

Lepidopteran-Active Cry1Da1 Amino Acid Sequence Variant Proteins

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Applications
5 62/064,994, filed October 16, 2014, and 62/065,017, filed October 17, 2014, which are each
incorporated herein by reference in their entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] A computer readable form of the Sequence Listing is filed herewith by electronic
submission. The Sequence Listing is incorporated by reference in its entirety, is contained in
10 the file created on October 13, 2015, having the file name P34223WO00_SEQ_PCT.txt, and
which is 327,235 bytes in size (as measured in the MS-Windows® operating system).

FIELD OF THE INVENTION

[0003] The invention generally relates to the field of insect inhibitory proteins. A novel class
of engineered proteins exhibiting insect inhibitory activity against agriculturally-relevant
15 pests of crop plants and seeds is disclosed. In particular, the disclosed class of engineered
inhibitory proteins has insecticidal activity against the Lepidopteran order of insect pests.
Plants, plant parts, and seeds containing a polynucleotide construct encoding one or more of
the disclosed engineered inhibitory proteins are provided.

BACKGROUND OF THE INVENTION

20 [0004] *Helicoverpa zea* is a significant Lepidopteran pest of major agricultural crops,
including corn, cotton, and soy. Known as the corn earworm (CEW), cotton bollworm
(CBW), and soy podworm (SPW), this polyphagous insect species is particularly difficult to
control with insecticidal proteins from *Bacillus thuringiensis* (*Bt*) or other bacterial species.
H. zea is considered at risk for resistance development to current insect control traits, given
25 its ability to feed on many different crops and the current absence of a high-dose control
strategy. Accordingly, new modes of action (MoA) are required to ensure the durability of
transgenic plants protected from *H. zea* feeding damage.

[0005] The Cry1Da1 protein is a Lepidopteran-active protein that was first described by
Hofte, *et al.* "Nucleotide sequence and deduced amino acid sequence of a new Lepidoptera-

specific crystal protein gene from *Bacillus thuringiensis*.” *Nucleic Acids Res.* 18(18) (1990): 5545. This protein exhibits excellent insecticidal activity towards *Spodoptera* species including *Spodoptera frugiperda* (fall armyworm, FAW), a pest of several row crops, including corn, cotton and soybean. However, Cry1Da1 exhibits low-to-moderate activity
 5 towards a variety of other major Lepidopteran pests, including bollworms (*e.g.*, *Helicoverpa armigera* and *H. zea*), borers (*e.g.*, *Ostrinia nubilalis* and *Diatraea grandiosella*) and soybean looper (*Pseudoplusia includens*). Because of its narrow insecticidal spectrum and its inability to provide commercial-level protection against a range of important Lepidopteran agricultural pests such as CEW, the Cry1Da1 insecticidal protein has limited value as a transgenic plant
 10 insect control trait. As a result, no current commercial varieties of insect-protected crops utilize Cry1Da1 as a plant-incorporated protectant.

[0006] Despite its narrow insecticidal spectrum, Cry1Da1 is an interesting insecticidal protein because it appears that the Cry1Da1 protein uses an alternative MoA for controlling certain Lepidopteran pests. Evidence for this comes from studies with multiple resistant
 15 insect colonies. For example, field-derived colonies of *Plutella xylostella* (diamondback moth) and *Pectinophora gossypiella* (pink bollworm) that are resistant to Cry1Ac intoxication retain full sensitivity to the Cry1Da1 protein (Tabashnik, *et al.* “Cross-Resistance of Pink Bollworm (*Pectinophora gossypiella*) to *Bacillus thuringiensis* toxins.” *Appl. Environ. Microbiol.* 66 (2000): 4582-4584; Tabashnik, *et al.* “Cross-Resistance to
 20 *Bacillus thuringiensis* Toxin Cry1Ja in a Strain of Diamondback Moth Adapted to Artificial Diet.” *J. Invert. Pathol.* 76: (2000): 81-83.). These lines of evidence indicate that Cry1Da1 recognizes Lepidopteran midgut receptors distinct from those recognized by Lepidopteran-active proteins currently deployed in transgenic crops, including Cry1Ac, Cry1Ab, Cry1A.105, Cry1Fa, Cry2Ae, and Cry2Ab2. In view of this apparent novel MoA,
 25 optimization of Cry1Da1-like proteins for improved activity against a broader spectrum of *Helicoverpa* species while maintaining or increasing their insecticidal activity towards *Spodoptera* would create a high-value plant-incorporated protectant for insect resistance management.

SUMMARY OF THE INVENTION

30 [0007] In the present invention, several amino acid sequence variants of the TIC844 and Cry1Da scaffold proteins have been identified that exhibit markedly improved activity (compared to the Cry1Da1 native toxin) towards *H. zea* while retaining excellent activity

towards *S. frugiperda*. The improved variants of TIC844 and Cry1Da have been engineered to be expressed in crop plants (e.g., corn, soybean, cotton, sugarcane), and provide novel options for *in-planta* resistance management and Lepidopteran insect pest control in view of the apparent unique mode-of-action of Cry1Da coupled with the engineered improvement in activity against *H. zea*.

[0008] The engineered Lepidopteran toxic proteins described herein (referred to as “engineered toxin proteins”, “engineered toxic proteins”, or “engineered insecticidal proteins”) are derivatives of the naturally occurring *Bacillus thuringiensis* insecticidal toxin Cry1Da1 (SEQ ID NO:2) or the chimeric homolog of Cry1Da1, TIC844 (SEQ ID NO:14), which comprises the Cry1Da1 core toxin but substitutes the Cry1Ab3 protoxin for the native Cry1Da1 protoxin domain. The engineered insecticidal proteins of the present invention each contain at least one amino acid substitution, one amino acid addition, or one amino acid deletion compared to the scaffold proteins set forth in any of SEQ ID NO:2 or SEQ ID NO:14. The engineered insecticidal proteins of the present invention are particularly toxic to insects of the *Helicoverpa zea* (corn earworm, soy podworm, cotton bollworm) and *Spodoptera frugiperda* (fall armyworm) species. While the scaffold proteins TIC844 (SEQ ID NO:14) and Cry1Da1 (SEQ ID NO:2) display low toxicity to *H. zea*, the engineered insecticidal proteins of the present invention exhibit surprising and unexpectedly improved insecticidal activity and an enhanced insecticidal spectrum against Lepidopteran insect pests including *H. zea*.

[0009] In certain embodiments, an engineered insecticidal protein comprising an amino acid sequence as set forth in any of SEQ ID NO:44, SEQ ID NO: 40, SEQ ID NO: 12, SEQ ID NO:26, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:42, or an insect inhibitory fragment thereof is disclosed. In certain embodiments, the engineered insecticidal protein exhibits inhibitory activity against an insect species of the order Lepidoptera. The target Lepidopteran pest species inhibited by the Lepidopteran toxic proteins of the present invention include at least fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), bertha armyworm (*Mamestra configurata*), black cutworm (*Agrotis ipsilon*), cabbage looper (*Trichoplusia ni*), soybean looper (*Chrysodeixis includens*), velvetbean caterpillar (*Anticarsia gemmatilis*), green cloverworm (*Hypena scabra*), tobacco budworm (*Heliothis virescens*), granulate cutworm (*Agrotis subterranea*), armyworm (*Pseudaletia unipuncta*), western cutworm (*Agrotis orthogonia*), European corn borer (*Ostrinia nubilalis*), navel orangeworm

(*Amyelois transitella*), corn root webworm (*Crambus caliginosellus*), sod webworm (*Herpetogramma licarsisalis*), sunflower moth (*Homoeosoma electellum*), lesser cornstalk borer (*Elasmopalpus lignosellus*), codling moth (*Cydia pomonella*), grape berry moth (*Endopiza viteana*), oriental fruit moth (*Grapholita molesta*), sunflower bud moth (*Suleima helianthana*), diamondback moth (*Plutella xylostella*), pink bollworm (*Pectinophora gossypiella*), pink stem borer (*Sesamia inferens*), gypsy moth (*Lymantria dispar*), cotton leaf worm (*Alabama argillacea*), fruit tree leaf roller (*Archips argyrospila*), European leafroller (*Archips rosana*), Asiatic rice borer, or rice stem borer (*Chilo suppressalis*), rice leaf roller (*Cnaphalocrocis medinalis*), corn root webworm (*Crambus caliginosellus*), bluegrass webworm (*Crambus teterrellus*), southwestern corn borer (*Diatraea grandiosella*), sugarcane borer (*Diatraea saccharalis*), spiny bollworm (*Earias insulana*), spotted bollworm (*Earias vittella*), Old World cotton bollworm (*Helicoverpa armigera*), corn earworm, soy podworm or cotton bollworm (*Helicoverpa zea*), sod webworm (*Herpetogramma licarsisalis*), European grape vine moth (*Lobesia botrana*), citrus leafminer (*Phyllocnistis citrella*), large white butterfly (*Pieris brassicae*), imported cabbageworm, or small white butterfly (*Pieris rapae*), tobacco cutworm, or cluster caterpillar (*Spodoptera litura*), and tomato leafminer (*Tuta absoluta*).

[0010] Also disclosed herein is a polynucleotide encoding an engineered insecticidal protein or pesticidal fragment thereof, wherein the polynucleotide is operably linked to a heterologous promoter and the engineered insecticidal protein comprises the amino acid sequence as set forth in any of SEQ ID NO:44, SEQ ID NO: 40, SEQ ID NO: 12, SEQ ID NO:26, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:42.

[0011] In another embodiment, disclosed herein is a polynucleotide encoding an engineered insecticidal protein, wherein the polynucleotide comprises a nucleotide sequence that optionally hybridizes under stringent conditions to the reverse complement of the polynucleotide sequence as set forth in any of SEQ ID NO: 43, SEQ ID NO: 39, SEQ ID NO: 11, SEQ ID NO: 11, SEQ ID NO: 25, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO:31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37 or SEQ ID NO: 41; or encodes the engineered insecticidal protein comprising an amino acid sequence as set forth in any of SEQ ID NO:44, SEQ ID NO: 40, SEQ ID NO: 12, SEQ ID NO:26, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:16, SEQ ID NO:18, SEQ ID

NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:42.

[0012] Also provided herein is a host cell comprising the polynucleotide set forth in any of SEQ ID NO: 43, SEQ ID NO: 39, SEQ ID NO: 11, SEQ ID NO: 11, SEQ ID NO: 25, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO:31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37 or SEQ ID NO: 41, wherein the host cell is selected from the group consisting of a bacterial host cell or a plant host cell. Contemplated bacterial host cells include bacterial host cells selected from the group consisting of *Agrobacterium*, *Rhizobium*, *Bacillus*, *Brevibacillus*, *Escherichia*, *Pseudomonas*, *Klebsiella*, and *Erwinia*, wherein the *Bacillus* species is a *Bacillus cereus* or a *Bacillus thuringiensis*, said *Brevibacillus* is a *Brevibacillus laterosperous*, and said *Escherichia* is an *Escherichia coli*. Further, contemplated plant host cells include monocots or dicots.

[0013] In yet another embodiment, provided herein is an insect inhibitory composition comprising an engineered insecticidal protein comprising an amino acid sequence as set forth in any of SEQ ID NO:44, SEQ ID NO: 40, SEQ ID NO: 12, SEQ ID NO:26, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:42 or an insect inhibitory fragment thereof. It is contemplated that this insect inhibitory composition can further comprise at least one insect inhibitory agent different from the engineered insecticidal protein. Contemplated insect inhibitory agents include an insect inhibitory protein, an insect inhibitory dsRNA molecule, and an insect inhibitory chemistry. It is contemplated that the at least one other pesticidal agent can exhibit activity against one or more pest species of the orders Lepidoptera, Coleoptera, Hemiptera, Homoptera, or Thysanoptera.

[0014] Also disclosed herein are is a seed comprising an insect inhibitory effective amount of an engineered insecticidal protein comprising the amino acid sequence as set forth in any of SEQ ID NO:44, SEQ ID NO: 40, SEQ ID NO: 12, SEQ ID NO:26, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:42; or a polynucleotide set forth in any of SEQ ID NO: 43, SEQ ID NO: 39, SEQ ID NO: 11, SEQ ID NO: 11, SEQ ID NO: 25, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21,

SEQ ID NO: 23, SEQ ID NO:31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37 or SEQ ID NO: 41.

[0015] A method for controlling a Lepidopteran pest, the method comprising contacting the Lepidopteran pest with an inhibitory amount of an engineered insecticidal protein is also disclosed herein in another embodiment.

[0016] In yet another embodiment, disclosed herein is a transgenic plant cell, plant or plant part comprising an engineered insecticidal protein. Methods are provided for controlling a Lepidopteran pest, comprising exposing the pest to the transgenic plant cell, plant or plant part comprising an engineered insecticidal protein. Commodity products derived from the plant cell, plant or plant part comprising an engineered insecticidal protein wherein the product comprises a detectable amount of the engineered insecticidal protein are also contemplated. Contemplated commodity products include plant biomass, oil, meal, animal feed, flour, flakes, bran, lint, hulls, and processed seed.

[0017] Another method disclosed herein is a method of producing a seed comprising the engineered insecticidal protein, the method comprising: planting at least one seed comprising the engineered insecticidal protein; growing plants from said seed; and harvesting seed from the plants, wherein said harvested seed comprises the engineered insecticidal protein.

[0018] Yet another method disclosed in this application is a method of inhibiting Lepidopteran pests from feeding on a crop plant comprising modifying one or more amino acid residue(s) of SEQ ID NO: 2 or SEQ ID NO:14 through substitution of the one or more amino acid residue(s) to produce a modified SEQ ID NO:2 or SEQ ID NO:14; and making available a Lepidopteran-inhibiting amount of the modified SEQ ID NO: 2 or SEQ ID NO:14 within, on the surface, or in the vicinity of tissues of said crop plant; wherein the SEQ ID NO:2 or SEQ ID NO:14 modified amino acid residue is selected from the group consisting of serine at position 282 replaced by lysine or valine, tyrosine at position 316 replaced by serine, isoleucine at position 368 replaced by proline or arginine, serine at 374 replaced by arginine, asparagine at position 375 replaced by histidine, and isoleucine at position 432 replaced by leucine.

[0019] Recombinant polynucleotide molecules that encode the engineered insecticidal proteins of the present invention are also provided. Contemplated recombinant polynucleotide molecules comprise a polynucleotide sequence selected from the group consisting of SEQ ID NO: 43, SEQ ID NO: 39, SEQ ID NO: 11, SEQ ID NO: 11, SEQ ID NO: 25, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO:31, SEQ ID

NO: 33, SEQ ID NO: 35, SEQ ID NO: 37 or SEQ ID NO: 41; and optionally a polynucleotide sequence encoding an insect inhibitory agent different from the engineered insecticidal protein.

- [0020] Another method disclosed in this application is method for increasing the
 5 Lepidopteran activity and enhancing the Lepidopteran inhibitory spectrum of a scaffold protein, the method comprising modifying one or more amino acid residue(s) of SEQ ID NO: 2 or SEQ ID NO: 14 through substitution of the amino acid residue(s) to produce an engineered insecticidal protein, wherein the SEQ ID NO:2 or SEQ ID NO:14 modified amino acid residue is selected from the group consisting of serine at position 282 replaced by lysine
 10 or valine, tyrosine at position 316 replaced by serine, isoleucine at position 368 replaced by proline or arginine, serine at 374 replaced by arginine, asparagine at position 375 replaced by histidine, and isoleucine at position 432 replaced by leucine. In certain embodiments of this method, the engineered insecticidal protein has at least an eight-fold increase in *Helicoverpa zea* lethality relative to the scaffold protein
- 15 [0021] Other embodiments, features, and advantages of the invention will be apparent from the following detailed description, the examples, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0022] Figure 1 illustrates the MIC₅₀ values of the scaffold protein TIC844 (SEQ ID NO: 14)
 20 compared to the engineered insecticidal protein TIC844_8 (SEQ ID NO: 26) for two different *Helicoverpa zea* (CEW) colonies, Union City and Benzon.

BRIEF DESCRIPTION OF THE SEQUENCES

- [0023] SEQ ID NO:1 is a nucleotide sequence encoding a Cry1Da1 protein.
 25 [0024] SEQ ID NO:2 is an amino acid sequence of a Cry1Da1 protein toxin.
 [0025] SEQ ID NO:3 is a nucleotide sequence encoding a Cry1Da1_3 protein.
 [0026] SEQ ID NO:4 is an amino acid sequence of a Cry1Da1_3 protein toxin.
 [0027] SEQ ID NO:5 is a nucleotide sequence encoding a Cry1Da1_4 protein.
 [0028] SEQ ID NO:6 is an amino acid sequence of a Cry1Da1_4 protein toxin.
 30 [0029] SEQ ID NO:7 is a nucleotide sequence encoding a Cry1Da1_5 protein.
 [0030] SEQ ID NO:8 is an amino acid sequence of a Cry1Da1_5 protein toxin.
 [0031] SEQ ID NO:9 is a nucleotide sequence encoding a Cry1Da1_6 protein.

- [0032] SEQ ID NO:10 is an amino acid sequence of a Cry1Da1_6 protein toxin.
- [0033] SEQ ID NO:11 is a nucleotide sequence encoding a Cry1Da1_7 protein.
- [0034] SEQ ID NO:12 is an amino acid sequence of a Cry1Da1_7 protein toxin.
- [0035] SEQ ID NO:13 is a nucleotide sequence encoding a TIC844 protein.
- 5 [0036] SEQ ID NO:14 is an amino acid sequence of a TIC844 protein toxin.
- [0037] SEQ ID NO:15 is a nucleotide sequence encoding a TIC844_2 protein.
- [0038] SEQ ID NO:16 is an amino acid sequence of a TIC844_2 protein toxin.
- [0039] SEQ ID NO:17 is a nucleotide sequence encoding a TIC844_4 protein.
- [0040] SEQ ID NO:18 is an amino acid sequence of a TIC844_4 protein toxin.
- 10 [0041] SEQ ID NO:19 is a nucleotide sequence encoding a TIC844_5 protein.
- [0042] SEQ ID NO:20 is an amino acid sequence of a TIC844_5 protein toxin.
- [0043] SEQ ID NO:21 is a nucleotide sequence encoding a TIC844_6 protein.
- [0044] SEQ ID NO:22 is an amino acid sequence of a TIC844_6 protein toxin.
- [0045] SEQ ID NO:23 is a nucleotide sequence encoding a TIC844_7 protein.
- 15 [0046] SEQ ID NO:24 is an amino acid sequence of a TIC844_7 protein toxin.
- [0047] SEQ ID NO:25 is a nucleotide sequence encoding a TIC844_8 protein.
- [0048] SEQ ID NO:26 is an amino acid sequence of a TIC844_8 protein toxin.
- [0049] SEQ ID NO:27 is a polynucleotide sequence designed for use in expressing a Cry1Da1 protein in plants.
- 20 [0050] SEQ ID NO:28 is an amino acid sequence of a Cry1Da1 protein toxin.
- [0051] SEQ ID NO:29 is a polynucleotide sequence designed for use in expressing a Cry1Da1_2.nno protein in plants.
- [0052] SEQ ID NO:30 is an amino acid sequence of a Cry1Da1_2.nno protein toxin.
- [0053] SEQ ID NO:31 is a polynucleotide sequence designed for use in expressing a Cry1Da1_3.nno protein in plants.
- 25 [0054] SEQ ID NO:32 is an amino acid sequence of a Cry1Da1_3.nno protein toxin.
- [0055] SEQ ID NO:33 is a polynucleotide sequence designed for use in expressing a Cry1Da1_4.nno protein in plants.
- [0056] SEQ ID NO:34 is an amino acid sequence of a Cry1Da1_4.nno protein toxin.
- 30 [0057] SEQ ID NO:35 is a polynucleotide sequence designed for use in expressing a Cry1Da1_5.nno protein in plants.
- [0058] SEQ ID NO:36 is an amino acid sequence of a Cry1Da1_5.nno protein toxin.
- [0059] SEQ ID NO:37 is a polynucleotide sequence designed for use in expressing a Cry1Da1_6.nno protein in plants.

- [0060] SEQ ID NO:38 is an amino acid sequence of a Cry1Da1_6.nno protein toxin.
- [0061] SEQ ID NO:39 is a polynucleotide sequence designed for use in expressing a Cry1Da1_7.nno protein in plants.
- [0062] SEQ ID NO:40 is an amino acid sequence of a Cry1Da1_7.nno protein toxin.
- 5 [0063] SEQ ID NO:41 is a polynucleotide sequence designed for use in expressing a TIC844_9.nno protein in plants.
- [0064] SEQ ID NO:42 is an amino acid sequence of a TIC844_9.nno protein toxin.
- [0065] SEQ ID NO:43 is a polynucleotide sequence designed for use in expressing a TIC844_11.nno protein in plants.
- 10 [0066] SEQ ID NO:44 is an amino acid sequence of a TIC844_11.nno protein toxin.

DETAILED DESCRIPTION OF THE INVENTION

- [0067] Engineered insecticidal proteins that exhibit surprisingly higher levels of toxic activity
- 15 against Lepidopteran species and a broader insecticidal spectrum compared to other previously known Lepidopteran insecticidal proteins are provided herein. These engineered insecticidal proteins are derived from insecticidal scaffold proteins, which serve as templates for various amino acid modifications. Examples of such insecticidal scaffold proteins include but are not limited to Cry1Da1 and TIC844 (a homolog of Cry1Da1). TIC844 comprises the
- 20 Cry1Da1 core toxin (*i.e.*, domains I, II and III) but utilizes the Cry1Ab3 protoxin domain to ensure good expression in *Bacillus thuringiensis* (*Bt*). Expression of Cry1Da1 in *Bt* is poor when using the native Cry1Da1 protoxin domain. However, as demonstrated in this application, the expression of Cry1Da1 core toxin is remarkably improved in acrySTALLIFEROUS strains of *Bt* when the native protoxin domain is removed and the Cry1Da1 core toxin coding
- 25 segment is fused in frame with a segment encoding the Cry1Ab3 protoxin domain. Notably, the scaffold proteins TIC844 (SEQ ID NO:14) and Cry1Da1 (SEQ ID NO:2) do not exhibit the commercially useful Lepidopteran inhibitory spectrum and improved Lepidopteran inhibitory activity observed in the engineered insecticidal proteins.

- [0068] The engineered insecticidal proteins disclosed herein are related by amino acid
- 30 modifications such that the modified proteins exhibit enhanced Lepidopteran inhibitory spectrum and/or improved Lepidopteran inhibitory activity compared to the parent scaffold protein, TIC844 or Cry1Da1. The phrases “more active”, “improved activity”, “enhanced specificity”, “increased toxic potency”, “increased toxicity”, “improved Lepidopteran

inhibitory activity”, “greater Lepidopteran inhibitory activity”, and “enhanced Lepidopteran inhibitory spectrum” refer to a comparison of the activity of an engineered insecticidal protein to the activity of a scaffold protein (TIC844 or Cry1Da1) against a Lepidopteran insect, wherein the activity attributed to the engineered insecticidal protein is greater than the activity attributed to the scaffold protein. In certain embodiments, the engineered insecticidal proteins provided herein exhibit an enhanced Lepidopteran inhibitory spectrum and/or improved or greater Lepidopteran inhibitory activity when compared to the activities of the scaffold TIC844 or Cry1Da1 protein where the Lepidopteran pest species include, but are not limited to, *Helicoverpa zea* and *Spodoptera frugiperda*.

- 5
- 10 [0069] As used herein, the terms and phrases “active” or “activity”; “pesticidal activity” or “pesticidal”; or “insecticidal activity”, “insect inhibitory”, “insecticidal”, or “an insect inhibitory amount”, refer to efficacy of a toxic agent, such as an insecticidal protein, in inhibiting (inhibiting growth, feeding, fecundity, or viability), suppressing (suppressing growth, feeding, fecundity, or viability), controlling (controlling the pest infestation,
- 15 controlling the pest feeding activities on a particular crop containing an effective amount of a disclosed engineered insecticidal protein) or killing (causing the morbidity, mortality, or reduced fecundity of) a pest. Similarly, a “Lepidopteran inhibitory amount” refers to an amount of a toxic agent, such as an insecticidal protein, that results in any measurable inhibition of Lepidopteran viability, Lepidopteran growth, Lepidopteran development,
- 20 Lepidopteran reproduction, Lepidopteran feeding behavior, Lepidopteran mating behavior and/or any measurable decrease in the adverse effects caused to a plant by Lepidopteran feeding. These terms are intended to include the result of providing a pesticidally effective amount of a toxic agent to a pest where the exposure of the pest to the toxic agent results in morbidity, mortality, reduced fecundity, or stunting. These terms also include repulsion of
- 25 the pest from the plant, a tissue of the plant, a plant part, seed, plant cells, or from the particular geographic location where the plant may be growing, as a result of providing a pesticidally effective amount of the toxic agent in or on the plant. In general, pesticidal activity refers to the ability of a toxic agent to be effective in inhibiting the growth, development, viability, feeding behavior, mating behavior, fecundity, or any measurable
- 30 decrease in the adverse effects caused by an insect feeding on this protein, protein fragment, protein segment or polynucleotide of a particular target pest, including but not limited to insects of the order Lepidoptera. The toxic agent can be produced by the plant or can be applied to the plant or to the environment within the location where the plant is located.

[0070] A pesticidally effective amount of a toxic agent, when provided in the diet of a target pest, exhibits pesticidal activity when the toxic is ingested by the pest. A toxic agent can be a pesticidal protein or one or more chemical agents known in the art. Pesticidal or insecticidal chemical agents and pesticidal or insecticidal protein agents can be used alone or in combinations with each other. Chemical agents include but are not limited to dsRNA molecules targeting specific genes for suppression in a target pest, organochlorides, organophosphates, carbamates, pyrethroids, neonicotinoids, and ryanoids. Pesticidal or insecticidal protein agents include the engineered insecticidal proteins set forth in this application, as well as other proteinaceous toxic agents including those that target Lepidopteran pest species, as well as protein toxins that are used to control other plant pests such as Cry proteins available in the art for use in controlling Coleopteran, Hemipteran and Homopteran species.

[0071] The term “segment” or “fragment” is used herein to describe consecutive amino acid or nucleic acid sequences that are shorter than the complete amino acid or nucleic acid sequence describing the engineered insecticidal proteins.

[0072] It is intended that reference to a pest, particularly a pest of a crop plant, means insect pests of crop plants, particularly those Lepidopteran insect pests that are controlled by the disclosed engineered insecticidal proteins. However, reference to a pest can also include Coleopteran, Hemipteran and Homopteran insect pests of plants, as well as nematodes and fungi, when toxic agents targeting these pests are co-localized or present together with the disclosed engineered insecticidal proteins.

[0073] The disclosed engineered insecticidal proteins exhibit insecticidal activity towards insect pests from the Lepidopteran insect species, including adults, pupae, larvae, and neonates. The insects of the order Lepidoptera include, but are not limited to, armyworms, cutworms, loopers, and heliothines in the Family Noctuidae, *e.g.*, fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), bertha armyworm (*Mamestra configurata*), black cutworm (*Agrotis ipsilon*), cabbage looper (*Trichoplusia ni*), soybean looper (*Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatilis*), green cloverworm (*Hypena scabra*), tobacco budworm (*Heliothis virescens*), granulate cutworm (*Agrotis subterranea*), armyworm (*Pseudaletia unipuncta*), western cutworm (*Agrotis orthogonia*); borers, casebearers, webworms, coneworms, cabbageworms and skeletonizers from the Family Pyralidae, *e.g.*, European corn borer (*Ostrinia nubilalis*), navel orangeworm (*Amyelois transitella*), corn root webworm (*Crambus caliginosellus*), sod webworm (*Herpetogramma licarsisalis*), sunflower moth (*Homoeosoma electellum*), lesser cornstalk

borer (*Elasmopalpus lignosellus*); leafrollers, budworms, seed worms, and fruit worms in the Family Tortricidae, e.g., codling moth (*Cydia pomonella*), grape berry moth (*Endopiza viteana*), oriental fruit moth (*Grapholita molesta*), sunflower bud moth (*Suleima helianthana*); and many other economically important Lepidoptera, e.g., diamondback moth (5 *Plutella xylostella*), pink bollworm (*Pectinophora gossypiella*) and gypsy moth (*Lymantria dispar*). Other insect pests of order Lepidoptera include, e.g., *Alabama argillacea* (cotton leaf worm), *Archips argyrospila* (fruit tree leaf roller), *Archips rosana* (European leafroller) and other *Archips* species, *Chilo suppressalis* (Asiatic rice borer, or rice stem borer), *Cnaphalocrocis medinalis* (rice leaf roller), *Crambus caliginosellus* (corn root webworm), 10 *Crambus teterrellus* (bluegrass webworm), *Diatraea grandiosella* (southwestern corn borer), *Diatraea saccharalis* (surgarcane borer), *Earias insulana* (spiny bollworm), *Earias vittella* (spotted bollworm), *Helicoverpa armigera* (American bollworm), *Helicoverpa zea* (corn earworm or cotton bollworm), *Heliothis virescens* (tobacco budworm), *Herpetogramma licarsisalis* (sod webworm), *Lobesia botrana* (European grape vine moth), *Phyllocnistis* 15 *citrella* (citrus leafminer), *Pieris brassicae* (large white butterfly), *Pieris rapae* (imported cabbageworm, or small white butterfly), *Plutella xylostella* (diamondback moth), *Spodoptera exigua* (beet armyworm), *Spodoptera litura* (tobacco cutworm, cluster caterpillar), and *Tuta absoluta* (tomato leafminer).

[0074] Reference in this application to an “isolated DNA molecule”, or an equivalent term or 20 phrase, is intended to mean that the DNA molecule is one that is present alone or in combination with other compositions, but not within its natural environment. For example, nucleic acid elements such as a coding sequence, intron sequence, untranslated leader sequence, promoter sequence, transcriptional termination sequence, and the like, that are naturally found within the DNA of the genome of an organism are not considered to be 25 “isolated” so long as the element is within the genome of the organism and at the location within the genome in which it is naturally found. However, each of these elements, and subparts of these elements, would be “isolated” within the scope of this disclosure so long as the element is not within the genome of the organism and at the location within the genome in which it is naturally found. Similarly, a nucleotide sequence encoding an insecticidal 30 protein or any naturally occurring insecticidal variant of that protein would be an isolated nucleotide sequence so long as the nucleotide sequence was not within the DNA of the bacterium from which the sequence encoding the protein is naturally found. A synthetic nucleotide sequence encoding the amino acid sequence of a naturally occurring insecticidal protein would be considered to be isolated for the purposes of this disclosure. For the

purposes of this disclosure, any transgenic nucleotide sequence, *i.e.*, the nucleotide sequence of the DNA inserted into the genome of the cells of a plant or bacterium, or present in an extrachromosomal vector, would be considered to be an isolated nucleotide sequence whether it is present within the plasmid or similar structure used to transform the cells, within the genome of the plant or bacterium, or present in detectable amounts in tissues, progeny, biological samples or commodity products derived from the plant or bacterium.

[0075] As described further in the Examples, repetitive rounds of engineering, testing and selecting of over two thousand (2000) amino acid sequence variants of TIC844 and Cry1Da1 resulted in the identification of certain amino acid residues that may be substituted, inserted or deleted from the given scaffold protein to produce engineered insecticidal proteins that exhibit an expanded Lepidopteran inhibitory spectrum and/or improved Lepidopteran inhibitory activity (*i.e.*, more toxic; less insecticidal protein required to obtain same level of mortality) when compared to the spectrum and activity of the baseline scaffold proteins, TIC844 or Cry1Da1. These repetitive rounds of engineering, testing and selecting also resulted in the identification of neutral amino acid residue substitutions, insertions or deletions in the TIC844 and Cry1Da1 scaffold proteins that do not change the proteins' insect inhibitory spectrum or activity. The specific amino acid residues in the TIC844 and Cry1Da1 scaffold that can be modified to yield an enhanced Lepidopteran inhibitory spectrum and/or improved Lepidopteran inhibitory activity relative to TIC844 or Cry1Da1 are identified herein. In certain embodiments, the engineered insecticidal protein provided herein can exhibit about an eight fold or greater Lepidopteran inhibitory activity against a Lepidopteran pest species than a scaffold protein of SEQ ID NO:14 (TIC844) or SEQ ID NO:2 (Cry1Da1).

[0076] The "engineering" in these repetitive rounds included identifying relevant residues in the scaffold protein to modify to create a modified test protein, and cloning and expressing the resultant modified test proteins. The atomic structure of the scaffold proteins was used to guide and complement semi-random approaches of selecting amino acid residues to modify for engineering. The "testing" in these repetitive rounds included comparing the Lepidopteran species activities of a modified test protein to its parent scaffold protein. The "selecting" in these repetitive rounds included identifying modified test proteins with improved activity (improved variants) and the relevant residues which were engineered to create these improved variants (these improved variants are referred to herein as "engineered insecticidal proteins").

[0077] Examples of methods for testing and selecting engineered insecticidal proteins include administering identical amounts of a modified test protein and of a scaffold protein (TIC844

or Cry1Da1) to an insect pest under controlled assay conditions and measuring and comparing the potency of the modified test and scaffold proteins. Another method for testing and selecting engineered insecticidal proteins includes determining the protein doses (*e.g.*, protein concentration in diet) of a modified test protein and of a scaffold protein (TIC844 or Cry1Da1) which elicit equivalent insect population responses under controlled assay conditions (*i.e.*, obtaining a dose response curve). A statistically robust dose response value used for comparison would be the median lethal concentration (LC₅₀) required to kill 50% of a test population or the molting inhibition concentration (“MIC₅₀”), the median concentration required to inhibit molting by 50%).

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- 10 **[0078]** In certain embodiments, the engineered insecticidal proteins described herein include at least one amino acid modification of the following relative positions of TIC844 (SEQ ID NO:14) or Cry1Da1 (SEQ ID NO:2): serine at position 282 replaced by lysine or valine, tyrosine at position 316 replaced by serine, isoleucine at position 368 replaced by proline or arginine, serine at 374 replaced by arginine, asparagine at position 375 replaced by histidine, and isoleucine at position 432 replaced by leucine. The engineered insecticidal proteins can also include at least two, three, four, or more of these amino acid substitutions or deletions within the same engineered insecticidal protein sequence.

- 15
- 20 **[0079]** The engineered insecticidal proteins that include these amino acid modifications include the proteins set forth as SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:44, and insect inhibitory fragments thereof. Each of these engineered insecticidal proteins has a measured mass of about 132k-Daltons. Individual characteristics of the insecticidal scaffold proteins TIC844 and Cry1Da1 and the engineered insecticidal proteins derived therefrom are reported in Table 1.
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Table 1. Characteristics of TIC844, Cry1Da1 and the Engineered Insecticidal Proteins.

<u>Protein</u> (Name/SEQ ID NO.)	<u>Molecular Weight</u> (in Daltons)	<u>Amino Acid Length</u>	<u>Isoelectric Point</u>	<u>Charge at PH 7.0</u>	<u>No. of Strongly Basic (-) Amino Acids</u>	<u>No. of Strongly Acidic Amino Acids</u>	<u>No. of Hydrophobic Amino Acids</u>	<u>No. of Polar Amino Acids</u>
Cry1Da1/ NO:2	132481.87	1165	5.087	-39.319	113	156	388	347
Cry1Da1_3 / NO:4	132405.77	1165	5.087	-39.318	113	156	388	347
Cry1Da1_4 / NO:6	132550.98	1165	5.112	-38.319	114	156	388	346
Cry1Da1_5 / NO:8	132448.80	1165	5.112	-38.318	114	156	387	347
Cry1Da1_6 / NO:10	132430.82	1165	5.112	-38.319	114	156	387	346
Cry1Da1_7 / NO:12	132401.78	1165	5.087	-39.318	113	156	388	346
TIC844/ NO:14	129182.91	1139	5.026	-39.540	110	153	382	340
TIC844_2/ NO:16	129129.85	1139	5.048	-39.373	110	153	382	339
TIC844_4/ NO:18	129106.81	1139	5.026	-39.539	110	153	382	340
TIC844_5/ NO:20	1291118.08	1069	5.325	-27.535	105	136	363	321
TIC844_6/ NO:22	129252.02	1139	5.050	-38.540	111	153	382	339
TIC844_7/ NO:24	129149.84	1139	5.050	-38.539	111	153	381	340

TIC844_8/ NO:26	129102.82	1139	5.026	-39.539	110	153	382	339
Cry1Da1/ NO:28	132481.87	1165	5.087	-39.319	113	156	388	347
Cry1Da1_2. nno/NO:30	132552.95	1166	5.087	-39.319	113	156	389	347
Cry1Da1_3. nno/NO:32	132476.85	1166	5.087	-39.318	113	156	389	347
Cry1Da1_4. nno/NO:34	132622.06	1166	5.112	-38.319	114	156	389	346
Cry1Da1_5. nno/NO:36	132519.88	1166	5.112	-38.318	114	156	388	347
Cry1Da1_6. nno/NO:38	132501.90	1166	5.112	-39.319	114	156	388	346
Cry1Da1_7. nno/NO:40	132472.86	1166	5.087	-39.318	113	156	389	346
TIC844_9.n no/NO:42	129253.99	1140	5.026	-39.540	110	153	383	340
TIC844_11. nno/NO:44	129173.90	1140	5.026	-39.539	110	153	383	339

[0080] Fragments of the engineered insecticidal proteins described herein can be truncated forms wherein one or more amino acids are deleted from the N-terminal end, C-terminal end, the middle of the protein, or combinations thereof without a loss of insect inhibitory activity.

- 5 These fragments should retain the insect inhibitory activity of the parent engineered insecticidal protein.

- [0081]** Proteins that resemble the engineered insecticidal proteins can be identified by comparison to each other using various computer based algorithms known in the art. For example, amino acid sequence identities of proteins related to the engineered insecticidal
- 10 proteins can be analyzed using a Clustal W alignment using these default parameters: Weight matrix: blosum, Gap opening penalty: 10.0, Gap extension penalty: 0.05, Hydrophilic gaps: On, Hydrophilic residues: GPSNDQERK, Residue-specific gap penalties: On (Thompson, *et al* (1994) Nucleic Acids Research, 22:4673-4680). Percent amino acid identity is further calculated by the product of 100% multiplied by (amino acid identities/length of subject
 - 15 protein). Other alignment algorithms are also available in the art and provide results similar to those obtained using a Clustal W alignment.

[0082] As described further in the Examples of this application, synthetic or artificial sequences encoding the scaffold proteins and the engineered insecticidal proteins were designed for use in plants. Exemplary synthetic nucleotide sequences that were designed for use in plants are set forth in SEQ ID NO:27 (Cry1Da1.nno), SEQ ID NO:29 (Cry1Da1_2.nno), SEQ ID NO:31 (Cry1Da1_3.nno), SEQ ID NO:33 (Cry1Da1_4.nno), SEQ ID NO:35 (Cry1Da1_5.nno), SEQ ID NO:37 (Cry1Da1_6.nno), SEQ ID NO:39 (Cry1Da1_7.nno), SEQ ID NO:41 (TIC844_9.nno) and SEQ ID NO:43 (TIC844_11.nno).

[0083] Expression cassettes and vectors containing these synthetic or artificial nucleotide sequences were constructed and introduced into corn, cotton and soybean plant cells in accordance with transformation methods and techniques known in the art. Transformed cells were regenerated into transformed plants that were observed to be expressing the engineered insecticidal protein or the scaffold protein. To test pesticidal activity, bioassays were performed in the presence of Lepidopteran pest larvae using plant leaf disks obtained from the transformed plants.

[0084] Recombinant nucleic acid molecule compositions that encode the engineered insecticidal proteins are contemplated. For example, an engineered insecticidal protein can be expressed with recombinant DNA constructs in which a polynucleotide molecule with an ORF encoding the engineered insecticidal protein is operably linked to genetic expression elements such as a promoter and any other regulatory element necessary for expression in the system for which the construct is intended. Non-limiting examples include a plant-functional promoter operably linked to the synthetic engineered insecticidal protein encoding sequences for expression of the engineered insecticidal protein in plants or a *Bt*-functional promoter operably linked to an engineered insecticidal protein encoding sequence for expression of the protein in a *Bt* bacterium or other *Bacillus* species. Other elements can be operably linked to the engineered insecticidal protein encoding sequences including, but not limited to, enhancers, introns, untranslated leaders, encoded protein immobilization tags (HIS-tag), translocation peptides (*i.e.*, plastid transit peptides, signal peptides), polypeptide sequences for post-translational modifying enzymes, ribosomal binding sites, and RNAi target sites. Exemplary recombinant polynucleotide molecules provided herein include, but are not limited to, a heterologous promoter operably linked to a polynucleotide such as SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39 and SEQ ID NO:43, that encodes the polypeptide or protein having the amino acid sequence as set forth in SEQ ID

NO:4 (Cry1Da1_3), SEQ ID NO:6 (Cry1Da1_4), SEQ ID NO:8 (Cry1Da1_5), SEQ ID NO:10 (Cry1Da1_6), SEQ ID NO:12 (Cry1Da1_7), SEQ ID NO:16 (TIC844_2), SEQ ID NO:18 (TIC844_4), SEQ ID NO:20 (TIC844_5), SEQ ID NO:22 (TIC844_6), SEQ ID NO:24 (TIC844_7), SEQ ID NO:26 (TIC844_8), SEQ ID NO:32 (Cry1Da1_3.nno), SEQ ID
 5 NO:34 (Cry1Da1_4.nno), SEQ ID NO:36 (Cry1Da1_5.nno), SEQ ID NO:38 (Cry1Da1_6.nno), SEQ ID NO:40 (Cry1Da1_7.nno) and SEQ ID NO:44 (TIC844_11.nno).

A heterologous promoter can also be operably linked to synthetic DNA coding sequences encoding a plastid targeted engineered insecticidal protein and untargeted engineered insecticidal protein. It is contemplated that the codons of a recombinant nucleic acid
 10 molecule encoding for an engineered insecticidal protein disclosed herein can be substituted by synonymous codons (known in the art as a silent substitution).

[0085] A recombinant DNA molecule or construct comprising an engineered insecticidal protein encoding sequence can further comprise a region of DNA that encodes for one or more toxic agents which can be configured to concomitantly express or co-express with a
 15 DNA sequence encoding an engineered insecticidal protein, a protein different from an engineered insecticidal protein, an insect inhibitory dsRNA molecule, or an ancillary protein. Ancillary proteins include, but are not limited to, co-factors, enzymes, binding-partners, or other agents that function to aid in the effectiveness of an insect inhibitory agent, for example, by aiding its expression, influencing its stability in plants, optimizing free energy
 20 for oligomerization, augmenting its toxicity, and increasing its spectrum of activity. An ancillary protein may facilitate the uptake of one or more insect inhibitory agents, for example, or potentiate the toxic effects of the toxic agent.

[0086] A recombinant DNA molecule or construct can be assembled so that all proteins or dsRNA molecules are expressed from one promoter or each protein or dsRNA molecule is
 25 under separate promoter control or some combination thereof. The proteins of this invention can be expressed from a multi-gene expression system in which an engineered insecticidal protein is expressed from a common nucleotide segment which also contains other open reading frames and promoters, depending on the type of expression system selected. For example, a bacterial multi-gene expression system can utilize a single promoter to drive
 30 expression of multiply-linked/tandem open reading frames from within a single operon (*i.e.*, polycistronic expression). In another example, a plant multi-gene expression system can utilize multiply-unlinked expression cassettes, each expressing a different protein or other toxic agent such as one or more dsRNA molecules.

[0087] Recombinant nucleic acid molecules or recombinant DNA constructs comprising an engineered insecticidal protein encoding sequence can be delivered to host cells by vectors, e.g., a plasmid, baculovirus, synthetic chromosome, virion, cosmid, phagemid, phage, or viral vector. Such vectors can be used to achieve stable or transient expression of an engineered insecticidal protein encoding sequence in a host cell, or subsequent expression of the encoded polypeptide. An exogenous recombinant polynucleotide or recombinant DNA construct that comprises an engineered insecticidal protein sequence encoding sequence and that is introduced into a host cell is referred herein as a “transgene”.

[0088] Transgenic bacteria, transgenic plant cells, transgenic plants, and transgenic plant parts that contain a polynucleotide that encodes any one or more of the engineered insecticidal proteins are provided herein. The term “bacterial cell” or “bacterium” can include, but is not limited to, an *Agrobacterium*, a *Bacillus*, an *Escherichia*, a *Salmonella*, a *Pseudomonas*, or a *Rhizobium* cell. The term “plant cell” or “plant” can include but is not limited to a dicotyledonous cell or a monocotyledonous cell. Contemplated plants and plant cells include, but are not limited to, alfalfa, banana, barley, bean, broccoli, cabbage, brassica, carrot, cassava, castor, cauliflower, celery, chickpea, Chinese cabbage, citrus, coconut, coffee, corn, clover, cotton, a cucurbit, cucumber, Douglas fir, eggplant, eucalyptus, flax, garlic, grape, hops, leek, lettuce, Loblolly pine, millets, melons, nut, oat, olive, onion, ornamental, palm, pasture grass, pea, peanut, pepper, pigeonpea, pine, potato, poplar, pumpkin, Radiata pine, radish, rapeseed, rice, rootstocks, rye, safflower, shrub, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugar beet, sugarcane, sunflower, sweet corn, sweet gum, sweet potato, switchgrass, tea, tobacco, tomato, triticale, turf grass, watermelon, and wheat plant cell or plant. In certain embodiments, transgenic plants and transgenic plant parts regenerated from a transgenic plant cell are provided. In certain embodiments, the transgenic plants can be obtained from a transgenic seed, by cutting, snapping, grinding or otherwise disassociating the part from the plant. In certain embodiments, the plant part can be a seed, a boll, a leaf, a flower, a stem, a root, or any portion thereof, or a non-regenerable portion of a transgenic plant part. As used in this context, a “non-regenerable” portion of a transgenic plant part is a portion that cannot be induced to form a whole plant or that cannot be induced to form a whole plant that is capable of sexual and/or asexual reproduction. In certain embodiments, a non-regenerable portion of a plant part is a portion of a transgenic seed, boll, leaf, flower, stem, or root.

[0089] Methods of making transgenic plants that comprise Lepidoptera-inhibitory amounts of an engineered insecticidal proteins are provided. Such plants can be made by introducing a

polynucleotide that encodes the engineered insecticidal proteins provided in this application into a plant cell, and selecting a plant derived from said plant cell that expresses an insect or Lepidoptera-inhibitory amount of the engineered insecticidal protein. Plants can be derived from the plant cells by regeneration, seed, pollen, or meristem transformation techniques.

5 Methods for transforming plants are known in the art.

[0090] Plants expressing the engineered insecticidal proteins can be crossed by breeding with transgenic events expressing other insecticidal proteins and/or expressing other transgenic traits such as other insect control traits, herbicide tolerance genes, genes conferring yield or stress tolerance traits, and the like, or such traits can be combined in a single vector so that
10 the traits are all linked.

[0091] Processed plant products, wherein the processed product comprises a detectable amount of an engineered insecticidal protein, an insect inhibitory segment or fragment thereof, or any distinguishing portion thereof, are also disclosed in this application. In certain embodiments, the processed product is selected from the group consisting of plant parts, plant
15 biomass, oil, meal, sugar, animal feed, flour, flakes, bran, lint, hulls, processed seed, and seed. In certain embodiments, the processed product is non-regenerable. The plant product can comprise commodity or other products of commerce derived from a transgenic plant or transgenic plant part, where the commodity or other products can be tracked through commerce by detecting nucleotide segments or expressed RNA or proteins that encode or
20 comprise distinguishing portions of an engineered insecticidal protein.

[0092] Methods of controlling insects, in particular Lepidoptera infestations of crop plants, with the engineered insecticidal proteins are also disclosed in this application. Such methods can comprise growing a plant comprising an insect- or Lepidoptera-inhibitory amount of the engineered insecticidal protein. In certain embodiments, such methods can further comprise
25 any one or more of: (i) applying any composition comprising or encoding an engineered insecticidal protein to a plant or a seed that gives rise to a plant; and (ii) transforming a plant or a plant cell that gives rise to a plant with a polynucleotide encoding an engineered insecticidal protein. In general, it is contemplated that engineered insecticidal protein can be provided in a composition, provided in a microorganism, or provided in a transgenic plant to
30 confer insect inhibitory activity against Lepidopteran insects.

[0093] In certain embodiments, the engineered insecticidal protein is the insecticidally active ingredient of an insect inhibitory composition prepared by culturing recombinant *Bacillus* or any other recombinant bacterial cell transformed to express an engineered insecticidal protein under conditions suitable for expression. Such a composition can be prepared by desiccation,

- lyophilization, homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of such recombinant cells expressing/producing the engineered insecticidal protein. Such a process can result in a *Bacillus* or other entomopathogenic bacterial cell extract, cell suspension, cell homogenate, cell lysate, cell supernatant, cell filtrate, or cell pellet. By obtaining the engineered insecticidal protein so produced, a composition that includes the engineered insecticidal protein can include bacterial cells, bacterial spores, and parasporal inclusion bodies and can be formulated for various uses, including as agricultural insect inhibitory spray products or as insect inhibitory formulations in diet bioassays.
- 5
- 10 **[0094]** In an embodiment, in order to reduce the likelihood of resistance development, an insect inhibitory composition or transgenic plant comprising an engineered insecticidal protein can further comprise at least one additional toxic agent that exhibits insect inhibitory activity against the same Lepidopteran insect species, but which is different from the engineered insecticidal protein. Possible additional toxic agents for such a composition
- 15 include an insect inhibitory protein and an insect inhibitory dsRNA molecule. One example for the use of such ribonucleotide sequences to control insect pests is described in Baum, *et al.* (U.S. Patent Publication 2006/0021087 A1). Such additional polypeptide(s) for the control of Lepidopteran pests may be selected from the group consisting of an insect inhibitory protein, such as, but not limited to, Cry1A (U.S. Patent No. 5,880,275), Cry1Ab,
- 20 Cry1Ac, Cry1A.105, Cry1Ae, Cry1B (U.S. Patent Publication No. 10/525,318), Cry1C (U.S. Patent No. 6,033,874), Cry1D, Cry1E, Cry1F, and Cry1A/F chimeras (U.S. Patent Nos. 7,070,982; 6,962,705; and 6,713,063), Cry1G, Cry1H, Cry1I, Cry1J, Cry1K, Cry1L, Cry2A, Cry2Ab (U.S. Patent No. 7,064,249), Cry2Ae, Cry4B, Cry6, Cry7, Cry8, Cry9, Cry15, Cry43A, Cry43B, Cry51Aa1, ET66, TIC400, TIC800, TIC834, TIC1415, Vip3A, VIP3Ab,
- 25 VIP3B, AXMI-001, AXMI-002, AXMI-030, AXMI-035, AND AXMI-045 (U.S. Patent Publication 2013-0117884 A1), AXMI-52, AXMI-58, AXMI-88, AXMI-97, AXMI-102, AXMI-112, AXMI-117, AXMI-100 (U.S. Patent Publication 2013-0310543 A1), AXMI-115, AXMI-113, AXMI-005 (U.S. Patent Publication 2013-0104259 A1), AXMI-134 (U.S. Patent Publication 2013-0167264 A1), AXMI-150 (U.S. Patent Publication 2010-0160231
- 30 A1), AXMI-184 (U.S. Patent Publication 2010-0004176 A1), AXMI-196, AXMI-204, AXMI-207, AXMI-209 (U.S. Patent Publication 2011-0030096 A1), AXMI-218, AXMI-220 (U.S. Patent Publication 2014-0245491 A1), AXMI-221z, AXMI-222z, AXMI-223z, AXMI-224z, AXMI-225z (U.S. Patent Publication 2014-0196175 A1), AXMI-238 (U.S. Patent Publication 2014-0033363 A1), AXMI-270 (U.S. Patent Publication 2014-0223598 A1),

AXMI-345 (U.S. Patent Publication 2014-0373195 A1), DIG-3 (U.S. Patent Publication 2013-0219570 A1), DIG-5 (U.S. Patent Publication 2010-0317569 A1), DIG-11 (U.S. Patent Publication 2010-0319093 A1), AfIP-1A and derivatives thereof (U.S. Patent Publication 2014-0033361 A1), AfIP-1B and derivatives thereof (U.S. Patent Publication 2014-0033361 A1), PIP-1A/PIP-1B (U.S. Patent Publication 2014-0007292 A1), PSEEN3174 (U.S. Patent Publication 2014-0007292 A1), AECFG-592740 (U.S. Patent Publication 2014-0007292 A1), Pput_1063 (U.S. Patent Publication 2014-0007292 A1), Pput_1064 (U.S. Patent Publication 2014-0007292 A1), GS-135 and derivatives thereof (U.S. Patent Publication 2012-0233726 A1), GS153 and derivatives thereof (U.S. Patent Publication 2012-0192310 A1), GS154 and derivatives thereof (U.S. Patent Publication 2012-0192310 A1), GS155 and derivatives thereof (U.S. Patent Publication 2012-0192310 A1), SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2012-0167259 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2012-0047606 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2011-0154536 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2011-0112013 A1, SEQ ID NO:2 and 4 and derivatives thereof as described in U.S. Patent Publication 2010-0192256 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2010-0077507 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2010-0077508 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent Publication 2009-0313721 A1, SEQ ID NO:2 or 4 and derivatives thereof as described in U.S. Patent Publication 2010-0269221 A1, SEQ ID NO:2 and derivatives thereof as described in U.S. Patent No. 7,772,465 (B2), CF161_0085 and derivatives thereof as described in WO2014/008054 A2, Lepidopteran toxic proteins and their derivatives as described in US Patent Publications US2008-0172762 A1, US2011-0055968 A1, and US2012-0117690 A1; SEQ ID NO:2 and derivatives thereof as described in US7510878(B2), SEQ ID NO:2 and derivatives thereof as described in U.S. Patent No. 7812129(B1); and the like.

[0095] In other embodiments, an insect inhibitory composition or transgenic plant can further comprise at least one additional toxic agent that exhibits insect inhibitory activity to an insect pest that is not inhibited by the engineered insecticidal proteins of the present invention (such as Coleopteran, Hemipteran and Homopteran pests), in order to expand the spectrum of insect inhibition obtained.

[0096] Such additional toxic agent for the control of Coleopteran pests may be selected from the group consisting of an insect inhibitory protein, such as, but not limited to, Cry3Bb (U.S. Patent No. 6,501,009), Cry1C variants, Cry3A variants, Cry3, Cry3B, Cry34/35, 5307,

AXMI134 (U.S. Patent Publication 2013-0167264 A1) AXMI-184 (U.S. Patent Publication 2010-0004176 A1), AXMI-205 (U.S. Patent Publication 2014-0298538 A1), axmi207 (U.S. Patent Publication 2013-0303440 A1), AXMI-218, AXMI-220 (U.S. Patent Publication 20140245491A1), AXMI-221z, AXMI-223z (U.S. Patent Publication 2014-0196175 A1),
 5 AXMI-279 (U.S. Patent Publication 2014-0223599 A1), AXMI-R1 and variants thereof (U.S. Patent Publication 2010-0197592 A1, TIC407, TIC417, TIC431, TIC807, TIC853, TIC901, TIC1201, TIC3131, DIG-10 (U.S. Patent Publication 2010-0319092 A1), eHIPs (U.S. Patent Application Publication No. 2010/0017914), IP3 and variants thereof (U.S. Patent Publication 2012-0210462 A1), and α -Hexatoxin-Hv1a (U.S. Patent Application Publication US2014-
 10 0366227 A1).

[0097] Such additional toxic agent for the control of Hemipteran pests may be selected from the group consisting of Hemipteran-active proteins such as, but not limited to, TIC1415 (US Patent Publication 2013-0097735 A1), TIC807 (U.S. Patent No. 8609936), TIC834 (U.S. Patent Publication 2013-0269060 A1), AXMI-036 (U.S. Patent Publication 2010-0137216
 15 A1), and AXMI-171 (U.S. Patent Publication 2013-0055469 A1). Additional polypeptides for the control of Coleopteran, Lepidopteran, and Hemipteran insect pests can be found on the *Bacillus thuringiensis* toxin nomenclature website maintained by Neil Crickmore (on the world wide web at btnomenclature.info).

[0098] Engineered insecticidal protein-encoding sequences and sequences having a
 20 substantial percentage identity to the engineered insecticidal proteins can be identified using methods known to those of ordinary skill in the art such as polymerase chain reaction (PCR), thermal amplification and hybridization. For example, the engineered insecticidal proteins can be used to produce antibodies that bind specifically to related proteins, and can be used to screen for and to find other proteins that are closely related.

[0099] Furthermore, nucleotide sequences encoding the engineered insecticidal proteins can be used as probes and primers for screening to identify other members of the class using thermal-cycle or isothermal amplification and hybridization methods. For example, oligonucleotides derived from sequences as set forth in SEQ ID NO: 3 can be used to determine the presence or absence of an engineered insecticidal transgene in a
 25 deoxyribonucleic acid sample derived from a commodity product. Given the sensitivity of certain nucleic acid detection methods that employ oligonucleotides, it is anticipated that oligonucleotides derived from sequences as set forth in any of SEQ ID NO: 3 can be used to detect the respective engineered insecticidal protein in commodity products derived from
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pooled sources where only a fraction of the commodity product is derived from a transgenic plant containing any of SEQ ID NO: 3.

[0100] Other features and advantages of the invention will be apparent from the following Examples and claims.

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EXAMPLES

[0101] In view of the foregoing, those of skill in the art should appreciate that changes can be made in the specific aspects which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. Thus, specific structural and functional details disclosed herein are not to be interpreted as limiting. It should be
10 understood that the entire disclosure of each reference cited herein is incorporated within the disclosure of this application.

EXAMPLE 1

Design of Modified Test Proteins and Sample Preparation for Insect Bioassay Testing

[0102] This Example illustrates the methods undertaken to identify relevant amino acid
15 residues in the scaffold proteins to modify to create modified test proteins, and the cloning and expressing of the resultant modified test proteins.

[0103] Several molecular engineering techniques were employed in a tiered approach to construct improved variants of Cry1Da1 having an enhanced Lepidopteran inhibitory spectrum and/or improved Lepidopteran inhibitory activity compared to the scaffold proteins of Cry1Da1 and TIC844, a homolog of Cry1Da1. The first tier, or initial round of design,
20 was primarily hypothesis driven. The second and third tiers were statistically-driven rounds of design. For example, in the second tier of design, statistically non-deleterious mutations were combined with putative beneficial mutations to produce double mutations which satisfied defined statistical criteria. In the third tier of design, all the data from the previous
25 tests was analyzed using multiple statistical methods. Only mutations showing statistically significant improvement in more than one statistical method were selected to the final pool of mutations. The variants designed in this tier contained one or two more positive mutations from variants previously confirmed positive. Thus, the third tier design significantly enriched the active variants compared to the first and second tier. As demonstrated in the subsequent
30 Examples, the use of the three-tiered design strategy identified both single and synergistic

mutations that provided significant improvement in activity against CEW for certain improved variants relative to the TIC844 and Cry1Da1 scaffolds.

[0104] The methods which were utilized to create the modified test proteins included, but were not limited to, semi-random modifications, directed modifications of variances in alignment of TIC844/Cry1Da1 with other native *Bacillus thuringiensis* (*Bt*) proteins, and structure/function assisted design. Examples of utilized molecular engineering techniques include the following.

[0105] *Receptor binding.* Susceptibility of Lepidopteran pests, specifically Corn Earworm (CEW, *Helicoverpa zea*) to Cry1Da1/TIC844 improved variants may be attributable to different targeted gut receptors. Designs which were utilized to improve binding to receptors in the gut, thus increasing toxicity, included: (1) mutating every position in the apical loops of domain II to all amino-acid types; and (2) swapping all possible combinations of the apical loops of domain II with those from other Cry1Da1 homologs (*e.g.*, Cry1Db1, Cry1Dc1) and CEW-active three-domain toxins (*e.g.*, Cry1Bb1, Cry1Ja1 and Cry2Ab2).

[0106] *Alignment based approaches.* Alignment of Cry1Da1 with other homologs (*e.g.*, Cry1Db1 and Cry1Dc1) was used to identify regions of variability. As a result of the alignment, one hundred fifty (150) positions and two hundred ninety five (295) unique single mutations were identified. These positions were located throughout the three domains. Positions within four (4) amino acids from one another were grouped together. Only mutations from the same parental sequences were nominated for every group of positions, rendering one hundred thirty two (132) unique variants.

[0107] *Surface mutagenesis approaches.* The polynucleotides encoding the surface positions in domains II and III of the scaffold proteins were mutagenized by a scan. Amino acid residues were modified to alanine where an alanine was not already present in the scaffold protein. At surface positions where the native residues were lysine, arginine mutations were introduced in addition to the alanine mutations. The rationale for the lysine to arginine mutations was based on the observation that Lepidopteran-active toxins tend to have very few lysine and many arginine and, therefore, it was hypothesized that changing the surface lysine positions in domains II and III to arginine would increase the Lepidopteran activity of the modified test protein.

[0108] *Alteration of proteolytic events.* The proteolytic process was hypothesized to be an important aspect of the activity of three-domain toxins in the Lepidopteran insect guts. In order to test this, several sets of mutations were made to potentially alter any proteolytic cleavage. Potential cleavage sites are located at the N-terminus and between domain III and

the protoxin. The mutational positions included predicted loop regions from the N-terminus to the beginning of helix 4 and from the C-terminus of domain III to ~40 amino acids into the protoxin. Generally, glycine residues were hypothesized to promote proteolysis either through proteolytic site recognition or by increasing the protein flexibility, thereby rendering it more susceptible to proteolytic cleavage. Further, trypsin and chymotrypsin are two proteases that are widely accepted as viable proteases in Lepidopteran midguts. Lysine residues provide recognition sites for trypsin and tyrosine residues provide recognition sites for chymotrypsin. Thus, selected mutational positions in the potential cleavage sites were mutated to either glycine, lysine or tyrosine.

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- 10 [0109] *Potential hot-spot mutations from other CEW-active toxins.* Activity and absence of activity data against CEW for a large set of proteins (including chimeras, fragments and native sequences) was analyzed. Information gained from a statistical analysis of this data was utilized to identify potential specific mutations or positions for mutation that would be likely to increase CEW activity in the resultant modified test proteins.
- 15 [0110] The modified test proteins which resulted from the molecular engineering methodologies described above were cloned using methods known in the art into a recombinant *Bt* plasmid expression vector downstream of a sporulation specific expression promoter and transformed into an acrySTALLIFEROUS *Bt* host cell.

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EXAMPLE 2

Testing of Modified Test Proteins in Diet Bioassays Against Lepidopteran Pests

[0111] This Example illustrates the testing of the modified test proteins created from the engineering efforts described in Example 1.

- 25 [0112] From the engineering efforts described in Example 1, about two thousand five hundred (2,500) recombinant *Bt* strains were produced which expressed more than two thousand three hundred (2,300) different modified test proteins. These modified test proteins were expressed in *Bt* and assayed for toxicity to various species of Lepidoptera. Feeding assays were conducted with neonate larvae (<24 hour post hatch) of various Lepidopteran species, including corn earworm (CEW, *Helicoverpa zea*) and fall armyworm (FAW, *Spodoptera frugiperda*). Insect eggs for the CEW testing were obtained from two different laboratory colonies: Benson Research, Carlisle, PA and Monsanto Company, Union City, TN. All of the expressed modified test proteins were tested on CEW and some of those modified test proteins demonstrating improved activity against CEW compared to their
- 30

parent scaffold proteins were tested on FAW, in addition to performing additional bioassays to confirm CEW activity.

- [0113] Various protocols for bioassays and scoring insects for mortality and stunting are known in the art. Variations of methods, such as those described in PCT Patent Application
5 Publication No. WO 2012/139004 and in U.S. Patent No. 7,927,598, were used.

EXAMPLE 3

Modified Test Proteins Exhibiting Improved CEW Activity

- 10 [0114] This Example illustrates the discovery of an enhanced Lepidopteran inhibitory spectrum and/or improved or greater Lepidopteran inhibitory activity for some of the modified test proteins when compared to the activities of the scaffold TIC844 or Cry1Da1 proteins in multiple testing rounds.

- [0115] The modified test proteins created from the engineering efforts described in Example
15 1 and tested in insect bioassay as described in Example 2 were tested in repetitive rounds in which the Lepidopteran species activities of the modified test proteins were compared to their respective parent scaffold proteins (*i.e.*, TIC844 or Cry1Da1). In a first round, three hundred and seventy (370) different modified test proteins demonstrated increased toxicity against CEW relative to TIC844 or Cry1Da1 in diet bioassays. In each of these diet
20 bioassays, identical amounts of the protein (either modified test protein or scaffold protein) was provided to CEW under controlled single-dose assay conditions. The potency of the modified test proteins and scaffold proteins was determined by measuring and comparing the observed mortality and stunting of each of the modified test protein bioassays to the observed mortality and stunting of the parent scaffold protein bioassays.

- 25 [0116] Of the three hundred and seventy (370) modified test proteins which demonstrated increased toxicity against CEW when compared to the scaffold proteins in single-dose assay screens, about one hundred eighty (180) of them were further tested in FAW bioassays to determine whether these modified test proteins maintained or exhibited increased FAW activity compared to their scaffold protein parents. About forty (40) to fifty (50) of these
30 modified test proteins exhibited similar or better FAW activity than their parent scaffold proteins. These further-screened modified test proteins were also tested in additional CEW bioassays to confirm CEW activity. These rounds of selecting and testing modified test proteins which demonstrated improved CEW activity while maintaining or improving FAW activity resulted in a final list of improved variants (referred to herein as the “engineered

- insecticidal proteins”). Table 2 identifies these engineered insecticidal proteins and the amino acid mutations in each engineered insecticidal protein. Table 2 also demonstrates the activity of the scaffold and the engineered insecticidal proteins against CEW and FAW (insecticidal activity is demonstrated in LC₅₀ value (the toxin concentration required to kill 50% of an insect population during a fixed exposure duration. The lower the LC₅₀ value, the greater the toxicity) and the MIC₅₀ value (the concentration required to inhibit molting to a specific instar of 50% of the larvae during a fixed exposure duration). This Table demonstrates that the engineered insecticidal proteins have improved CEW-activity, while maintaining or improving FAW activity.

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Table 2. Amino Acid Mutations and Activity Data for Scaffold Proteins and Engineered Insecticidal Proteins.

<u>Protein</u> (Name/SEQ ID NO.)	<u>Amino Acid Mutations*</u>	LC ₅₀ ($\mu\text{g}/\text{cm}^2$) against CEW Benzon colony with spore-crystal bioassay prep	MIC ₅₀ ($\mu\text{g}/\text{cm}^2$) against CEW Benzon colony with spore-crystal bioassay prep
Cry1Da1/ NO:2, 28	None (scaffold protein)	NA**	~3.0
Cry1Da1_3/ NO:4	Cry1Da1 + Y316S	NA**	NA**
Cry1Da1_4/ NO:6	Cry1Da1 + S374R	NA**	NA**
Cry1Da1_5/ NO:8	Cry1Da1 + Y316S_I368R	NA**	NA**
Cry1Da1_6/ NO:10	Cry1Da1 + S282K_Y316S_I368P	NA**	NA**
Cry1Da1_7/ NO:12	Cry1Da1 + S282V_Y316S_I368P	NA**	NA**

TIC844/ NO:14	None (scaffold protein)	41.90	3.73
TIC844_2/ NO:16	TIC844+Y316S_N375H_I432L	0.81	0.65
TIC844_4/ NO:18	TIC844+Y316S	0.98	0.57
TIC844_5/ NO:20	TIC844+S282K_Y316S_I368P	0.32	0.33
TIC844_6/ NO:22	TIC844+S374R	4.09	1.39
TIC844_7/ NO:24	TIC844+Y316S_I368R	0.93	0.61
TIC844_8/ NO:26	TIC844+ S282V_Y316S_I368P	0.221	.064

*The amino acid mutations are identified using the standard IUPAC amino acid code. See IUPAC-IUB Joint Commission on Biochemical Nomenclature. Nomenclature and Symbolism for Amino Acids and Peptides. Eur. J. Biochem. 138:9-37(1984). The first amino acid sequence abbreviation indicates the original amino acid in the given scaffold protein, the number represents the position of the amino acid, and the second amino acid sequence abbreviation indicates the amino acid placed in that position in the improved variant protein.

**The core toxin of CryIDa1 is identical to the core toxin of TIC844.

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[0117] Further demonstrating the enhanced Lepidopteran inhibitory spectrum and improved Lepidopteran inhibitory activity of the engineered insecticidal proteins, the lethality of engineered insecticidal protein TIC844_8 relative to its parent scaffold protein is demonstrated in Figure 1. The bar chart of Figure 1 demonstrates the MIC₅₀ values of TIC844_8 compared to the scaffold protein TIC844 for two different CEW colonies, Union City and Benzon. The bioassay results depicted in Figure 1 were calculated from sucrose gradient-purified bioassay preparations. The reason these secondary bioassays were run with sucrose gradient-purified preparation of the proteins opposed to spore-crystal preparations of the proteins was to ensure that the improved activity of TIC844_8 persisted with more extensive purification. Further, the Union City colony was tested to confirm the improved activity observed on the Benzon colony. As demonstrated in Figure 1, the mutations in three residues for TIC844_8 (S282V_Y316S_I368P), imparted an 8-fold improvement in CEW

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lethality, relative to TIC844, for the Union City colony and a 50-fold improvement in CEW lethality, relative to TIC844, for the Benzon colony.

[0118] Even further demonstrating enhanced Lepidopteran inhibitory spectrum and improved Lepidopteran inhibitory activity of the engineered insecticidal proteins, the insect activity profiles for TIC844 and TIC844_8 from diet bioassay studies, conducted against a broad spectrum of Lepidopteran insect species, are shown in Table 3. The insects tested against in the bioassay studies in Table 3 include black cutworm (BCW, *Agrotis ipsilon*), corn earworm (CEW, *Helicoverpa zea*), fall armyworm (FAW, *Spodoptera frugiperda*), southern armyworm (SAW, *Spodoptera eridania*), cabbage looper (CLW, *Trichoplusia ni*), European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWC, *Diatraea grandiosella*), tobacco budworm (TBW, *Heliothis virescens*), velvetbean caterpillars (VBC, *Anticarsia gemmatilis*), soybean looper (SBL, *Chrysodeixis includes*), and sugarcane borer (SCB, *Diatraea saccharalis*). This Table 3 demonstrates the enhanced Lepidopteran inhibitory spectrum of TIC844_8 compared to the parent scaffold protein TIC844, specifically with improved activity against CEW and VBC.

Table 3. Insect activity spectrum for TIC844 and TIC844_8.

SEQ ID NO.	Protein	BCW	CEW	FAW	SAW	CLW	ECB	SWC	TBW	VBC	SBL	SCB
26	TIC844_8		*	*	*	*				*	*	*
14	TIC844			*	*	*					*	*

* Active against the indicated insect species.

[0119] The enhanced Lepidopteran inhibitory spectrum of the engineered insecticidal proteins is further demonstrated in Table 4 which depicts the insect activity profile for certain engineered insecticidal proteins from diet bioassay studies. The insects tested against in the bioassay studies in Table 4 include Old World cotton bollworm (CBW, *Helicoverpa armigera*), tobacco cutworm (TCW, *Spodoptera litura*), beet armyworm (BAW, *Spodoptera exigua*), pink bollworm (PBW, *Pectinophora gossypiella*), pink stem borer (PSB, *Sesamia inferens*) and spotted bollworm (SBW, *Earias vitella*). The results depicted in Table 4 demonstrates the enhanced Lepidopteran inhibitory spectrum of the listed engineered

insecticidal proteins compared to the scaffold protein Cry1Da1, specifically with improved activity against CBW, PBW (Cry1Ac resistant), PBW (field collected) and SBW.

5 **Table 4. Insect Activity Profile Comparison for Cry1Da1 and Engineered Insecticidal Proteins.**

SEQ ID NO.	Protein	CBW	TCW	BAW	PBW (Lab raised)	PBW (Cry1Ac resistant)	PBW (Field collected)	PSB	SBW
2	Cry1Da1		+	+	+	+	+	+	
12	Cry1Da1_7	+	+	+	+	+	+		+
18	TIC844_4	+	+	+	+	+			+
20	TIC844_5	+	+	+	+	+			+
24	TIC844_7	+	+	+	+	+			

+ Active against the indicated insect species.

EXAMPLE 4

Synthesis of genes encoding engineered insecticidal proteins and scaffold proteins for expression in plants

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[0120] This Example illustrates the synthesis of polynucleotides encoding engineered insecticidal proteins and scaffold proteins for expression in plants.

[0121] Nucleotide sequences encoding scaffold proteins and engineered insecticidal proteins for expression in plants were designed and synthesized according to methods generally described in U.S. Patent No. 5,500,365, avoiding certain inimical problem sequences such as ATTTA and A/T rich plant polyadenylation sequences while preserving the amino acid sequence of the original scaffold or engineered insecticidal protein. The nucleotide sequences for these genes encoding engineered insecticidal proteins and scaffold proteins for expression in plants are listed below in Table 5.

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Table 5. Polynucleotide Sequences Designed for Use in Plants Encoding Scaffold and Engineered Insecticidal Proteins.

NUCLEOTIDE SEQ ID NO.	PROTEIN	VARIANT
27	Cry1Da1.nno	None (scaffold protein)
29	Cry1Da1_2.nno	Cry1Da1 + A2**
31	Cry1Da1_3.nno	Cry1Da1 + Y316S + A2
33	Cry1Da1_4.nno	Cry1Da1 + S374R + A2
35	Cry1Da1_5.nno	Cry1Da1 + S374R + A2
37	Cry1Da1_6.nno	Cry1Da1 + S282K_Y316S_I368P + A2
39	Cry1Da1_7.nno	Cry1Da1 + S282V_Y316S_I368P + A2
41	TIC844_9.nno	TIC844 + A2
43	TIC844_11.nno	TIC844 + S282V_Y316S_I368P + A2

**Variant designation “A2” indicates insertion of an alanine residue at amino acid position 2 compared to the native sequence for cloning purposes into plant expression vectors.

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EXAMPLE 5

Expression cassettes for expression of engineered insecticidal proteins in plants

[0122] This Example illustrates the construction of expression cassettes comprising polynucleotide sequences designed for use in plants which encode scaffold and engineered insecticidal proteins.

[0123] A variety of plant expression cassettes were constructed with the polynucleotide sequences encoding scaffold and engineered insecticidal proteins designed for plant expression provided in Table 5. Such expression cassettes are useful for transient expression in plant protoplasts or transformation of plant cells. Typical expression cassettes were designed with respect to the eventual placement of the protein within the cell. One set of expression cassettes was designed in a manner to allow the protein to be translated and remain in the cytosol. Another set of expression cassettes was designed to have a transit peptide contiguous with the toxin protein to allow targeting to an organelle of the cell such as

the chloroplast or plastid. All expression cassettes were designed to begin at the 5' end with a promoter, which can be comprised of multiple promoter elements, enhancer elements, or other expression elements known to those of ordinary skill in the art operably linked to boost the expression of the transgene. The promoter sequence was usually followed contiguously with one or more leader sequences 3' to the promoter. An intron sequence was usually provided 3' to the leader sequence to improve expression of the transgene. A coding sequence for the toxin or transit peptide and coding sequence for the toxin was usually located 3' to the operably linked promoter, leader and intron configuration. A 3'UTR sequence was usually provided 3' of the coding sequence to facilitate termination of transcription and to provide sequences important for the polyadenylation of the resulting transcript. All of the elements described above were operably linked and arranged sequentially, often with additional sequences provided for the construction of the expression cassette.

EXAMPLE 6

Transformation vectors containing a scaffold or engineered insecticidal protein expression cassette

[0124] This Example illustrates the incorporation of scaffold or engineered insecticidal proteins into plant tissues.

[0125] Methods for producing a transgenic plant which expresses a nucleic acid segment encoding a scaffold protein or an engineered insecticidal protein can be done utilizing variations of methods 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 one or more of the engineered insecticidal proteins or scaffold proteins. 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 polypeptide *in vivo*. Vectors, plasmids, cosmids, and DNA segments for use in transforming such cells will generally comprise operons, genes, or gene-derived sequences, either native or synthetically-derived, and particularly those encoding the disclosed engineered insecticidal proteins. These DNA constructs can further include structures such as promoters, enhancers, polylinkers, or other gene sequences which can have regulating activity upon the particular genes of interest. The resultant transgenic plant, plant parts and plant cells are tested for the expression and bioactivity of the encoded protein.

[0126] Examples of methods which can be modified for obtaining transgenic plants that express Lepidopteran-active proteins include those describing, for example, Cry1A proteins (U.S. Patent No. 5,880,275), Cry1B (U.S. Patent Application No. 10/525318), Cry1C (U.S. Patent No. 6,033,874), Cry1A/F chimeras (U.S. Patent Nos. 7,070,982; 6,962,705, and 5 6,713,063), and a Cry2Ab protein (U.S. Patent No. 7,064,249).

EXAMPLE 7

Lepidopteran activity of engineered insecticidal proteins in stably transformed corn

10 [0127] This Example illustrates the inhibitory activity exhibited by the engineered insecticidal proteins against Lepidopteran pests when expressed in corn plants and provided as a diet to the respective insect pest.

[0128] R0 transgenic corn plants expressing Cry1Da1 and Cry1Da1_7.nno proteins were produced using vectors containing the expression cassettes described in Example 6. F1 15 transgenic corn plants were grown from seed produced by pollinating ears of non-transformed wild-type commercial germplasm plants with pollen from R0 transformants.

[0129] The transformed cells were induced to form plants by methods known in the art. Bioassays using plant leaf disks were performed analogous to those described in U.S. Patent No. 8,344,207. A non-transformed plant was used to obtain tissue for a negative control. 20 Multiple transformation events from each binary vector were assessed, and the results were tabulated.

[0130] The insecticidal activity of transgenic corn plants expressing Cry1Da1 and Cry1Da1_7.nno proteins at F1 and R0 is provided in Table 6, in addition to activity against transgenic corn plants expressing Cry1Da1 and Cry1Da1_7.nno proteins at F1 in the field. 25 Specifically, Table 6 demonstrates the Lepidopteran activity profile for Cry1Da1_7.nno compared to the parent scaffold protein Cry1Da1 when tested against CEW, FAW, and SWC. As can be seen in Table 6, unlike Cry1Da1, Cry1Da1_7.nno demonstrates activity against both CEW and FAW in R0 and F1 bioassay and F1 field tests.

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Table 6. Insect activity profile for Cry1Da1 and Cry1Da1_7.nno expressed in corn plants.

Protein (SEQ ID NO.)	CEW			FAW			SWC		
	R0	F1	Field	R0	F1	Field	R0	F1	Field
Cry1Da1 (28)	-	NT	NT	+	NT	NT	-	NT	NT
Cry1Da1_7.nno (40)	+	+	+	+	+	+	-	-	-

+ Active against insect species; - Inactive against insect species; NT Not Tested

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EXAMPLE 8

Lepidopteran activity of engineered insecticidal proteins in stably transformed cotton

[0131] This Example illustrates the inhibitory activity exhibited by the engineered insecticidal proteins against Lepidopteran pests when expressed in cotton plants and provided as a diet to the respective insect pest.

[0132] Cotton plants expressing Cry1Da1_7.nno and TIC844_11.nno proteins were produced using vectors containing the expression cassettes described in Example 6. The transformed cells were induced to form plants by methods known in the art. Cotton leaf tissue was used in bioassay as described in Example 7 and tested against CBW, FAW, Tobacco budworm (TBW, *Heliothis virescens*), and SBL. Table 7 shows the activity observed against these Lepidopteran species in stably transformed R₀ generation cotton. As can be seen in Table 7, Cry1Da1_7.nno and TIC844_11.nno demonstrated activity against two or more Lepidopteran pest species in stably transformed R₀ generation cotton.

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Table 7. Bioassay activity profile of Cry1Da1_7.nno, and TIC844_11.nno expressed in R₀ generation cotton.

Toxin	CBW	FAW	TBW	SBL
Cry1Da1_7.nno (SEQ ID NO:40)	+	+	+	+
TIC844_11.nno (SEQ ID NO:44)	+	+	-	+

+ Active against insect species; - Inactive against insect species.

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[0133] Selected transformed events were used to produce R₁ plants. R₁ plants expressing Cry1Da1_7.nno were assayed for resistance to CBW, FAW and SBL. Leaf, square and boll tissues were used in bioassay, in addition to field tests conducted in screenhouses. Table 8 shows the activity observed in these tests. As demonstrated in Table 8, Cry1Da1_7.nno demonstrated activity against CBW, FAW and SBL in bioassay and field tests.

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Table 8. Insect activity profile of Cry1Da1_7.nno expressed in R₁ generation cotton.

Toxin	CBW			FAW			SBL	Screenhouse	
	Leaf	Square	Boll	Leaf	Square	Boll	Leaf	CBW	FAW
Cry1Da1_7.nno (SEQ ID NO:40)	+	+	+	+	+	+	+	+	+

+ Active against insect species; - Inactive against insect species.

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EXAMPLE 9

Lepidopteran activity of engineered insecticidal proteins in stably transformed soybean

[0134] This Example illustrates the inhibitory activity exhibited by the engineered insecticidal proteins against Lepidopteran pests when expressed in soybean plants and provided as a diet to the respective insect pest.

[0135] Soybean plants expressing Cry1Da1_7.nno, TIC844_9.nno and TIC844_11.nno proteins were produced using vectors containing the expression cassettes described in Example 6. Leaf tissue was harvested and used in bioassay as described in Example 7 or, alternatively, lyophilized tissue was used in the insect diet for bioassay. Bioassay was

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performed against various Lepidopteran species, including SAW, SBL and Soybean Pod Worm (SPW, *Helicoverpa zea*). Table 9 shows the activity observed against these Lepidopteran pests in stably transformed R₀ generation soybeans. As can be seen in Table 9, Cry1Da1_7.nno and TIC844_11.nno demonstrated activity against SPW, SAW and SBL.

5 TIC844_9.nno (TIC844 plus a bonus alanine for cloning) did not demonstrate activity against SPW.

Table 9. Bioassay activity profile of Cry1Da1_7.nno, TIC844_9.nno and TIC844_11.nno expressed in R₀ generation soybean.

Toxin	SPW	SAW	SBL
Cry1Da1_7.nno (SEQ ID NO:40)	+	+	+
TIC844_11.nno (SEQ ID NO:44)	+	+	+
TIC844_9.nno (SEQ ID NO:42)	-	+	+

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+ Active against insect species; - Inactive against insect species.

[0136] Selected transformed events were used to produce R₁ plants. R₁ plants expressing Cry1Da1_7.nno were assayed for resistance to SAW, SBL, SPW and Velvetbean caterpillar (VBC, *Anticarsia gemmatilis*). Leaf tissue was harvested from the R₁ generation plants and used in a feeding bioassay. Table 10 shows the activity observed in these tests. As demonstrated in Table 10, Cry1Da1_7.nno demonstrated activity against SPW, SAW and SBL.

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Table 10. Bioassay activity profile of Cry1Da1_7.nno expressed in R₁ generation soybean.

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Toxin	SPW	SAW	SBL	VBC
Cry1Da1_7.nno (SEQ ID NO:40)	+	+	+	-

+ Active against insect species; - Inactive against insect species.

[0137] Table 11 shows the results of field tests conducted in screenhouses with stably transformed R₁ generation soybean plants expressing Cry1Da1_7.nno. Species used to infest

25

plants in the screenhouses include Black armyworm (BLAW, *Spodoptera cosmioides*), Bean shoot moth (BSM, *Crociosema aporema*), South American podworm (SAPW, *Helicoverpa gelotopoeon*), Sunflower looper (SFL, *Rachiplusia nu*) and VBC. Table 11 shows the activity observed in these tests. As demonstrated in Table 11, Cry1Da1_7.nno demonstrated

5 activity against BLAW, SAPW and SFL.

Table 11. Activity profile of Cry1Da1_7.nno expressed in R₁ generation soybean tested in screenhouse field tests.

Toxin	BLAW	BSM	SAPW	SFL	VBC
Cry1Da1_7.nno (SEQ ID NO:40)	+	-	+	+	-

10

+ Active against insect species; - Inactive against insect species.

[0138] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of the foregoing

15 illustrative embodiments, it will be apparent to those of skill in the art that variations, changes, modifications, and alterations may be applied to the composition, methods, and in the steps or in the sequence of steps of the methods described herein, without departing from the true concept, spirit, and scope of the invention. More specifically, it will be apparent that

20 certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as defined by the appended claims.

Claims

1. An engineered insecticidal protein comprising the amino acid sequence as set forth in SEQ ID NO:44.
2. The engineered insecticidal protein of claim 1, wherein the engineered insecticidal protein exhibits inhibitory activity against an insect species of the order Lepidoptera.
3. The engineered insecticidal protein of claim 2, wherein the Lepidoptera is selected from the group consisting of a *Spodoptera* and a *Helicoverpa*.
4. The engineered insecticidal protein of claim 2, wherein the Lepidoptera is selected from the group consisting of *Helicoverpa zea* and *Spodoptera frugiperda*.
5. The engineered insecticidal protein of claim 1, encoded by a polynucleotide operably linked to a heterologous promoter.
6. The engineered insecticidal protein of claim 5, wherein said engineered insecticidal protein is encoded by a polynucleotide comprising a nucleotide sequence that hybridizes to the reverse complement of the polynucleotide sequence as set forth in SEQ ID NO:43.
7. A host cell comprising the polynucleotide of SEQ ID NO:43, wherein said polynucleotide is present within a host cell selected from the group consisting of a bacterial host cell and a plant host cell.
8. The host cell of claim 7, wherein said bacterial host cell is selected from the group consisting of *Agrobacterium*, *Rhizobium*, *Bacillus*, *Brevibacillus*, *Escherichia*, *Pseudomonas*, *Klebsiella*, and *Erwinia*.
9. The host cell of claim 7, wherein said plant host cell is selected from the group of plants consisting of a monocot plant cell and a dicot plant cell.
10. An insect inhibitory composition comprising the engineered insecticidal protein of claim 1, wherein said protein is present in an insect inhibitory amount.

11. The insect inhibitory composition of claim 10, wherein said composition further comprises a second insect inhibitory agent different from said engineered insecticidal protein.
12. The insect inhibitory composition of claim 11, wherein said second insect inhibitory agent is selected from the group consisting of an insect inhibitory protein and an insect inhibitory dsRNA molecule.
13. The insect inhibitory composition of claim 12, wherein said second pesticidal agent exhibits activity against one or more pest species selected from the group of orders consisting of Lepidoptera, Coleoptera, Hemiptera, Homoptera, and Thysanoptera.
14. A seed comprising the full length polynucleotide set forth in SEQ ID NO: 43, wherein said polynucleotide is operably linked to a heterologous promoter.
15. A method of controlling a Lepidopteran pest comprising contacting said Lepidopteran pest with an insect inhibitory amount of the engineered insecticidal protein of claim 1.
16. A transgenic plant or plant part comprising the engineered insecticidal protein of claim 1 and a polynucleotide having the nucleotide sequence as set forth in SEQ ID NO:43, wherein said engineered insecticidal protein is present in an insect inhibitory amount.
17. A method of controlling a Lepidopteran pest, said method comprising exposing said pest to the transgenic plant or plant part of claim 16.
18. A commodity product comprising a detectable amount of the engineered insecticidal protein of claim 1 or a detectable amount of a polynucleotide having the sequence as set forth in SEQ ID NO:43.
19. The commodity product of claim 18, wherein said commodity product is selected from the group consisting of plant biomass, oil, meal, animal feed, flour, flakes, bran, lint, hulls, and processed seed.
20. A method of producing a seed comprising the engineered insecticidal protein of claim 1, the method comprising:

a. planting at least one seed comprising a polynucleotide having the sequence as set forth in SEQ ID NO:43;

b. growing a plant from said seed; and

c. harvesting progeny seed from said plant,

wherein said harvested seed comprises said polynucleotide.