of a composition that contains a Cry1Ab insecticidal protein and further contains a DIG-3 insecticidal protein.

[0020] An embodiment of the invention comprises a maize plant comprising a plant-expressible gene encoding a DIG-3 core toxin-containing protein and a plant-expressible gene encoding a Cry1Ab core toxin-containing protein, and seed of such a plant.

[0021] A further embodiment of the invention comprises a maize plant wherein a plant-expressible gene encoding a DIG-3 insecticidal protein and a plant-expressible gene encoding a Cry1Ab insecticidal protein have been introgressed into said maize plant, and seed of such a plant.

[0022] As described in the Examples, competitive receptor binding studies using DIG-3 and radiolabeled Cry1Ab proteins show that the DIG-3 protein does not compete for binding in ECB tissues to which Cry1Ab binds. These results also indicate that the combination of Cry1Ab and DIG-3 proteins can be an effective means to mitigate the development of resistance in ECB populations to either of these proteins. Thus, based in part on the data described herein, co-production (stacking) of DIG-3 with Cry1Ab for high dose can be used in IRM stacks for controlling ECB.

[0023] Other proteins can be added to this pair. For example, the subject invention also relates in part to triple stacks or "pyramids" of three (or more) toxins, with Cry1Ab and DIG-3 being the base pair. In some preferred pyramid embodiments, the selected toxins have three separate sites of action against ECB. Some preferred "three sites of action" pyramid combinations include the subject base pair of proteins plus Cry1Fa as the third protein for targeting ECB. These particular triple stacks would, according to the subject invention, advantageously and surprisingly provide three sites of action against ECB. This can help to reduce or eliminate the requirement for refuge acreage. By "separate sites of action," it is meant any of the given proteins do not cause cross-resistance with each other.

[0024] Thus, one deployment option is to use the subject pair of proteins in combination with a third toxin/gene, and to use this triple stack to mitigate the development of resistance in ECB to any of these toxins. Accordingly, the subject invention also relates in part to

triple stacks or "pyramids" of three (or more) toxins. In some preferred pyramid embodiments, the selected toxins have three separate sites of action against ECB.

[0025] Included among deployment options of the subject invention would be to use two, three, or more proteins of the subject proteins in crop-growing regions where ECB can (or is known to) develop resistant populations.

[0026] Cry1Fa is deployed in the Herculex[®] and SmartStax[™] products, for example. The subject pair of genes (Cry1Ab and DIG-3) could be combined into, for example, a Cry1Fa product such as Herculex[®] and/or SmartStax[™]. Accordingly, the subject pair of proteins could be significant in reducing the selection pressure on these and other proteins. The subject pair of proteins could thus be used as in the three gene combinations for corn.

[0027] As discussed above, additional toxins/genes can also be added according to the subject invention. For example, for use of Cry1Ab with Cry1Be to target ECB, see WO 2011/084631. For use of Cry1Ab with Cry2Aa to target ECB, see WO 2011/075590. Thus, Cry1Be and/or Cry2Aa could be used (optionally with Cry1Fa) in multiple protein stacks with the subject pair of proteins.

[0028] Plants (and acreage planted with such plants) that produce any of the subject combinations of proteins are included within the scope of the subject invention. Additional toxins/genes can also be added, but the particular stacks discussed above advantageously and surprisingly provide multiple sites of action against ECB. This can help to reduce or eliminate the requirement for refuge acreage. A field thus planted of over ten acres is thus included within the subject invention.

[0029] GENBANK can also be used to obtain the sequences for any of the genes and proteins discussed herein. Patents can also be used. For example, U.S. Patent No. 5,188,960 and U.S. Patent No. 5,827,514 describe Cry1Fa core toxin containing proteins suitable for use in carrying out the present invention. U.S. Patent No. 6,218,188 describes plant-optimized DNA sequences encoding Cry1Fa core toxin-containing proteins that are suitable for use in the present invention.

[0030] Insects related to ECB can also be targeted. These can include stem borers and/or stalk-boring insects. The southwestern corn borer (*Diatraea grandiosella* – of the suborder Heterocera) is one example. The sugarcane borer is also a *Diatraea* species (*Diatraea saccharalis*). Combinations of proteins described herein can be used to target larval stages of the target insect. Adult lepidopterans, for example, butterflies and moths, primarily feed on flower nectar and are a significant effector of pollination. Nearly all lepidopteran larvae,

i.e., caterpillars, feed on plants, and many are serious pests. Caterpillars feed on or inside foliage or on the roots or stem of a plant, depriving the plant of nutrients and often destroying the plant's physical support structure. Additionally, caterpillars feed on fruit, fabrics, and stored grains and flours, ruining these products for sale or severely diminishing their value.

[0031] Some chimeric toxins of the subject invention comprise a full N-terminal core toxin portion of a *Bt* toxin and, at some point past the end of the core toxin portion, the protein has a transition to a heterologous protoxin sequence. The N-terminal, insecticidally active, toxin portion of a *Bt* toxin is referred to as the "core" toxin. The transition from the core toxin segment to the heterologous protoxin segment can occur at approximately the toxin/protoxin junction or, in the alternative, a portion of the native protoxin (extending past the core toxin portion) can be retained, with the transition to the heterologous protoxin portion occurring downstream.

[0032] Typical, full-length three domain *B.t. Cry* proteins are approximately 130 kDa to 150 kDa. Cry1Ab is one example. DIG-3 is also a three-domain toxin – approximately 142 kDa in size.

[0033] As an example, one chimeric toxin of the subject invention, is a full core toxin portion of Cry1Ab (approximately amino acids 1 to 601) and/or a heterologous protoxin (approximately amino acids 602 to the C-terminus). In one preferred embodiment, the portion of a chimeric toxin comprising the protoxin is derived from a Cry1Ab protein toxin. In a preferred embodiment, the portion of a chimeric toxin comprising the protoxin is derived from a Cry1Ab protein toxin.

[0034] A person skilled in this art will appreciate that *Bt* toxins (even within a certain class such as Cry1B) can vary to some extent in length and the precise location of the transition from core toxin portion to protoxin portion. Typical full-length Cry toxins are about 1150 to about 1200 amino acids in length. The transition from core toxin portion to protoxin portion will typically occur at between about 50% to about 60% of the full length toxin. The chimeric toxin of the subject invention will include the full expanse of this N-terminal core toxin portion. Thus, the chimeric toxin will comprise at least about 50% of the full length Cry1 protein. This will typically be at least about 590 amino acids (and could include 600-650 or so residues). With regard to the protoxin portion, the full expanse of the Cry1Ab protoxin portion extends from the end of the core toxin portion to the C-terminus of the molecule.

[0035] Genes and toxins. The genes and toxins useful according to the subject invention include not only the full length sequences disclosed but also fragments of these sequences, variants, mutants, and fusion proteins which retain the characteristic pesticidal activity of the toxins specifically exemplified herein. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences which encode the same toxins or which encode equivalent toxins having pesticidal activity. As used herein, the term "equivalent toxins" refers to toxins having the same or essentially the same biological activity against the target pests as the claimed toxins.

[0036] As used herein, the boundaries represent approximately 95% (Cry1Ab's, for examples), 78% (Cry1A's and Cry1B's), and 45% (Cry1's) sequence identity, per "Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins," N. Crickmore, D.R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D.H. Dean. Microbiology and Molecular Biology Reviews (1998) Vol 62: 807-813. These cut offs can also be applied to the core toxins only.

[0037] It should be apparent to a person skilled in this art that genes encoding active toxins can be identified and obtained through several means. The specific genes or gene portions exemplified herein may be obtained from the isolates deposited at a culture depository. These genes, or portions or variants thereof, may also be constructed synthetically, for example, by use of a gene synthesizer. Variations of genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Genes that encode active fragments may also be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these protein toxins.

[0038] Fragments and equivalents which retain the pesticidal activity of the exemplified toxins would be within the scope of the subject invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding the same, or essentially the same, toxins. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino acid substitutions, deletions, additions, or insertions which do not materially affect pesticidal

activity. Fragments of genes encoding proteins that retain pesticidal activity are also included in this definition.

[0039] A further method for identifying the genes encoding the toxins and gene portions useful according to the subject invention is through the use of oligonucleotide probes. These probes are detectable nucleotide sequences. These sequences may be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO93/16094. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample have substantial homology. Preferably, hybridization is conducted under stringent conditions by techniques well-known in the art, as described, for example, in Keller, G. H., M. M. Manak (1987) DNA Probes, Stockton Press, New York, N.Y., pp. 169-170. Some examples of salt concentrations and temperature combinations are as follows (in order of increasing stringency): 2X SSPE or SSC at room temperature; 1X SSPE or SSC at 42° C; 0.1X SSPE or SSC at 42° C; 0.1X SSPE or SSC at 65° C. Detection of the probe provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying toxin-encoding genes of the subject invention. The nucleotide segments which are used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0040] Variant toxins. Certain toxins of the subject invention have been specifically exemplified herein. Since these toxins are merely exemplary of the toxins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent toxins (and nucleotide sequences coding for equivalent toxins) having the same or similar pesticidal activity of the exemplified toxin. Equivalent toxins will have amino acid homology with an exemplified toxin. This amino acid homology will typically be greater than 75%, preferably be greater than 90%, and most preferably be greater than 95%. The amino acid homology will be highest in critical regions of the toxin which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be

placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Below is a listing of examples of amino acids belonging to each class.

Table 1: Examples of Amino Acids within the Four Classes of Amino Acids

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

[0041] In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin.

[0042] Recombinant hosts. The genes encoding the toxins of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. Conjugal transfer and recombinant transfer can be used to create a *Bt* strain that expresses both toxins of the subject invention. Other host organisms may also be transformed with one or both of the toxin genes then used to accomplish the synergistic effect. With suitable microbial hosts, *e.g.*, *Pseudomonas*, the microbes can be applied to the situs of the pest, where they will proliferate and be ingested. The result is control of the pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest.

[0043] Where the *Bt* toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the

polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

[0044] A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, *e.g.*, genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, *e.g.*, genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Rhodopseudomonas spheroides*, *Xanthomonas campestris*, *Rhizobium melioli*, *Alcaligenes entrophus*, and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C. diffluens*, *C. laurentii*, *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odoratus*, *Kluyveromyces veronae*, and *Aureobasidium pollulans*. Of particular interest are the pigmented microorganisms.

[0045] A wide variety of methods is available for introducing a *Bt* gene encoding a toxin into a microorganism host under conditions which allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867, which is incorporated herein by reference.

[0046] Treatment of cells. *Bacillus thuringiensis* or recombinant cells expressing the *Bt* toxins can be treated to prolong the toxin activity and stabilize the cell. The pesticide microcapsule that is formed comprises the *Bt* toxin or toxins within a cellular structure that has been stabilized and will protect the toxin when the microcapsule is applied to the environment of the target pest. Suitable host cells may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxic substances are unstable or the level of

application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi.

[0047] The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

[0048] Treatment of the microbial cell, e.g., a microbe containing the *Bt* toxin gene or genes, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability of protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Lugol iodine, Bouin's fixative, various acids and Helly's fixative (See: Humason, Gretchen L., *Animal Tissue Techniques*, W. H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host environment. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like. Methods for treatment of microbial cells are disclosed in U.S. Pat. Nos. 4,695,455 and 4,695,462, which are incorporated herein by reference.

[0049] The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of cell treatment should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of treatment should retain at least a substantial portion of the bio-availability or bioactivity of the toxin.

[0050] Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the *Bt* gene or genes into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls,

pigmentation, and intracellular packaging or formation of inclusion bodies; survival in aqueous environments; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

[0051] Growth of cells. The cellular host containing the *Bt* insecticidal gene or genes 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 *Bt* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

[0052] The *Bt* cells producing the toxins of the invention can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle the bacteria can be harvested by first separating the *Bt* spores and crystals from the fermentation broth by means well known in the art. The recovered *Bt* spores and crystals can be formulated into a wettable powder, liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers, and other components to facilitate handling and application for particular target pests. These formulations and application procedures are all well known in the art.

[0053] Formulations. Formulated bait granules containing an attractant and spores, crystals, and toxins of the *Bt* isolates, or recombinant microbes comprising the genes obtainable from the *Bt* isolates disclosed herein, can be applied to the soil. Formulated product can also be applied as a seed-coating or root treatment or total plant treatment at later stages of the crop cycle. Plant and soil treatments of *Bt* cells may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, gels, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

[0054] As would be appreciated by a person skilled in the art, the pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1%

by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

[0055] The formulations can be applied to the environment of the lepidopteran pest, e.g., foliage or soil, by spraying, dusting, sprinkling, or the like.

[0056] Plant transformation. A preferred recombinant host for production of the insecticidal proteins of the subject invention is a transformed plant. Genes encoding *Bt* toxin proteins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *Escherichia coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *inter alia*. Accordingly, the DNA fragment having the sequence encoding the *Bt* toxin protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516, Lee and Gelvin (2008), Hoekema (1985), Fraley *et al.*, (1986), and An *et al.*, (1985), and is well established in the art.

[0057] Once the inserted DNA has been integrated in the plant genome, it is relatively stable. The transformation vector normally contains a selectable marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as Bialaphos,

Kanamycin, G418, Bleomycin, or Hygromycin, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

[0058] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the Right and Left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters *et al.*, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[0059] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[0060] In a preferred embodiment of the subject invention, plants will be transformed with genes wherein the codon usage has been optimized for plants. See, for example, U.S. Patent No. 5,380,831, which is hereby incorporated by reference. While some truncated toxins are exemplified herein, it is well-known in the *Bt* art that 130 kDa-type (full-length) toxins have an N-terminal half that is the core toxin, and a C-terminal half that is the protoxin "tail." Thus, appropriate "tails" can be used with truncated / core toxins of the subject invention. See e.g. U.S. Patent No. 6,218,188 and U.S. Patent No. 6,673,990. In addition, methods for creating synthetic *Bt* genes for use in plants are known in the art (Stewart and Burgin, 2007). One non-limiting example of a preferred transformed plant is a fertile maize plant comprising a plant expressible gene encoding a Cry1Ab protein, and further comprising a second plant expressible gene encoding a Cry1Be protein.

[0061] Transfer (or introgression) of the Cry1Ab- and Cry1Be-determined trait(s) into inbred maize lines can be achieved by recurrent selection breeding, for example by backcrossing. In this case, a desired recurrent parent is first crossed to a donor inbred (the non-recurrent parent) that carries the appropriate gene(s) for the Cry1A- and Cry1Be-determined traits. The progeny of this cross is then mated back to the recurrent parent followed by selection in the resultant progeny for the desired trait(s) to be transferred from the non-recurrent parent. After three, preferably four, more preferably five or more generations of backcrosses with the recurrent parent with selection for the desired trait(s), the progeny will be heterozygous for loci controlling the trait(s) being transferred, but will be like the recurrent parent for most or almost all other genes (see, for example, Poehlman & Sleper (1995) *Breeding Field Crops*, 4th Ed., 172-175; Fehr (1987) *Principles of Cultivar Development*, Vol. 1: Theory and Technique, 360-376).

[0062] Insect Resistance Management (IRM) Strategies. Roush *et al.*, for example, outlines two-toxin strategies, also called "pyramiding" or "stacking," for management of insecticidal transgenic crops. (The Royal Society. *Phil. Trans. R. Soc. Lond. B.* (1998) 353, 1777-1786).

[0063] On their website, the United States Environmental Protection Agency (epa.gov/oppbppd1/biopesticides/pips/bt_corn_refuge_2006.htm) publishes the following requirements for providing non-transgenic (*i.e.*, *non-B.t.*) refuges (a section of non-Bt crops / corn) for use with transgenic crops producing a single Bt protein active against target pests.

"The specific structured requirements for corn borer-protected Bt (Cry1Ab or Cry1F) corn products are as follows:

Structured refuges: 20% non-Lepidopteran Bt corn refuge in Corn Belt;
50% non-Lepidopteran Bt refuge in Cotton Belt

Blocks

Internal (*i.e.*, within the Bt field)

External (*i.e.*, separate fields within ½ mile (¼ mile if possible) of the Bt field to maximize random mating)

In-field Strips

Strips must be at least 4 rows wide (preferably 6 rows) to reduce the effects of larval movement"

[0064] In addition, the National Corn Growers Association, on their website:

(ncga.com/insect-resistance-management-fact-sheet-bt-corn)

[0065] also provides similar guidance regarding the refuge requirements. For example:

"Requirements of the Corn Borer IRM:

- Plant at least 20% of your corn acres to refuge hybrids
- In cotton producing regions, refuge must be 50%
- Must be planted within 1/2 mile of the refuge hybrids
- Refuge can be planted as strips within the Bt field; the refuge strips must be at least 4 rows wide
- Refuge may be treated with conventional pesticides only if economic thresholds are reached for target insect
- Bt-based sprayable insecticides cannot be used on the refuge corn
- Appropriate refuge must be planted on every farm with Bt corn"

[0066] As stated by Roush *et al.* (on pages 1780 and 1784 right column, for example), stacking or pyramiding of two different proteins each effective against the target pests and with little or no cross-resistance can allow for use of a smaller refuge. Roush suggests that for a successful stack, a refuge size of less than 10% refuge, can provide comparable resistance management to about 50% refuge for a single (non-pyramided) trait. For currently available pyramided *Bt* corn products, the U.S. Environmental Protection Agency requires significantly less (generally 5%) structured refuge of non-*Bt* corn be planted than for single trait products (generally 20%).

[0067] There are various ways of providing the IRM effects of a refuge, including various geometric planting patterns in the fields (as mentioned above) and in-bag seed mixtures, as discussed further by Roush *et al.* (*supra*), and U.S. Patent No. 6,551,962.

[0068] The above percentages, or similar refuge ratios, can be used for the subject double or triple stacks or pyramids. For triple stacks with three sites of action against a single target

pest, a goal would be zero refuge (or less than 5% refuge, for example). This is particularly true for commercial acreage – of over 10 acres for example.

[0069] 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.

[0070] Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

[0071] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. All temperatures are in degrees Celsius.

EXAMPLES

Example 1 – ^{125}I Labeling of Cry1Ab Protein

Iodination of Cry1Ab core toxin. Cry1Ab toxin (SEQ ID NO:1) was trypsin activated and iodinated using Iodo-Beads (Pierce). Briefly, two Iodo-Beads were washed twice with 500 μl of phosphate buffered saline, PBS (20 mM sodium phosphate, 0.15 M NaCl, pH 7.5), and placed into a 1.5 ml centrifuge tube behind lead shielding. To this was added 100 μl of PBS. In a hood and through the use of proper radioactive handling techniques, 0.5 mCi Na ^{125}I (17.4 Ci/mg, Amersham) was added to the PBS solution with the Iodo-Bead. The components were allowed to react for 5 minutes at room temperature, then 10 μg of highly pure truncated Cry1Ab protein was added to the solution and allowed to react for an additional 5 minutes. The reaction was terminated by removing the solution from the iodo-beads and applying it to a 0.5 ml desalting Zeba spin column (InVitrogen) equilibrated in 20 mM CAPS buffer, pH 10.5 + 1 mM DTT. The iodo-bead was washed twice with 10 μl of PBS each and the wash solution also applied to the desalting column. The radioactive solution was eluted through the desalting column by centrifugation at 1,000 x g for 2 min. Radio-purity of the radio-iodinated Cry1Ab was determined by SDS-PAGE, phosphor-imaging and gamma counting. Briefly, 2 μl of the radioactive protein was

separated by SDS-PAGE using 4-20% tris glycine polyacrylamide gels (1 mm thick, InVitrogen). After separation, the gels were dried using a BioRad gel drying apparatus following the manufacturer's instructions. The dried gels were imaged by wrapping them in Mylar film (12 μm thick), and exposing them under a Molecular Dynamics storage phosphor screen (35 cm x 43 cm), for 1 hour. The plates were developed using a Molecular Dynamics Storm 820 phosphorimager and the imaged analyzed using ImageQuant TM software. The specific activity was approximately 4 $\mu\text{Ci}/\mu\text{g}$ protein.

Example 2 - BBMV Preparation Protocol

Preparation and Fractionation of Solubilized BBMV's. Last instar *Ostrinia nubilalis* larvae were fasted overnight and then dissected in the morning after chilling on ice for 15 minutes. The midgut tissue was removed from the body cavity, leaving behind the hindgut attached to the integument. The midgut was placed in 9X volume of ice cold homogenization buffer (300 mM mannitol, 17 mM tris. base, pH 7.5), supplemented with Protease Inhibitor Cocktail¹ (Sigma P-2714) diluted as recommended by the supplier. The tissue was homogenized with 15 strokes of a glass tissue homogenizer. BBMV's were prepared by the MgCl_2 precipitation method of Wolfersberger (1993). Briefly, an equal volume of a 24 mM MgCl_2 solution in 300 mM mannitol was mixed with the midgut homogenate, stirred for 5 minutes and allowed to stand on ice for 15 min. The solution was centrifuged at 2,500 x g for 15 min at 4° C. The supernatant was saved and the pellet suspended into the original volume of 0.5-X diluted homogenization buffer and centrifuged again. The two supernatants were combined, centrifuged at 27,000 x g for 30 min at 4 °C to form the BBMV fraction. The pellet was suspended into 10 ml homogenization buffer supplemented with protease inhibitors, and centrifuged again at 27,000 x g for 30 min at 4 °C to wash the BBMV's. The resulting pellet was suspended into BBMV Storage Buffer (10 mM HEPES, 130 mM KCl, 10% glycerol, pH 7.4) to a concentration of about 3 mg/ml protein. Protein concentration was determined by using the Bradford method (1976) with bovine serum albumin (BSA) as the standard. Alkaline phosphatase determination was made prior to freezing the samples using the Sigma assay following manufacturer's instructions. The specific activity of this marker enzyme in the BBMV fraction typically

¹ Final concentration of cocktail components (in μM) are AEBSF (500), EDTA (250 mM), Bestatin (32), E-64 (0.35), Leupeptin (0.25), and Aprotinin (0.075).

increased 7-fold compared to that found in the midgut homogenate fraction. The BBMV's were aliquoted into 250 µl samples, flash frozen in liquid N₂ and stored at -80 °C.

Example 3 - Method to Measure Binding of ¹²⁵I Cry1Ab Protein to BBMV Proteins

Binding of ¹²⁵I Cry1Ab Protein to BBMV's. To determine the optimal amount of BBMV protein to use in the binding assays, a saturation curve was generated. ¹²⁵I radiolabeled Cry1Ab protein (0.5 nM) was incubated for 1 hour at 28 °C with various amounts of BBMV protein, ranging from 0-500 µg/ml in binding buffer (8 mM NaHPO₄, 2 mM KH₂PO₄, 150 mM NaCl, 0.1% bovine serum albumin, pH 7.4). Total volume was 0.5 ml. Bound ¹²⁵I Cry1Ab protein was separated from unbound by sampling 150 µl of the reaction mixture in triplicate from a 1.5 ml centrifuge tube into a 500 µl centrifuge tube and centrifuging the samples at 14,000 x g for 6 minutes at room temperature. The supernatant was gently removed, and the pellet gently washed three times with ice cold binding buffer. The bottom of the centrifuge containing the pellet was cut out and placed into a 13 x 75-mm glass culture tube. The samples were counted for 5 minutes each in the gamma counter. The counts contained in the sample were subtracted from background counts (reaction with out any protein) and was plotted versus BBMV protein concentration. The optimal amount of protein to use was determined to be 0.15 mg/ml of BBMV protein.

To determine the binding kinetics, a saturation curve was generated. Briefly, BBMV's (150 µg/ml) were incubated for 1 hr. at 28 °C with increasing concentrations of ¹²⁵I Cry1Ab toxin, ranging from 0.01 to 10 nM. Total binding was determined by sampling 150 µl of each concentration in triplicate, centrifugation of the sample and counting as described above. Non-specific binding was determined in the same manner, with the addition of 1,000 nM of the homologous trypsinized non-radioactive Cry1Ab toxin added to the reaction mixture to saturate all non-specific receptor binding sites. Specific binding was calculated as the difference between total binding and non-specific binding.

Homologous (Cry1Ab) and heterologous (DIG-3) competition binding assays were conducted using 150 µg/ml BBMV protein and 0.5 nM of the ¹²⁵I radiolabeled Cry1Ab protein. Cry1Ab and DIG-3 (SEQ ID NO:2) were trypsin activated and used as competitor proteins. The concentration of the competitive non-radiolabeled Cry1Ab or DIG-3 toxin added to the reaction mixture ranged from 0.03 to 1,000 nM and were added at the same time as the radioactive ligand, to assure true binding competition. Incubations were carried

out for 1 hr. at 28 °C and the amount of ^{125}I Cry1Ab protein bound to its receptor toxin measured as described above with non-specific binding subtracted. One hundred percent total binding was determined in the absence of any competitor ligand. Results were plotted on a semi-logarithmic plot as percent total specific binding versus concentration of competitive ligand added.

Example 4 – Summary of Results

Figure 1 shows percent specific binding of ^{125}I Cry1Ab (0.5 nM) in BBMV's from *Ostrinia nubilalis* versus competition by unlabeled homologous Cry1Ab (●) and heterologous DIG-3 (■). The displacement curve for homologous competition by Cry1Ab results in a sigmoidal shaped curve showing 50% displacement of the radioligand at about 0.5 nM of Cry1Ab. DIG-3 does not displace any of the binding of ^{125}I Cry1Ab from its binding site at concentrations of 100 nM or lower (200-fold higher than the concentration of ^{125}I Cry1Ab in the assay). Only at 300 nM do we observe about 25% displacement of the binding of ^{125}I Cry1Ab by DIG-3. These results show that DIG-3 does not effectively compete for the binding of Cry1Ab to receptor sites located in BBMV's from *Ostrinia nubilalis*.

Reference List

- Heckel,D.G., Gahan,L.J., Baxter,S.W., Zhao,J.Z., Shelton,A.M., Gould,F., and Tabashnik,B.E. (2007). The diversity of Bt resistance genes in species of Lepidoptera. *J Invertebr Pathol* 95, 192-197.
- Luo,K., Banks,D., and Adang,M.J. (1999). Toxicity, binding, and permeability analyses of four bacillus thuringiensis cry1 delta-endotoxins using brush border membrane vesicles of spodoptera exigua and spodoptera frugiperda. *Appl. Environ. Microbiol.* 65, 457-464.
- Palmer, M., Buchkremer, M, Valeva, A, and Bhakdi, S. Cysteine-specific radioiodination of proteins with fluorescein maleimide. *Analytical Biochemistry* 253, 175-179. 1997.
Ref Type: Journal (Full)
- Sambrook,J. and Russell,D.W. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory).
- Schlenz, M. L., Babcock, J. M., and Storer, N. P. Response of Cry1F-resistant and Susceptible European Corn Borer and Fall Armyworm Colonies to Cry1A.105 and Cry12Ab2. DAI 0830, 2008. Indianapolis, Dow AgroSciences. Derbi Report.
- Sheets, J. J. and Storer, N. P. Analysis of Cry1Ac Binding to Proteins in Brush Border Membrane Vesicles of Corn Earworm Larvae (*Heliothis zea*). Interactions with Cry1F Proteins and Its Implication for Resistance in the Field. DAI-0417, 1-26. 2001. Indianapolis, Dow AgroSciences.
- Tabashnik,B.E., Liu,Y.B., Finson,N., Masson,L., and Heckel,D.G. (1997). One gene in diamondback moth confers resistance to four Bacillus thuringiensis toxins. *Proc. Natl. Acad. Sci. U. S. A* 94, 1640-1644.
- Tabashnik,B.E., Malvar,T., Liu,Y.B., Finson,N., Borthakur,D., Shin,B.S., Park,S.H., Masson,L., de Maagd,R.A., and Bosch,D. (1996). Cross-resistance of the diamondback moth indicates altered interactions with domain II of Bacillus thuringiensis toxins. *Appl. Environ. Microbiol.* 62, 2839-2844.
- Tabashnik,B.E., Roush,R.T., Earle,E.D., and Shelton,A.M. (2000). Resistance to Bt toxins. *Science* 287, 42.
- Woltersberger,M.G. (1993). Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the gypsy moth (*Lymantria dispar*). *Arch. Insect Biochem. Physiol* 24, 139-147.
- Xu,X., Yu,L., and Wu,Y. (2005). Disruption of a cadherin gene associated with resistance to Cry1Ac {delta}-endotoxin of Bacillus thuringiensis in *Helicoverpa armigera*. *Appl Environ Microbiol* 71, 948-954.

We claim:

1. A transgenic plant comprising a cry1Ab polynucleotide encoding a Cry1Ab insecticidal protein, and a DIG-3 polynucleotide encoding a DIG-3 insecticidal protein, wherein said DIG-3 polynucleotide hybridizes at 42° C in 1X SSC with the complement of a polynucleotide that encodes a core toxin of SEQ ID NO:2.
2. The transgenic plant of claim 1, said plant further comprising DNA encoding a third insecticidal protein, preferably selected from the group consisting of Cry1Fa, Cry1Be, and Cry2Aa.
3. The transgenic plant of claim 2, said plant further comprising DNA encoding a fourth insecticidal protein, preferably selected from the group consisting of Cry1Be and Cry2Aa where the third insecticidal protein is Cry1Fa protein.
4. Seed of a plant of any of claims 1-3.
5. A field of plants comprising non-*Bt* refuge plants and a plurality of plants of any of claims 1-3, wherein said refuge plants comprise less than 40% of all crop plants in said field.
6. The field of plants of claim 5, wherein said refuge plants comprise less than 30% of all the crop plants in said field.
7. The field of plants of claim 5, wherein said refuge plants comprise less than 20% of all the crop plants in said field.
8. The field of plants of claim 5, wherein said refuge plants comprise less than 10% of all the crop plants in said field.
9. The field of plants of claim 5, wherein said refuge plants comprise less than 5% of all the crop plants in said field.
10. The field of plants of claim 5, wherein said refuge plants are in blocks or strips.
11. A mixture of seeds comprising refuge seeds from non-*Bt* refuge plants, and a plurality of seeds of claim 4, wherein said refuge seeds comprise less than 40% of all the seeds in the mixture.
12. The mixture of seeds of claim 11, wherein said refuge seeds comprise less than 30% of all the seeds in the mixture.

13. The mixture of seeds of claim 11, wherein said refuge seeds comprise less than 20% of all the seeds in the mixture.
14. The mixture of seeds of claim 11, wherein said refuge seeds comprise less than 10% of all the seeds in the mixture.
15. The mixture of seeds of claim 11, wherein said refuge seeds comprise less than 5% of all the seeds in the mixture.
16. A method of managing development of resistance to a *Cry* protein by an insect, said method comprising planting seeds to produce a field of plants of any of claims 5-10.
17. A field of any of claims 5-10, wherein said plants occupy more than 10 acres.
18. A plant of any of claims 1-3, wherein said plant is selected from the group consisting of corn, soybeans, and cotton.
19. The plant of claim 18, wherein said plant is a maize plant.
20. A non-totipotent plant cell comprising a cry1Ab polynucleotide encoding a Cry1Ab insecticidal protein, and a DIG-3 polynucleotide encoding a DIG-3 insecticidal protein, wherein said DIG-3 polynucleotide hybridizes at 42° C in 1X SSC with the complement of a polynucleotide that encodes a core toxin of SEQ ID NO:2.
21. A method of controlling a corn borer insect, wherein said method comprises contacting said insect or the environment of said insect with an effective amount of a composition that contains a Cry1Ab insecticidal protein and further contains a DIG-3 insecticidal protein.
22. The method of claim 22, wherein said composition is a plurality of plant cells.
23. A method of producing the composition of claim 22, wherein said method comprises reproducing said cells.

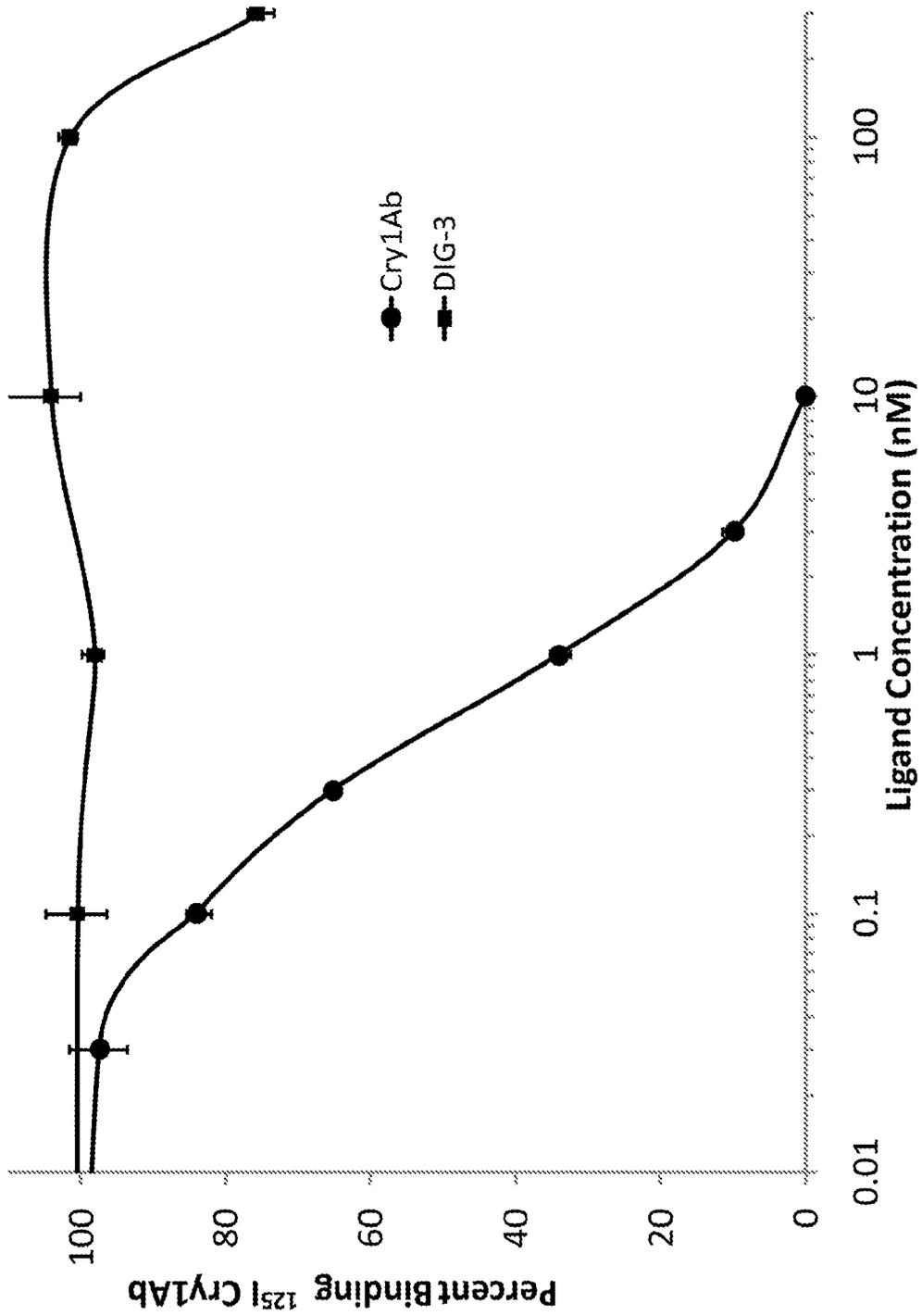


Figure 1.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/49491

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A01H 5/00, C12N 15/82 (2012.01) USPC - 800/302, 800/298, 435/480 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) USPC -- 800/302, 800/298, 435/480, 800/295, 435/468, 435/440, 435/476, 435/471 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase -- US, WO, EP; PubWEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files -- 654, 652, 349, 348, 340, 35, 65, 155; PCT Patentscope; Google Scholar -- transgenic plant, corn, seed, Cry1AB, DIG-3, Cry1FA, Cry2Aa, insecticidal proteins, filed planting, refuge crops/seeds, non-Bt crop, hybridization, sequences		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2010/0269223 A1 (LIRA et al.) 21 October 2010 (21.10.2010) para [0006]-[0010], [0026], [0028], [0032], [0038], [0049], [0057], [0078]-[0082], [0102], [0103], [0126], [0157], [0158], [0167]; SEQ ID NO: 2, abstract	21, 22 ----- 1-15, 18-20, 23
Y	WO 2011/084630 A1 (MEADE et al.) 14 July 2011 (14.07.2011) para [0014], [0024]-[0026], [0039], [0059], [0061], [0066], [0068]	1-15, 18-20, 23
Y	US 2010/0029725 A1 (COSGROVE et al.) 04 February 2010 (04.02.2010) para [0018], [0063], claim 58	11-15
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/>		
* 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 04 October 012 (04.10.2012)		Date of mailing of the international search report 23 OCT 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/49491

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 filed or furnished:

a. (means)

on paper

in electronic form

b. (time)

in the international application as filed

together with the international application in electronic form

subsequently to this Authority for the purposes of search

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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/49491

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.: 16 and 17
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:

- 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.:

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.