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, 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, 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. The DVP of claim 3, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.

5. The DVP of claim 4, wherein the DVP comprises an amino sequence as 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.

6. The DVP of claim 1, wherein the DVP is a homopolymer or heteropolymer of two or more DVPs, wherein the amino acid sequence of each DVP is the same or different.

7. The DVP of claim 1, wherein the DVP is a fused protein comprising two or more DVPs separated by a cleavable linker or non-cleavable linker, and wherein the amino acid sequence of each DVP may be the same or different.

8. The DVP or claim 7, wherein the cleavable linker is cleavable inside the gut or hemolymph of an insect.

9. A composition comprising a DVP of any one of claims 1-8, or combinations thereof, and an excipient.

10. 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, 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, or a complementary nucleotide sequence thereof.

11. The polynucleotide of claim 10, 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.

12. The polynucleotide of claim 10, 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.

13. The polynucleotide of claim 12, wherein the polynucleotide encodes a DVP having an amino sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219.

14. The polynucleotide of claim 13, wherein the polynucleotide encodes a DVP having an amino sequence as set forth in any one of SEQ ID NOs: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219.

15. 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.

16. The method of claim 15, 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.

17. The method of claim 15, 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.

18. The method of claim 17, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.

19. The method of claim 18, wherein the DVP comprises an amino sequence as 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.

20. The method of claim 15, wherein the DVP is a homopolymer or heteropolymer of two or more DVPs, wherein the amino acid sequence of each DVP is the same or different.

21. The method of claim 15, wherein the DVP is a fused protein comprising two or more DVPs separated by a cleavable linker or non-cleavable linker, and wherein the amino acid sequence of each DVP may be the same or different.

22. The method of claim 21, wherein the cleavable linker is cleavable inside the gut or hemolymph of an insect.

23. The method of claim 15, wherein the vector is a plasmid comprising an alpha-MF signal.

24. The method of claim 15, wherein the vector is transformed into a yeast cell.

25. The method of claim 24, wherein the yeast cell is selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces*.

26. The method of claim 25, wherein the yeast cell is selected from the group consisting of *Kluyveromyces lactis*, *Kluyveromyces marxianus, Saccharomyces cerevisiae*, and *Pichia pastoris*.

27. The method of claim 26, wherein the yeast cell is *Kluyveromyces lactis*.

28. The method of claim 15, wherein the DVP is secreted into the growth medium.

29. The method of claim 15, wherein expression of the DVP provides a yield of: at least 70 mg/L, at least 80 mg/L, at least 90 mg/L, at least 100 mg/L, at least 110 mg/L, at least 120 mg/L, at least 130 mg/L, at least 140 mg/L, at least 150 mg/L, at least 160 mg/L, at least 170 mg/L, at least 180 mg/L, at least 190 mg/L 200 mg/L, at least 500 mg/L, at least 750 mg/L, at least 1,000 mg/L, at least 1,250 mg/L, at least 1,500 mg/L, at least 1,750 mg/L, at least 2,000 mg/L, at least 2,500 mg/L, at least 3,000 mg/L, at least 3,500 mg/L, at least 4,000 mg/L, at least 4,500 mg/L, at least 5,000 mg/L, at least 5,500 mg/L, at least at least 6,000 mg/L, at least 6,500 mg/L, at least 7,000 mg/L, at least 7,500 mg/L, at least 8,000 mg/L, at least 8,500 mg/L, at least 9,000 mg/L, at least 9,500 mg/L, at least 10,000 mg/L, at least 11,000 mg/L, at least 12,000 mg/L, at least 12,500 mg/L, at least 13,000 mg/L, at least 14,000 mg/L, at least 15,000 mg/L, at least 16,000 mg/L, at least 17,000 mg/L, at least 17,500 mg/L, at least 18,000 mg/L, at least 19,000 mg/L, at least 20,000 mg/L, at least 25,000 mg/L, at least 30,000 mg/L, at least 40,000 mg/L, at least 50,000 mg/L, at least 60,000 mg/L, at least 70,000 mg/L, at least 80,000 mg/L, at least 90,000 mg/L, or at least 100,000 mg/L of DVP per liter of yeast culture medium.

30. The method of claim 15, wherein expression of the DVP in the medium results in the expression of a single DVP in the medium.

31. The method of claim 15, wherein expression of the DVP in the medium results in the expression of a DVP polymer comprising two or more DVP polypeptides in the medium.

32. The method of claim 15, wherein the vector comprises two or three expression cassettes, each expression cassette operable to encode the DVP of the first expression cassette.

33. The method of claim 15, 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.

34. The method of claim 15, wherein the expression cassette is operable to encode 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.

35. The method of claim 34, wherein the expression cassette is operable to encode a DVP having an amino acid sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219.

36. The method of claim 35, wherein the expression cassette is operable to encode a DVP having an amino acid sequence as set forth in any one of SEQ ID NOs: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219.

37. A method of combating, controlling, or inhibiting a pest comprising, applying a pesticidally effective amount of the composition of claim 9 to the locus of the pest, or to a plant or animal susceptible to an attack by the pest.

38. The method of claim 37, wherein the pest is selected from the group consisting of: 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.*

39. The method of claim 38, wherein the pest is selected from the group consisting of: *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.*

40. 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.

41. The vector of claim 40, wherein the polynucleotide is 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: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219.

42. The vector of claim 41, wherein the polynucleotide is 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: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219.

43. 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, 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; or complementary nucleotide sequence thereof.

44. The yeast cell of claim 43, 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.

45. The yeast cell of any one of claims 43, 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.

46. The yeast cell of any one of claims 45, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219.

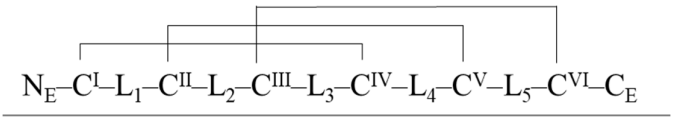
47. The yeast cell of any one of claims 46, wherein the DVP comprises an amino sequence as set forth in any one of SEQ ID NOs: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219.

48. The yeast cell of claim 43, wherein the yeast cell is selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces*.

49. The yeast cell of claim 48, wherein the yeast cell is selected from the group consisting of *Kluyveromyces lactis*, *Kluyveromyces marxianus, Saccharomyces cerevisiae*, and *Pichia pastoris*.

50. The yeast cell of claim 49, wherein the yeast cell is *Kluyveromyces lactis* or *Kluyveromyces marxianus*.

51. 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).

52. The recombinant CRP of claim 51, 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.

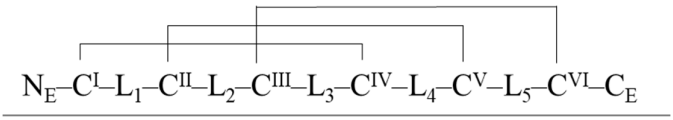
53. The recombinant CRP of claim 52, wherein the disulfide bond topology forms an ICK motif.

54. The recombinant CRP of claim 51, wherein the modifiable CRP is a wild-type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

55. The recombinant CRP of claim 54, wherein the modifiable CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 193, 195, or 198.

56. The recombinant CRP of claim 55, wherein the recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 6-14, 197, 199, or 201.

57. 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).

58. The method of claim 57, 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.

59. The method of claim 58, wherein the disulfide bond topology forms an ICK motif.

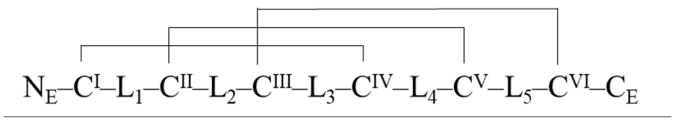
60. The method of claim 59, wherein the modifiable CRP is a wild-type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

61. The method of claim 60, wherein the modifiable CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 193, 195, or 198.

62. The method of claim 61, wherein the recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 6-14, 197, 199, or 201.

63. 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).

64. The method of claim 63, 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.

65. The method of claim 64, wherein the disulfide bond topology forms an ICK motif.

66. The method of claim 65, wherein the modifiable CRP is a wild type μ-DGTX-Dc1a; a DVP; a Kappa-ACTX, an ApsIII, or a variant thereof.

~~67. The method of claim 66, wherein the modifiable CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 1-2, 193, 195, or 198.~~

~~68. The method of claim 67, wherein the recombinant CRP comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 6-14, 197, 199, or 201.~~

~~69. 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 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.~~

~~70. 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 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.~~

~~71. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid 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.~~

~~72. 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 set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.~~

~~73. 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 to the amino acid sequence set forth in any one of SEQ ID NOs: 6-11, 15-~~

16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.

74. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219, or a pharmaceutically acceptable salt thereof.

75. 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 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.

76. 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 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.

77. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid 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.

78. 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 set forth in any one of SEQ ID NOs: 213, or 217-219, or a pharmaceutically acceptable salt thereof.

79. 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 to the amino acid sequence set forth in any one of SEQ ID NOs: 213, or 217-219, or a pharmaceutically acceptable salt thereof.

~~80. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set forth in any one of SEQ ID NOs: 213, or 217-219, or a pharmaceutically acceptable salt thereof.~~

~~81. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP comprising an amino acid set as forth in SEQ ID NOs: 213, or a pharmaceutically acceptable salt thereof.~~

~~82. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set as forth in SEQ ID NOs: 213, or a pharmaceutically acceptable salt thereof.~~

~~83. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP comprising an amino acid set as forth in SEQ ID NOs: 217, or a pharmaceutically acceptable salt thereof.~~

~~84. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set as forth in SEQ ID NOs: 217, or a pharmaceutically acceptable salt thereof.~~

~~85. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP comprising an amino acid set as forth in SEQ ID NOs: 218, or a pharmaceutically acceptable salt thereof.~~

~~86. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set as forth in SEQ ID NOs: 218, or a pharmaceutically acceptable salt thereof.~~

~~87. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP comprising an amino acid set as forth in SEQ ID NOs: 219, or a pharmaceutically acceptable salt thereof.~~

88. A diguetoxin variant polypeptide (DVP) having insecticidal activity against one or more insect species, said DVP consisting of an amino acid set as forth in SEQ ID NOs: 219, or a pharmaceutically acceptable salt thereof.

89. 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, 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, or a pharmaceutically acceptable salt thereof.

90. The fusion protein of claim 89, 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.

91. The fusion protein of claim 89, 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.

92 The fusion protein of claim 91, wherein the one or more DVPs comprise an amino sequence as set forth in any one of SEQ ID NOs: 6-11, 15-16, 20-22, 24-26, 29, 35, 45-48, 53, 128, 136, 139-140, 144, 146-147, 187-191, 207, 210-215, or 217-219.

93. The fusion protein of claim 92, wherein the one or more DVPs comprise an amino sequence as set forth in any one of SEQ ID NOs: 47, 53, 136, 139-140, 144, 146-147, 187-191, 210-215, or 217-219.

94. The fusion protein of claim 89, wherein the one or more DVPs is a homopolymer or heteropolymer of two or more DVPs, wherein the amino acid sequence of each DVP is the same or different.

~~95. The fusion protein of claim 89, wherein the one or more DVPs, the alpha-MF, or a combination thereof, are separated by a cleavable linker or non-cleavable linker.~~

~~96. The fusion protein of claim 95, wherein the cleavable linker is cleavable inside the gut or hemolymph of an insect.~~

~~97. The fusion protein of claim 89, wherein the alpha-MF peptide is an alpha-MF peptide derived from a yeast species.~~

~~98. The fusion protein of claim 97, wherein the yeast species is selected from any species of the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Yarrowia* or *Schizosaccharomyces*.~~

~~99. The fusion protein of claim 98, wherein the yeast species is selected from the group consisting of *Kluyveromyces lactis*, *Kluyveromyces marxianus, Saccharomyces cerevisiae*, and *Pichia pastoris*.~~

~~100. The fusion protein of claim 99, wherein the yeast species is *Kluyveromyces lactis* or *Kluyveromyces marxianus*.~~

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.

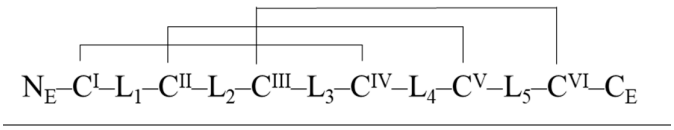
17. The yeast cell of claim 16, wherein if X_9 is G, T, A, S, M or V, or X_{11} is F, A, T, S, M or V, then a disulfide bond is removed.

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).

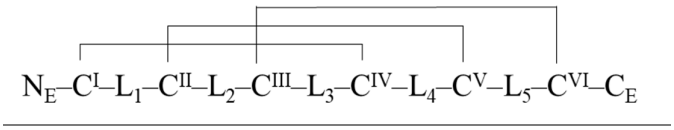
22. The recombinant CRP of claim 21, wherein the disulfide bond topology forms one of the following cystine knot motifs: an inhibitor cystine knot (ICK) motif; a growth factor cystine knot (GFCK) motif; or a cyclic cystine knot (CCK) motif.

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).

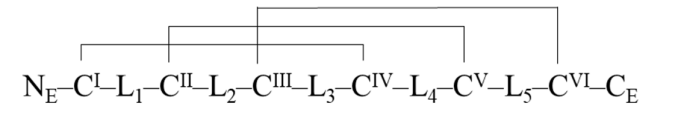
27. The method of claim 26, wherein the disulfide bond topology forms one of the following cystine knot motifs: an inhibitor cystine knot (ICK) motif; a growth factor cystine knot (GFCK) motif; or a cyclic cystine knot (CCK) motif.

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*.