CLAIMS

1. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP comprising an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to the amino acid sequence according to Formula (I): A-X_1-D-G-D-V-E-G-P-A-G-C-K-K-Y-D-X_2-E-C-X_3-X_4-G-E-C-C-Q-K-Q-Y-L-X_5-X_6-K-W-R-X_7-L-X_8-C-R-X_9-X_{10}-K-S-G-F-F-S-S-K-X_{11}-X_{12}-C-R-D-V, wherein the polypeptide comprises at least one amino acid substitution relative to the wild-type sequence of the diguetoxin as set forth in SEQ ID NO:2, wherein X_1 is K or L; X_2 is V, A, or E; X_3 is D, Y, or A; X_4 is S or A; X_5 is W, A, F; X_6 is Y, A, S, H, or K; X_7 is P or A; X_8 is D, A, K, S, T or M; X_9 is C, G, T, A, S, M, or V; X_{10} is L, A, N, V, S, E, I, or Q; X_{11} is C, F, A, T, S, M, or V; and X_{12} is V, A, or T, or a pharmaceutically acceptable salt thereof.

2. The DVP of claim 1, wherein if X_9 is G, T, A, S, M or V, or X_{11} is F, A, T, S, M or V, then a disulfide bond is removed.

3. The DVP of claim 1, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219, or a pharmaceutically acceptable salt thereof.

4. A composition comprising a DVP of any one of claims 1-3 and an excipient.

5. A polynucleotide operable to encode a DVP, said DVP comprising an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to the amino acid sequence according to Formula (I): A-X_1-D-G-D-V-E-G-P-A-G-C-K-K-Y-D-X_2-E-C-X_3-X_4-G-E-C-C-Q-K-Q-Y-L-X_5-X_6-K-W-R-X_7-L-X_8-C-R-X_9-X_{10}-K-S-G-F-F-S-S-K-X_{11}-X_{12}-C-R-D-V, wherein the polypeptide comprises at least one amino acid substitution relative to the wild-type sequence of the diguetoxin as set forth in SEQ ID NO:2, wherein X_1 is K or L; X_2 is V, A, or E; X_3 is D, Y, or A; X_4 is S or A; X_5 is W, A, F; X_6 is Y, A, S, H, or K; X_7 is P or A; X_8 is D, A, K, S, T or M; X_9 is C, G, T, A, S, M, or V; X_{10} is L, A, N, V, S, E, I, or Q; X_{11} is C, F, A, T, S, M, or V; and X_{12} is V, A, or T, or a complementary nucleotide sequence thereof.

6. The polynucleotide of claim 5, wherein if the polynucleotide encodes a DVP wherein if X_9 is G, T, A, S, M or V, or X_{11} is F, A, T, S, M or V, then a disulfide bond is removed.

7. The polynucleotide of claim 5, wherein the polynucleotide encodes a DVP having an amino sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219.

8. A method of producing a DVP, the method comprising:

(a) preparing a vector comprising a first expression cassette comprising a polynucleotide operable to encode a DVP, or complementary nucleotide sequence thereof, said DVP comprising an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to the amino acid sequence according to Formula (I): A-X_1-D-G-D-V-E-G-P-A-G-C-K-K-Y-D-X_2-E-C-X_3-X_4-G-E-C-C-Q-K-Q-Y-L-X_5-X_6-K-W-R-X_7-L-X_8-C-R-X_9-X_{10}-K-S-G-F-F-S-S-K-X_{11}-X_{12}-C-R-D-V, wherein the polypeptide comprises at least one amino acid substitution relative to the wild-type sequence of the diguetoxin as set forth in SEQ ID NO:2, and wherein X_1 is K or L; X_2 is V, A, or E; X_3 is D, Y, or A; X_4 is S or A; X_5 is W, A, F; X_6 is Y, A, S, H, or K; X_7 is P or A; X_8 is D, A, K, S, T or M; X_9 is C, G, T, A, S, M, or V; X_{10} is L, A, N, V, S, E, I, or Q; X_{11} is C, F, A, T, S, M, or V; and X_{12} is V, A, or T;

(b) introducing the vector into a yeast cell; and

(c) growing the yeast cell in a growth medium under conditions operable to enable expression of the DVP and secretion into the growth medium.

9. The method of claim 8, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219, or a pharmaceutically acceptable salt thereof.

10. The method of claim 8, wherein the yeast cell is selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces*.

11. The method of claim 10, wherein the yeast cell is selected from the group consisting of *Kluyveromyces lactis*, *Kluyveromyces marxianus, Saccharomyces cerevisiae*, and *Pichia pastoris*.

12. The method of claim 11, wherein the yeast cell is *Kluyveromyces lactis*.

13. The method of claim 8, wherein the vector comprises two or three expression cassettes, each expression cassette operable to encode the DVP of the first expression cassette, or a DVP of

a different expression cassette, and wherein each of the expression cassette encodes a DVP having an amino acid sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219.

14. A method of combating, controlling, or inhibiting a pest comprising, applying a pesticidally effective amount of the composition of claim 4 to the locus of the pest, or to a plant or animal susceptible to an attack by a pest, wherein the pest may be selected from the group consisting of: Achema Sphinx Moth (Hornworm) (Eumorpha achemon); Alfalfa Caterpillar (Colias eurytheme); Almond Moth (Caudra cautella); Amorbia Moth (Amorbia humerosana); Armyworm (Spodoptera spp., e.g. exigua, frugiperda, littoralis, Pseudaletia unipuncta); Artichoke Plume Moth (Platyptilia carduidactyla); Azalea Caterpillar (Datana major); Bagworm (Thyridopteryx); ephemeraeformis); Banana Moth (Hypercompe scribonia); Banana Skipper (Erionota thrax); Blackheaded Budworm (Acleris gloverana); California Oakworm (Phryganidia californica); Spring Cankerworm (Paleacrita merriccata); Cherry Fruitworm (Grapholita packardi); China Mark Moth (Nymphula stagnata); Citrus Cutworm (Xylomyges curialis); Codling Moth (Cydia pomonella); Cranberry Fruitworm (Acrobasis vaccinii); Cross-striped Cabbageworm (Evergestis rimosalis); Cutworm (Noctuid species, Agrotis ipsilon); Douglas Fir Tussock Moth (Orgyia pseudotsugata); Ello Moth (Hornworm) (Erinnyis ello); Elm Spanworm (Ennomos subsignaria); European Grapevine Moth (Lobesia botrana); European Skipper (Thymelicus lineola); Essex Skipper; Fall Webworm (Melissopus latiferreanus)); Filbert Leafroller (Archips rosanus)); Fruittree Leafroller (Archips argyrospilia)); Grape Berry Moth (Paralobesia viteana)); Grape Leafroller (Platynota stultana)); Grapeleaf Skeletonizer (Harrisina americana); Green Cloverworm (Plathypena scabra)); Greenstriped Mapleworm (Dryocampa rubicunda)); Gummosos-Batrachedra comosae (Hodges); Gypsy Moth (Lymantria dispar); Hemlock Looper (Lambdina fiscellaria); Hornworm (Manduca spp.); Imported Cabbageworm (Pieris rapae); Io Moth (Automeris io); Jack Pine Budworm (Choristoneura pinus); Light Brown Apple Moth (Epiphyas postvittana); Melonworm (Diaphania hyalinata); Mimosa Webworm (Homadaula anisocentra); Obliquebanded Leafroller (Choristoneura rosaceana); Oleander Moth (Syntomeida epilais); Omnivorous Leafroller (Playnota stultana); Omnivorous Looper (Sabulodes aegrotata); Orangedog (Papilio cresphontes); Orange Tortrix (Argyrotaenia citrana); Oriental Fruit Moth (Grapholita molesta); Peach Twig Borer (Anarsia lineatella); Pine Butterfly (Neophasia menapia); Podworm; Redbanded Leafroller (Argyrotaenia velutinana); Redhumped Caterpillar (Schizura concinna); Rindworm Complex (Various Leps.); Saddleback Caterpillar (Sibine stimulea); Saddle Prominent Caterpillar Heterocampa guttivitta); Saltmarsh Caterpillar

(Estigmene acrea); Sod Webworm (Crambus spp.); Spanworm (Ennomos subsignaria); Fall Cankerworm (Alsophila pometaria); Spruce Budworm (Choristoneura fumiferana); Tent Caterpillar (Various Lasiocampidae); Thecla-Thecla Basilides (Geyr) (Thecla basilides); Tobacco Hornworm (Manduca sexta); Tobacco Moth (Ephestia elutella); Tufted Apple Budmoth (Platynota idaeusalis); Twig Borer (Anarsia lineatella); Variegated Cutworm (Peridroma saucia); Variegated Leafroller (Platynota flavedana); Velvetbean Caterpillar (Anticarsia gemmatalis); Walnut Caterpillar (Datana integerrima); Webworm (Hyphantria cunea); Western Tussock Moth (Orgyia vetusta); Southern Cornstalk Borer (Diatraea crambidoides); Corn Earworm; Sweet potato weevil; Pepper weevil; Citrus root weevil; Strawberry root weevil; Pecan weevil); Filbert weevil; Ricewater weevil; Alfalfa weevil; Clover weevil; Tea shot-hole borer; Root weevil; Sugarcane beetle; Coffee berry borer; Annual blue grass weevil (Listronotus maculicollis); Asiatic garden beetle (Maladera castanea); European chafer (Rhizotroqus majalis); Green June beetle (Cotinis nitida); Japanese beetle (Popillia japonica); May or June beetle (Phyllophaga sp.); Northern masked chafer (Cyclocephala borealis); Oriental beetle (Anomala orientalis); Southern masked chafer (Cyclocephala lurida); Billbug (Curculionoidea); Aedes aegypti; Busseola fusca; Chilo suppressalis; Culex pipiens; Culex quinquefasciatus; Diabrotica virgifera; Diatraea saccharalis; Helicoverpa armigera; Helicoverpa zea; Heliothis virescens; Leptinotarsa decemlineata; Ostrinia furnacalis; Ostrinia nubilalis; Pectinophora gossypiella; Plodia interpunctella; Plutella xylostella; Pseudoplusia includens; Spodoptera exigua; Spodoptera frugiperda; Spodoptera littoralis; Trichoplusia ni; and Xanthogaleruca luteola.

15. A vector comprising a polynucleotide operable to encode a DVP having an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to an amino acid sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219.

16. A yeast cell comprising:

(a) a first expression cassette comprising a polynucleotide operable to encode a DVP, said DVP comprising an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to the amino acid sequence according to Formula (I): A-X_1-D-G-D-V-E-G-P-A-G-C-K-K-Y-D-X_2-E-C-X_3-X_4-G-E-C-C-Q-K-Q-Y-L-X_5-X_6-K-W-R-X_7-L-X_8-C-R-X_9-X_{10}-K-S-G-F-F-S-S-K-X_{11}-X_{12}-C-R-D-V, wherein the polypeptide comprises at least one amino acid substitution relative to the wild-type sequence of the diguetoxin as set forth in SEQ ID NO:2, wherein X_1 is K or L; X_2 is V, A, or E; X_3 is D, Y, or A; X_4 is S or A; X_5 is W, A, F; X_6 is Y, A, S, H, or K; X_7 is

P or A; X_8 is D, A, K, S, T or M; X_9 is C, G, T, A, S, M, or V; X_{10} is L, A, N, V, S, E, I, or Q; X_{11} is C, F, A, T, S, M, or V; and X_{12} is V, A, or T; or complementary nucleotide sequence thereof.

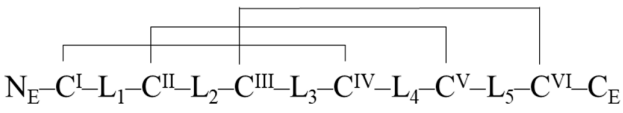
17. The yeast cell of claim 16, wherein if X_9 is G, T, A, S, M or V, or X_{11} is F, A, T, S, M or V, then a disulfide bond is removed.

18. The yeast cell of any one of claims 16, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219.

19. The yeast cell of claim 16, wherein the yeast cell is selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces*.

20. The yeast cell of claim 19, wherein the yeast cell is selected from the group consisting of *Kluyveromyces lactis*, *Kluyveromyces marxianus, Saccharomyces cerevisiae*, and *Pichia pastoris*.

21. A recombinant cysteine-rich protein (CRP), said recombinant CRP comprising a cystine knot (CK) architecture according to Formula (II):



Formula (II)

wherein C^I to C^{VI} are cysteine residues;

wherein cysteine residues C^I and C^{IV} are connected by a first disulfide bond; C^{II} and C^V are connected by a second disulfide bond; and C^{III} and C^{VI} are connected by a third disulfide bond;

wherein the first disulfide bond, the second disulfide bond, and the third disulfide bond have a disulfide bond topology that forms a cystine knot motif;

wherein the first disulfide bond, second disulfide bond, and third disulfide bond are the only disulfide bonds that form the cystine knot motif;

wherein N_E, L_1, L_2, L_3, L_4, L_5, and C_E are peptide subunits comprising an amino acid sequence having a length of 1 to 13 amino acid residues;

wherein N_E, L_3, C_E, or a combination thereof, are optionally absent;

wherein said recombinant CRP is created by modifying a modifiable CRP having: one or more non-CK disulfide bonds, wherein the one or more non-CK disulfide bonds are not the first disulfide bond, the second disulfide bond, or the third disulfide bond, and wherein the one or more non-CK disulfide bonds do not form the CK motif;

wherein the modifiable CRP is modified by removing one or more non-CK disulfide bonds from a modifiable CRP having one or more non-CK disulfide bonds;

wherein removing the one or more disulfide bonds from the modifiable CRP having one or more non-CK disulfide bonds, results in the recombinant CRP having the CK architecture according to Formula (II); and

wherein the recombinant CRP having the CK architecture according to Formula (II) has an increased level of expression relative to a level of expression of a modifiable CRP that does not have the CK architecture according to Formula (II).

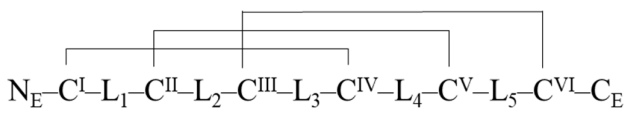
22. The recombinant CRP of claim 21, wherein the disulfide bond topology forms one of the following cystine knot motifs: an inhibitor cystine knot (ICK) motif; a growth factor cystine knot (GFCK) motif; or a cyclic cystine knot (CCK) motif.

23. The recombinant CRP of claim 22, wherein the disulfide bond topology forms an ICK motif.

24. The recombinant CRP of claim 21, wherein the modifiable CRP is a wild-type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

25. The recombinant CRP of claim 21, wherein the modifiable or recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 6-14, 193, 195, 197, 198, 199, or 201.

26. A method of making a recombinant cysteine-rich protein (CRP) comprising a cystine knot (CK) architecture according to Formula (II):



Formula (II)

wherein C^{I} to C^{VI} are cysteine residues;

wherein cysteine residues C^{I} and C^{IV} are connected by a first disulfide bond; C^{II} and C^{V} are connected by a second disulfide bond; and C^{III} and C^{VI} are connected by a third disulfide bond;

wherein the first disulfide bond, the second disulfide bond, and the third disulfide bond have a disulfide bond topology that forms a cystine knot motif;

wherein the first disulfide bond, second disulfide bond, and third disulfide bond are the only disulfide bonds that form the cystine knot motif;

wherein N_E, L_1, L_2, L_3, L_4, L_5, and C_E are peptide subunits comprising an amino acid sequence having a length of 1 to 13 amino acid residues;

wherein N_E, L_3, C_E, or a combination thereof, are optionally absent;

said method comprising:

(a) providing a modifiable CRP having one or more non-CK disulfide bonds, wherein the one or more non-CK disulfide bonds are not the first disulfide bond, the second disulfide bond, or the third disulfide bond, and wherein the one or more non-CK disulfide bonds do not form the CK motif; and

(b) modifying the modifiable CRP by removing one or more non-CK disulfide bonds from a modifiable CRP having one or more non-CK disulfide bonds;

wherein removing the one or more disulfide bonds from the modifiable CRP having one or more non-CK disulfide bonds, results in the recombinant CRP having the CK architecture according to Formula (II); and

wherein the recombinant CRP having the CK architecture according to Formula (II) has an increased level of expression relative to a level of expression of a modifiable CRP that does not have the CK architecture according to Formula (II).

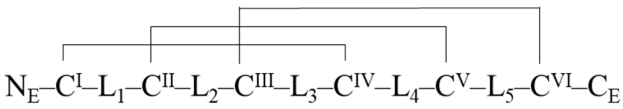
27. The method of claim 26, wherein the disulfide bond topology forms one of the following cystine knot motifs: an inhibitor cystine knot (ICK) motif; a growth factor cystine knot (GFCK) motif; or a cyclic cystine knot (CCK) motif.

28. The method of claim 26, wherein the modifiable CRP is a wild-type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

29. The method of claim 26, wherein the modifiable or recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 6-14, 193, 195, 197, ~~or~~ 198, 199, or 201.

30. A method of increasing the yield of a recombinant cysteine-rich protein (CRP), said method comprising:

(a) creating a recombinant CRP having a cystine knot (CK) architecture according to Formula (II):



Formula (II)

wherein C^I to C^{VI} are cysteine residues;

wherein cysteine residues C^I and C^{IV} are connected by a first disulfide bond; C^{II} and C^V are connected by a second disulfide bond; and C^{III} and C^{VI} are connected by a third disulfide bond;

wherein the first disulfide bond, the second disulfide bond, and the third disulfide bond have a disulfide bond topology that forms a cystine knot motif;

wherein the first disulfide bond, second disulfide bond, and third disulfide bond are the only disulfide bonds that form the cystine knot motif;

wherein N_E, L_1, L_2, L_3, L_4, L_5, and C_E are peptide subunits comprising an amino acid sequence having a length of 1 to 13 amino acid residues;

wherein N_E, L_3, C_E, or any combination thereof, are optionally absent;

wherein said recombinant CRP is created according to the following process:

(b) providing a modifiable CRP having one or more non-CK disulfide bonds, wherein the one or more non-CK disulfide bonds are not the first disulfide bond, the second disulfide bond, or the third disulfide bond, and wherein the one or more non-CK disulfide bonds do not form the CK motif;

(c) modifying the modifiable CRP by removing one or more non-CK disulfide bonds from the modifiable CRP having one or more non-CK disulfide bonds;

wherein removing the one or more disulfide bonds from the modifiable CRP having one or more non-CK disulfide bonds results in the recombinant CRP having the CK architecture according to Formula (II); and

wherein the recombinant CRP having the CK architecture according to Formula (II) has an increased level of expression relative to a level of expression of a modifiable CRP that does not have the CK architecture according to Formula (II).

31. The method of claim 30, wherein the disulfide bond topology forms one of the following cystine knot motifs: an inhibitor cystine knot (ICK) motif; a growth factor cystine knot (GFCK) motif; or a cyclic cystine knot (CCK) motif.

32. The method of claim 30, wherein the modifiable CRP is a wild-type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

33. The method of claim 30, wherein the modifiable or recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 6-14, 193, 195, 197, 198, 199, or 201.

34. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical, or 100% identical to the amino acid sequence set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219, or a pharmaceutically acceptable salt thereof.

35. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical, or 100% identical to the amino acid sequence set forth in any one of SEQ ID NOs: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.

36. A fusion protein comprising one or more DVPs operably linked to an alpha mating factor (alpha-MF) peptide; wherein said one or more DVPs have an amino acid sequence that is at least 80%, 85%, 90%, or at least 95% identical to the amino acid sequence according to Formula (I): A-X_1-D-G-D-V-E-G-P-A-G-C-K-K-Y-D-X_2-E-C-X_3-X_4-G-E-C-C-Q-K-Q-Y-L-X_5-X_6-K-W-R-X_7-L-X_8-C-R-X_9-X_{10}-K-S-G-F-F-S-S-K-X_{11}-X_{12}-C-R-D-V, wherein the DVP comprises at least one amino acid substitution relative to the wild-type sequence of the diguetoxin as set forth in SEQ ID NO:2, wherein X_1 is K or L; X_2 is V, A, or E; X_3 is D, Y, or A; X_4 is S or A; X_5 is W, A, F; X_6 is Y, A, S, H, or K; X_7 is P or A; X_8 is D, A, K, S, T or M; X_9 is C, G, T, A, S, M, or V;

X_{10} is L, A, N, V, S, E, I, or Q; X_{11} is C, F, A, T, S, M, or V; and X_{12} is V, A, or T, or a pharmaceutically acceptable salt thereof.

37. The fusion protein of claim 36, wherein if X_9 is G, T, A, S, M or V, or X_{11} is F, A, T, S, M or V, then a disulfide bond is removed.

38. The fusion protein of claim 36, wherein the one or more DVPs comprise an amino sequence as set forth in any one of SEQ ID NOs: 6-43, 45-51, 53, 128, 130, 136, 139-140, 144, 146-147, 187-191, 202-215, or 217-219.

39. The fusion protein of claim 36, wherein the one or more DVPs, the alpha-MF, or a combination thereof, are separated by a cleavable linker or non-cleavable linker.

40. The fusion protein of claim 36, wherein the alpha-MF peptide is an alpha-MF peptide derived from a yeast species, such as a species selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces.*