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(54) **Title:** INSECTICIDAL PROTEINS

(57) **Abstract:** Compositions and methods for controlling insect pests are disclosed. In particular, novel insect inhibitory proteins comprising two different components, both of which are required for biological activity against at least coleopteran insect pests are provided. Nucleic acid molecules encoding the novel insecticidal proteins are also provided. Methods of making the insecticidal proteins and methods of using the insecticidal proteins and nucleic acids encoding the insecticidal proteins of the invention, for example in transgenic plants to confer protection from insect damage, are also disclosed.

INSECTICIDAL PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to US Provisional Application No. 62/951,025, filed December 20, 2019, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[002] The present invention relates to the fields of molecular biology and pest control. More particularly the invention relates to novel insecticidal proteins comprising two different components, both of which are required for maximum biological activity. The invention further relates to nucleic acids whose expression results in the insecticidal proteins, and methods of making and methods of using the insecticidal proteins and corresponding nucleic acids to control insects.

BACKGROUND

[003] Insect pests are a major cause of crop losses. In the United States alone, billions of dollars are lost every year due to infestation by various genera of insects. In addition to losses in field crops, insect pests are also a burden to vegetable and fruit growers, to producers of ornamental flowers, and they are a nuisance to gardeners and homeowners.

[004] Species of corn rootworm are considered to be the most destructive corn pests. In the United States alone, three species, *Diabrotica virgifera virgifera*, the western corn rootworm, *D. longicornis barberi*, the northern corn rootworm and *D. undecimpunctata howardi*, the southern corn rootworm, cause over one billion dollars in damage to corn each year in the US corn belt. An important corn rootworm pest in the Southern US is the Mexican corn rootworm, *Diabrotica virgifera zeae*. In South America, *Diabrotica speciosa* is considered to be an important pest of corn. Western corn rootworm spread to Europe in 1992 and since 2008 has been causing economic damage throughout the major corn growing regions. Corn rootworm larvae cause the most substantial plant damage by feeding almost exclusively on corn roots. This injury has been shown to increase plant lodging, to reduce grain yield and vegetative yield as well as alter the nutrient content of the grain. Larval feeding also causes indirect effects on corn by opening avenues through the roots for bacterial and fungal infections which lead to root and stalk rot diseases. Adult corn rootworms are active in cornfields in late summer where they feed on ears, silks and pollen, thus interfering with normal pollination.

[005] Corn rootworms are mainly controlled by intensive applications of chemical pesticides, which are active through inhibition of insect growth, prevention of insect feeding or reproduction, or cause death. Good corn rootworm control can thus be reached, but these chemicals can sometimes also affect other, beneficial organisms. Another problem resulting from the wide use of chemical pesticides is the appearance of resistant insect populations. Yet another problem is due to the fact that corn rootworm larvae feed underground thus making it difficult to apply rescue treatments of insecticides. Therefore, most insecticide applications are made prophylactically at the time of planting. This practice results in a large environmental burden. This has been partially alleviated by various farm management practices, but there is an increasing need for alternative pest control mechanisms.

[006] Biological pest control agents, such as *Bacillus thuringiensis* (Bt) strains expressing pesticidal toxins like δ-endotoxins (delta-endotoxins; also called crystal toxins or Cry proteins), have been applied to crop plants on a small scale with satisfactory results against certain insect pests. The δ-endotoxins are proteins held within a crystalline matrix that are known to possess insecticidal activity when ingested by certain insects. Such Cry proteins from *Bacillus thuringiensis* have been expressed in transgenic crop plants and exploited commercially to control certain lepidopteran and coleopteran insect pests. For example, starting in 2003, transgenic corn hybrids that control corn rootworm by expressing a Cry3Bb1, a Cry34Ab1/Cry35Ab, a modified Cry3A (mCry3A) or an eCry3.1Ab protein have been available commercially in the US.

[007] Most of the nearly 200 Bt Cry proteins presently known have some degree of lepidopteran activity associated with them. The large majority of Bt insect inhibitory proteins which have been identified do not have coleopteran controlling activity. Therefore, it is particularly important, at least for commercial purposes, to identify additional coleopteran specific insect inhibitory proteins.

[008] Although the use of transgenic plants expressing Cry proteins has been shown to be extremely effective, insect pests that now have resistance against the Cry proteins expressed in certain transgenic plants are known. Therefore, there remains a need to identify new and effective pest control proteins that provide an economic benefit to farmers and that are environmentally acceptable. Particularly needed are proteins that are toxic to *Diabrotica* species, a major pest of corn, that have a different mode of action than the Cry proteins in existing insect control products as a way to mitigate the development of resistance. Furthermore, delivery of insect control proteins through products that minimize the burden on the environment, as through transgenic plants, are desirable.

SUMMARY

[009] In view of these needs, the present invention provides new insecticidal proteins that are encoded by nucleotide sequences that are at least identifiable in the genomes of bacteria, particularly in the Orders Shingobacteriales and Rhodobacterales, but may also be identifiable in the genomes of bacteria in other orders as well. Examples of such insecticidal proteins are exemplified herein and this class of insecticidal proteins, whether derived from Shingobacteriales, Rhodobacterales or from different orders is collectively designated sBin Insecticidal Proteins (sBin-IPs), and particularly sBin1-IPs and sBin2-IPs. The sBin1 and sBin2 proteins of the invention are the two components of a binary toxin that exhibits toxicity to at least coleopteran insect pests. The invention also provides variants of the sBin-IPs of the invention, and proteins which are substantially identical to the sBin-IPs of the invention and their variants. Examples of amino acid sequences of sBin-IPs of the invention, include, but are not limited to, any of SEQ ID NOs:1-6, where SEQ ID NOs:1, 3 and 5 are amino acid sequences of sBin1 proteins and SEQ ID NOs:2, 4 and 6 are amino acid sequences of sBin2 proteins. The sBin-IPs of the invention have toxicity to insect pests. For example, the proteins of the invention can be used to control economically important insect pests, including coleopteran insects such as species in the Genus *Diabrotica*. Such species include, without limitation, the western corn rootworm (WCR; *Diabrotica virgifera virgifera*), the northern corn rootworm (NCR; *D. longicornis barberi*), the southern corn rootworm (SCR; *D. undecimpunctata howardi*) and the Mexican corn rootworm (MCR; *D. virgifera zeae*).

[010] The invention further provides nucleic acid molecules comprising one or more nucleotide sequences that encode a sBin-IP or a variant sBin-IP and their complements, or nucleotide sequences that are substantially identical to a sBin-IP or a variant sBin-IP. Examples of nucleotide sequences that encode a sBin-IP or variant sBin-IP of the invention include, but are not limited to, any of SEQ ID NOs:7-24, where SEQ ID NOs:7, 9, 11, 13, 15, 17, 19, 21 and 23 are nucleotide sequences that encode a sBin1 protein and SEQ ID NOs: 8, 10, 12, 14, 16, 18, 20, 22 and 24 are nucleotide sequences that encode a sBin2 protein.

[011] Also provided by the invention are vectors comprising recombinant nucleic acids that encode a sBin-IP and/or variant sBin-IP of the invention; a plant or microorganism which includes and enables expression of such nucleic acids; plants transformed with such nucleic acids, for example transgenic corn plants; the progeny of such plants which contain the nucleic acids stably incorporated and hereditable in a Mendelian manner, and/or the seeds of such plants and such progeny. The invention also provides methods of breeding to introduce a transgene comprising a nucleic acid molecule of the invention into a progeny plant and into various germplasms.

[012] The invention also provides compositions and formulations containing a sBin-IP and/or variant sBin-IP of the invention, which are capable of inhibiting the ability of insect pests to survive, grow and/or reproduce, or of limiting insect-related damage or loss to crop plants, for example applying a sBin-IP, or variant thereof, as part of compositions or formulations to insect-infested areas or plants, or to prophylactically treat insect-susceptible areas or plants to confer protection against the insect pests.

[013] The invention further provides a method of making a sBin-IP, or variant thereof, and to methods of using the nucleic acids, for example in microorganisms to control insects or in transgenic plants to confer protection from insect damage. Such microorganisms can be, for example, an endophytic species that colonizes maize roots and delivers a sBin-IP of the invention to the maize rhizosphere, thus protecting the roots from corn rootworm feeding damage.

[014] The sBin-IPs and/or variant sBin-IPs of the invention can be used singly or in combination with other insect control agents and strategies to confer enhanced pest control efficiency against the same insect pest and/or to increase the spectrum of target insects with minimal environmental impact.

[015] Other aspects and advantages of the invention will become apparent to those skilled in the art from a study of the following description of the invention and non-limiting examples.

BRIEF DESRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 is a Seg_korCRW1 (sBin1Aa) amino acid sequence.

SEQ ID NO:2 is a Seg_korCRW2 (sBin2Aa) amino acid sequence.

SEQ ID NO:3 is a Dyad_SG02CRW1 (sBin1Ba) amino acid sequence.

SEQ ID NO:4 is a Dyad_SG02CRW2 (sBin2Ab) amino acid sequence.

SEQ ID NO:5 is a Parac_pantoCRW1 (sBin1Ca) amino acid sequence.

SEQ ID NO:6 is a Parac_pantoCRW2 (sBin2Ba) amino acid sequence.

SEQ ID NO:7 is a Seg_korCRW1 nucleotide sequence.

SEQ ID NO:8 is a Seg_korCRW2 nucleotide sequence.

SEQ ID NO:9 is a Dyad_SG02CRW1 nucleotide sequence.

SEQ ID NO:10 is a Dyad_SG02CRW2 nucleotide sequence.

SEQ ID NO:11 is a Parac_pantoCRW1 nucleotide sequence.

SEQ ID NO:12 is a Parac_pantoCRW2 nucleotide sequence.

SEQ ID NO:13 is a Seg_korCRW1 E. coli optimized nucleotide sequence.

SEQ ID NO:14 is a Seg_korCRW2 E. coli optimized nucleotide sequence.

SEQ ID NO:15 is a Dyad_SG02CRW1 E. coli optimized nucleotide sequence.

SEQ ID NO:16 is a Dyad_SG02CRW2 E. coli optimized nucleotide sequence.

SEQ ID NO:17 is a Parac_pantoCRW1 E. coli optimized nucleotide sequence.

SEQ ID NO:18 is a Parac_pantoCRW2 E. coli optimized nucleotide sequence.

SEQ ID NO:19 is a Seg_korCRW1 maize optimized nucleotide sequence.

SEQ ID NO20 is a Seg_korCRW2 maize optimized nucleotide sequence.

SEQ ID NO:21 is a Dyad_SG02CRW1 maize optimized nucleotide sequence.

SEQ ID NO:22 is a Dyad_SG02CRW2 maize optimized nucleotide sequence.

SEQ ID NO:23 is a Parac_pantoCRW1 maize optimized nucleotide sequence.

SEQ ID NO:24 is a Parac_pantoCRW2 maize optimized nucleotide sequence.

DETAILED DESCRIPTION

[016] This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

[017] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

[018] All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

[019] Nucleotide sequences provided herein are presented in the 5’ to 3’ direction, from left to right and are presented using the standard code for representing nucleotide bases as set forth in 37 C.F.R.

§§1.821 - 1.825 and the World Intellectual Property Organization (WIPO) Standard ST.25, for example: adenine (A), cytosine (C), thymine (T), and guanine (G).

[020] Amino acids are likewise indicated using the WIPO Standard ST.25, for example: alanine (Ala; A), arginine (Arg; R), asparagine (Asn; N), aspartic acid (Asp; D), cysteine (Cys; C), glutamine (Gln; Q), glutamic acid (Glu; E), glycine (Gly; G), histidine (His; H), isoleucine (Ile; 1), leucine (Leu; L), lysine (Lys; K), methionine (Met; M), phenylalanine (Phe; F), proline (Pro; P), serine (Ser; S), threonine (Thr; T), tryptophan (Trp; W), tyrosine (Tyr; Y), and valine (Val; V). As used herein, an "X" or "Xaa" in an amino acid sequence denotes that the amino acid in that position can be any of the 20 known amino acid or can be any of the enumerated amino acids recited herein.

[021] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

Definitions

[022] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[023] As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" is a reference to one or more plants and includes equivalents thereof known to those skilled in the art, and so forth.

[024] As used herein, the word "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative, "or."

[025] The term "about" is used herein to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent, preferably 10 percent up or down (higher or lower). With regard to a temperature the term "about" means ± 1 °C, preferably ± 0.5°C. Where the term "about" is used in the context of this

invention (e.g., in combinations with temperature or molecular weight values) the exact value (i.e., without “about”) is preferred.

[026] As used herein, phrases such as "between about X and Y", "between about X and about Y", "from X to Y" and “from about X to about Y” (and similar phrases) should be interpreted to include X and Y, unless the context indicates otherwise.

[027] As used herein, the term "amplified" means the construction of multiple copies of a nucleic acid molecule or multiple copies complementary to the nucleic acid molecule using at least one of the nucleic acid molecules as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., Diagnostic Molecular Microbiology: Principles and Applications, PERSING et al., Ed., American Society for Microbiology, Washington, D.C. (1993). The product of amplification is termed an “amplicon.”

[028] "Activity" of the insecticidal proteins of the invention is meant that the insecticidal proteins function as orally active insect control agents, have a toxic effect, and/or are able to disrupt or deter insect feeding, which may or may not cause death of the insect. When an insecticidal protein of the invention is delivered to the insect, the result is typically death of the insect, or the insect does not feed upon the source that makes the insecticidal protein available to the insect. “Pesticidal” is defined as a toxic biological activity capable of controlling a pest, such as an insect, nematode, fungus, bacteria, or virus, preferably by killing or destroying them. "Insecticidal" is defined as a toxic biological activity capable of controlling insects, preferably by killing them. A “pesticidal agent” is an agent that has pesticidal activity. An “insecticidal agent” is an agent that has insecticidal activity.

[029] The term “chimeric construct” or “chimeric gene” or “chimeric polynucleotide” or “chimeric nucleic acid” (or similar terms) as used herein refers to a construct or molecule comprising two or more polynucleotides of different origin assembled into a single nucleic acid molecule. The term “chimeric construct”, “chimeric gene”, “chimeric polynucleotide” or “chimeric nucleic acid” refers to any construct or molecule that contains, without limitation, (1) polynucleotides (*e.g.*, DNA) , including regulatory and coding polynucleotides that are not found together in nature (i.e., at least one of the polynucleotides in the construct is heterologous with respect to at least one of its other polynucleotides), or (2) polynucleotides encoding parts of proteins not naturally adjoined, or (3) parts of promoters that are not naturally adjoined. Further, a chimeric construct, chimeric gene, chimeric polynucleotide or chimeric nucleic acid may comprise regulatory polynucleotides and coding polynucleotides that are derived from different sources, or comprise regulatory polynucleotides and coding polynucleotides derived from the same source, but arranged in a manner different from that

found in nature. In some embodiments of the invention, the chimeric construct, chimeric gene, chimeric polynucleotide or chimeric nucleic acid comprises an expression cassette comprising a polynucleotide of the invention under the control of regulatory polynucleotides, particularly under the control of regulatory polynucleotides functional in plants or bacteria.

[030] A "coding sequence" is a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism to produce a protein.

[031] To "control" insects means to inhibit, through a toxic effect, the ability of insect pests to survive, grow, feed, and/or reproduce, or to limit insect-related damage or loss in crop plants. To "control" insects may or may not mean killing the insects, although it preferably means killing the insects.

[032] As used herein, a “codon optimized” sequence means a nucleotide sequence wherein the codons are chosen to reflect the particular codon bias that a host cell or organism may have. This is typically done in such a way so as to preserve the amino acid sequence of the polypeptide encoded by the nucleotide sequence to be optimized. In certain embodiments, the DNA sequence of the recombinant DNA construct includes sequence that has been codon optimized for the cell (e.g., an animal, plant, or fungal cell) in which the construct is to be expressed. For example, a construct to be expressed in a plant cell can have all or parts of its sequence (e.g., the first gene suppression element or the gene expression element) codon optimized for expression in a plant. See, for example, U.S. Pat. No. 6,121,014, incorporated herein by reference.

[033] The terms “comprises” or “comprising,” when used in this specification, specify the presence of stated features, integers, steps, operations, elements, or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, or groups thereof.

[034] As used herein, the transitional phrase “consisting essentially of” (and grammatical variants) means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim” and those that do not materially alter the basic and novel characteristic(s)” of the claimed invention. Thus, the term “consisting essentially of” when used in a claim of this invention is not intended to be interpreted to be equivalent to “comprising.”

[035] In the context of the invention, “corresponding to” or “corresponds to” means that when the amino acid sequences of variant or homolog proteins are aligned with each other, the amino acids that “correspond to” certain enumerated positions in the variant or homolog protein are those that align with these positions in a reference protein but that are not necessarily in these exact numerical positions relative to the particular reference amino acid sequence of the invention. For example, if SEQ ID NO:1 is the reference sequence and is aligned with SEQ ID NO:3, amino acid Phe (F) at

position 201 (F201) of SEQ ID NO:3 "corresponds to" a Phe (F) at position 200 (F200) of SEQ ID NO:1, or for example, Thr (T) at position 105 (T105) of SEQ ID NO:2 "corresponds to" Ser (S) at position 105 (S105) of SEQ ID NO:1.

[036] To "deliver" a composition or a toxic protein means that the composition or toxic protein comes in contact with an insect, which facilitates the oral ingestion of the composition or toxic protein, resulting in a toxic effect and control of the insect. The composition or toxic protein can be delivered in many recognized ways, including but not limited to, transgenic plant expression, formulated protein composition(s), sprayable protein composition(s), a bait matrix, or any other art-recognized protein delivery system.

[037] The term "domain" refers to a set of amino acids conserved at specific positions along an alignment of sequences of evolutionarily related proteins. While amino acids at other positions can vary between homologs, amino acids that are highly conserved at specific positions indicate amino acids that are likely essential in the structure, stability or function of a protein. Identified by their high degree of conservation in aligned sequences of a family of protein homologs, they can be used as identifiers to determine if any polypeptide in question belongs to a previously identified polypeptide group.

[038] "Effective insect-controlling amount" means that concentration of an insecticidal protein that inhibits, through a toxic effect, the ability of insects to survive, grow, feed and/or reproduce, or to limit insect-related damage or loss in crop plants. "Effective insect-controlling amount" may or may not mean killing the insects, although it preferably means killing the insects.

[039] "Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the nucleotide sequence of interest may have at least one of its components heterologous with respect to at least one of its other components. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular

organism, such as a plant, the promoter can also be specific to a particular tissue, or organ, or stage of development.

[040] An expression cassette comprising a nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. An expression cassette may also be one that comprises a native promoter driving its native gene, however it has been obtained in a recombinant form useful for heterologous expression. Such usage of an expression cassette makes it so it is not naturally occurring in the cell into which it has been introduced.

[041] An expression cassette also can optionally include a transcriptional and/or translational termination region (i.e., termination region) that is functional in plants. A variety of transcriptional terminators are available for use in expression cassettes and are responsible for the termination of transcription beyond the heterologous nucleotide sequence of interest and correct mRNA polyadenylation. The termination region may be native to the transcriptional initiation region, may be native to the operably linked nucleotide sequence of interest, may be native to the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the nucleotide sequence of interest, the plant host, or any combination thereof). Appropriate transcriptional terminators include, but are not limited to, the CAMV 35S terminator, the tml terminator, the nopaline synthase terminator and/or the pea rbcs E9 terminator. These can be used in both monocotyledons and dicotyledons. In addition, a coding sequence's native transcription terminator can be used. Any available terminator known to function in plants can be used in the context of this invention.

[042] The term "expression" when used with reference to a polynucleotide, such as a gene, ORF or portion thereof, or a transgene in plants, refers to the process of converting genetic information encoded in a gene into RNA (e.g., mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (i.e., via the enzymatic action of an RNA polymerase), and into protein where applicable (e.g. if a gene encodes a protein), through "translation" of mRNA. Gene expression can be regulated at many stages in the process. For example, in the case of antisense or dsRNA constructs, respectively, expression may refer to the transcription of the antisense RNA only or the dsRNA only. In embodiments, “expression” refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. “Expression” may also refer to the production of protein.

[043] A "gene" is a defined region that is located within a genome and comprises a coding nucleic acid sequence and typically also comprises other, primarily regulatory, nucleic acids responsible for the control of the expression, that is to say the transcription and translation, of the coding portion. A gene may also comprise other 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns. The regulatory nucleic acid sequence of the gene may

not normally be operatively linked to the associated nucleic acid sequence as found in nature and thus would be a chimeric gene.

[044] "Gene of interest" refers to any nucleic acid molecule which, when transferred to an organism, such as a bacteria or a plant, confers upon the bacteria or plant a desired trait such as antibiotic resistance, virus resistance, insect resistance, disease resistance, or resistance to other pests, herbicide tolerance, abiotic stress tolerance, male sterility, modified fatty acid metabolism, modified carbohydrate metabolism, improved nutritional value, improved performance in an industrial process or altered reproductive capability. The "gene of interest" may also be one that is transferred to bacteria or plants for the production of commercially valuable enzymes or metabolites in the plant.

[045] A "heterologous" nucleic acid sequence or nucleic acid molecule is a nucleic acid sequence or nucleic acid molecule not naturally associated with a host cell into which it is introduced, including non- naturally occurring multiple copies of a naturally occurring nucleic acid sequence. A heterologous nucleic acid sequence or nucleic acid molecule may comprise a chimeric sequence such as a chimeric expression cassette, where the promoter and the coding region are derived from multiple source organisms. The promoter sequence may be a constitutive promoter sequence, a tissue-specific promoter sequence, a chemically-inducible promoter sequence, a wound-inducible promoter sequence, a stress-inducible promoter sequence, or a developmental stage-specific promoter sequence.

[046] A "homologous" nucleic acid sequence is a nucleic acid sequence naturally associated with a host cell into which it is introduced.

[047] "Homologous recombination" is the reciprocal exchange of nucleic acid fragments between homologous nucleic acid molecules.

[048] A "hypothetical protein" as used herein refers to a protein whose existence has been predicted, but for which there is a lack of experimental evidence that it is expressed *in vivo*. Sequencing genomes or organisms such as bacteria or plants, often results in numerous predicted open reading frames to which functions cannot be readily assigned. These proteins, either orphan or conserved hypothetical proteins, make up about 20% to about 40% of proteins encoded in each newly sequenced genome. Even when there is enough evidence that the product of a gene is expressed, by techniques such as microarray and mass-spectrometry, it is difficult to assign a function to it given its lack of identity to protein sequences with annotated biochemical function. Typically, most protein sequences are inferred from computational analysis of genomic DNA sequence. Hypothetical proteins are typically created by gene prediction software during genome analysis. When bioinformatics tools used for the gene identification find large open reading frames without a characterized homolog in

a protein database, such tools typically return the designation "hypothetical protein" as an annotation remark.

[049] The term "motif" or "consensus sequence" or "signature" refers to a short conserved region in the sequence of evolutionarily related proteins. Motifs are frequently highly conserved parts of domains, but may also include only part of the domain, or be located outside of conserved domain (if all of the amino acids of the motif fall outside of a defined domain).

[050] The term “identity” or “identical” or "substantially identical," in the context of two nucleic acid or amino acid sequences, refers to two or more sequences or subsequences that have at least 60%, preferably at least 80%, more preferably 90%, even more preferably 95%, and most preferably at least 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues or bases in length, more preferably over a region of at least about 100 residues or bases, and most preferably the sequences are substantially identical over at least about 150 residues or bases. In an especially preferred embodiment, the sequences are substantially identical over the entire length of the coding regions. Furthermore, substantially identical nucleic acid or amino acid sequences perform substantially the same function.

[051] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[052] Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad Sci. USA 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally, Ausubel et al., infra).

[053] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (National Center for Biotechnology Information, U.S. National

Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894 USA). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., 1990). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad Sci. USA 89: 10915 (1989)).

[054] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90: 5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[055] Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

[056] "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize to its target subsequence, but not to other sequences.

[057] The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2× SSC wash at 65°C for 15 minutes (see, Sambrook, infra, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1× SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6× SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2× (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[058] The following are examples of sets of hybridization/wash conditions that may be used to clone homologous nucleotide sequences that are substantially identical to reference nucleotide sequences of the present invention: a reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with

washing in 2× SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1× SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5× SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1× SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1× SSC, 0.1% SDS at 65°C.

[059] A further indication that two nucleic acid sequences or proteins are substantially identical is that the protein encoded by the first nucleic acid is immunologically cross reactive with, or specifically binds to, the protein encoded by the second nucleic acid. Thus, a protein is typically substantially identical to a second protein, for example, where the two proteins differ only by conservative substitutions.

[060] The term "isolated" nucleic acid molecule, polynucleotide or protein is a nucleic acid molecule, polynucleotide or protein that no longer exists in its natural environment. An isolated nucleic acid molecule, polynucleotide or protein of the invention may exist in a purified form or may exist in a recombinant host such as in a transgenic bacteria or a transgenic plant. Therefore, a claim to an “isolated” nucleic acid molecule, as enumerated herein, encompasses a nucleic acid molecule when the nucleic acid molecule is comprised within a transgenic plant genome.

[061] A "nucleic acid molecule" or "nucleic acid sequence" is a segment of single- or double-stranded DNA or RNA that can be isolated from any source. In the context of the invention, the nucleic acid molecule is typically a segment of DNA. In some embodiments, the nucleic acid molecules of the invention are isolated nucleic acid molecules.

[062] "Operably linked" refers to the association of polynucleotides on a single nucleic acid fragment so that the function of one affects the function of the other. For example, a promoter is operably linked with a coding polynucleotide or functional RNA when it is capable of affecting the expression of that coding polynucleotide or functional RNA (i.e., that the coding polynucleotide or functional RNA is under the transcriptional control of the promoter). Coding polynucleotide in sense or antisense orientation can be operably linked to regulatory polynucleotides.

[063] As used herein “pesticidal,” insecticidal,” and the like, refer to the ability of a sBin-IP of the invention to control a pest organism or an amount of a sBin-IP that can control a pest organism as defined herein. Thus, a pesticidal sBin-IP can kill or inhibit the ability of a pest organism (e.g., insect pest) to survive, grow, feed, or reproduce.

[064] The terms "protein," "peptide" and "polypeptide" may be used interchangeably herein.

[065] A "plant" is any plant at any stage of development, particularly a seed plant. Exemplary plants include, but are not limited to corn (*Zea mays*), canola (*Brassica napus, Brassica rapa ssp.*), alfalfa

(*Medicago saliva*), rice (*Oryza sativa*, including without limitation Indica and/or Japonica varieties), rape (*Brassica napus*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tobacum*), potato (*Solanum tuberosum*), peanut (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), apple (*Malus pumila*), blackberry (*Rubus*), strawberry (*Fragaria*), walnut (*Juglans regia*), grape (*Vitis vinifera*), apricot (*Prunus armeniaca*), cherry (*Prunus*), peach (*Prunus persica*), plum (*Prunus domestica*), pear (*Pyrus communis*), watermelon (*Citrullus vulgaris*), duckweed (*Lemna* spp.), oats (*Avena sativa*), barley (*Hordium vulgare*), vegetables, ornamentals, conifers, and turfgrasses (*e.g.*, for ornamental, recreational or forage purposes), and biomass grasses (*e.g.*, switchgrass and miscanthus).

[066] Vegetables include without limitation Solanaceous species (*e.g.*, tomatoes; *Lycopersicon esculentum*), lettuce (*e.g.*, *Lactuca sativa*), carrots (*Caucus carota*), cauliflower (*Brassica oleracea*), celery (*Apium graveolens*), eggplant (*Solanum melongena*), asparagus (*Asparagus officinalis*), ochra (*Abelmoschus esculentus*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), members of the genus Cucurbita such as hubbard squash (*C. hubbard*), butternut squash (*C. moschata*), zucchini (*C. pepo*), crookneck squash (*C. crookneck*), *C. argyrosperma , C. argyrosperma ssp sororia, C. digitata, C. ecuadorensis, C. foetidissima, C. lundelliana*, and *C. martinezii*, and members of the genus *Cucumis* such as cucumber (*Cucumis sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*).

[067] Ornamentals include without limitation azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), and chrysanthemum.

[068] Conifers, which may be employed in practicing the present invention, include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and

cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

[069] Turfgrass include but are not limited to zoysiagrasses, bentgrasses, fescue grasses, bluegrasses, St. Augustinegrasses, bermudagrasses, bufallograsses, ryegrasses, and orchardgrasses.

[070] Also included are plants that serve primarily as laboratory models, *e.g.*, Arabidopsis.

[071] A "plant cell" is a structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in the form of an isolated single cell or a cultured cell, or as a part of a higher organized unit such as, for example, plant tissue, a plant organ, or a whole plant.

[072] "Plant cell culture" means cultures of plant units such as, for example, protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, ovules, embryo sacs, zygotes and embryos at various stages of development.

[073] "Plant material" refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

[074] A "plant organ" is a distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

[075] "Plant tissue" as used herein means a group of plant cells organized into a structural and functional unit. Any tissue of a plant *in planta* or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

[076] A “polynucleotide” refers to a polymer composed of many nucleotide monomers covalently bonded in a chain. Such “polynucleotides” includes DNA, RNA, modified oligo nucleotides (e.g., oligonucleotides comprising bases that are not typical to biological RNA or DNA, such as 2'-O-methylated oligonucleotides), and the like. In some embodiments, a nucleic acid or polynucleotide can be single-stranded, double-stranded, multi-stranded, or combinations thereof. Unless otherwise indicated, a particular nucleic acid or polynucleotide of the present invention optionally comprises or encodes complementary polynucleotides, in addition to any polynucleotide explicitly indicated.

[077] "Polynucleotide of interest" refers to any polynucleotide which, when transferred to an organism, e.g., a plant, confers upon the organism a desired characteristic such as insect resistance, disease resistance, herbicide tolerance, antibiotic resistance, improved nutritional value, improved performance in an industrial process, production of commercially valuable enzymes or metabolites or altered reproductive capability.

[078] A "promoter" is an untranslated DNA sequence upstream of the coding region that contains the binding site for RNA polymerase and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

[079] As used herein, the term "recombinant" refers to a form of nucleic acid (e.g., DNA or RNA) or protein or an organism that would not normally be found in nature and as such was created by human intervention. As used herein, a "recombinant nucleic acid molecule" is a nucleic acid molecule comprising a combination of polynucleotides that would not naturally occur together and is the result of human intervention, e.g., a nucleic acid molecule that is comprised of a combination of at least two polynucleotides heterologous to each other, or a nucleic acid molecule that is artificially synthesized, for example, a polynucleotide synthesize using an assembled nucleotide sequence, and comprises a polynucleotide that deviates from the polynucleotide that would normally exist in nature, or a nucleic acid molecule that comprises a transgene artificially incorporated into a host cell's genomic DNA and the associated flanking DNA of the host cell's genome. Another example of a recombinant nucleic acid molecule is a DNA molecule resulting from the insertion of a transgene into a plant‘s genomic DNA, which may ultimately result in the expression of a recombinant RNA or protein molecule in that organism. As used herein, a "recombinant plant" is a plant that would not normally exist in nature, is the result of human intervention, and contains a transgene or heterologous nucleic acid molecule incorporated into its genome. As a result of such genomic alteration, the recombinant plant is distinctly different from the related wild-type plant.

[080] “Regulatory elements" refer to sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

[081] “sBin Insecticidal Proteins” (sBin-IPs) are proteins encoded by genes found at least in the genomes of bacteria in the Orders Shingobacteriales and Rhodobacterales that are components of binary toxins that are active against at least insect pests in the Genus *Diabrotica*. In some embodiments, the genomes of the bacteria are of a *Dyadobacter* species, a *Segetibacter* species, or a *Paracoccus* species. In some embodiments, the *Dyadobacter* species is selected from the group consisting of *D. alkalitolerans, D. arcticus, D. beijingensis, D. crusticola, D. endophyticus, D. fermentans, D. ginsengisoli, D. hamtensis, D. jejuensis, D. koreensis, D. psychrophilus, D. sediminis, D. soli* and *D. tibetensis*. In some embodiments, the *Segetibacter* species is selected from the group consisting of *S. aerophilus* and *S. koreensis*. In some embodiments, the *Paracoccus* species is selected from the group consisting of P. *alcaliphilus, P. alkenifer, P. aminophilus, P. aminovorans, P. bengalensis, P. carotinifaciens, P. denitrificans, P. ferrooxidans, P. haeundaensis, P. halotolerans, P.*

homiensis, P. kawasakiensis, P. kocurii, P. kondratievae, P. koreensis, P. marcusii, P. methylutens, P. pantotrophus, P. seriniphilus, P. solventivorans, P. thiocyanatus, P. thiophilus, P. versutus, P. yeei and *P. zeaxanthinifaciens*.

[082] The terms "substitution," "insertion" or "addition," and "deletion" are used herein with reference to amino acid or nucleotide sequences. A "substitution" refers to a replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively. An "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring sequence. A "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent. Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger. Substitutions, deletions, insertions, or any combination thereof may be used to arrive at a final variant polypeptide. Generally, a few amino acids are changed to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. In certain embodiments, amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of an isoleucine with a valine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids. In embodiments, substitutions can be made in accordance with known "conservative substitutions." A "conservative substitution" refers to the substitution of an amino acid in one class by an amino acid in the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature. In contrast, in certain embodiments, substitutions are non-conservative. A "non-conservative substitution" refers to the substitution of an amino acid in one class with an amino acid from another class.

[083] "Transformation" is a process for introducing heterologous nucleic acid into a host cell or organism. In particular embodiments, "transformation" means the stable integration of a DNA molecule into the genome (nuclear or plastid) of an organism of interest.

[084] "Transformed / transgenic / recombinant" refer to a host organism such as a bacterium or a plant into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host or the nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof. A "non-transformed", "non-transgenic",

or "non- recombinant" host refers to a wild-type organism, e.g., a bacterium or plant, which does not contain the heterologous nucleic acid molecule.

[085] This invention provides compositions and methods for controlling harmful insect pests. Particularly, the invention relates to insecticidal proteins and variants thereof, designated herein as Rhizobiaceae Insecticidal Proteins (sBin-IPs), that have activity against at least coleopteran insects, for example, *Diabrotica virgifera virgifera* (western corn rootworm; WCR), *Diabrotica barberi* (northern corn rootworm; NCR), and/or *Diabrotica undecimpunctata howardi* (southern corn rootworm; SCR) and/or other *Diabrotica* species including *Diabrotica virgifera zeae* (Mexican corn rootworm; MCR) and *Diabrotica speciosa* (curcurbit beetle). The inventor of the instant invention found that certain proteins, described in the art as hypothetical proteins, which are purportedly encoded in the genomes of gram negative bacteria in at least the Orders Shingobacteriales and Rhodobacterales, surprisingly are components of binary insecticidal toxins. More particularly, coding sequences for the hypothetical proteins that were found to be insecticidal are in genomes of bacteria in genomes of gram-negative bacteria in *Segetibacter*, *Dyadobacter*, *Paracoccus*, and related genera. Even more particularly, the coding sequences for the hypothetical proteins exemplified herein as insecticidal proteins include, without limitation, those sequences in the genomes of a strain of *Segetibacter koreensis* (SEQ ID NO:7 and SEQ ID NO:8), a strain of *Dyadobacter sp.* SG02 (SEQ ID NO:9 and SEQ ID NO:10) and a strain of *Paracoccus pantotrophus* (SEQ ID NO:11 and SEQ ID NO:12). Upon synthesis of a nucleic acid molecule that encodes a protein described above and expressing the protein in a transgenic *E. coli* bacterium, the inventor determined that the proteins described in the art as hypothetical proteins surprisingly have insecticidal activity, particularly against *Diabrotica* insect pests. Such insecticidal proteins are generally designated herein as sBin Insecticidal Proteins (sBin-IPs) and those specially exemplified herein are designated as, sBin1Aa (SEQ ID NO:1), sBin2Aa (SEQ ID NO:2), sBin1Ba (SEQ ID NO:3), sBin2Ab (SEQ ID NO:4), sBin1Ca (SEQ ID NO:5) and sBin2Ba (SEQ ID NO:6). The skilled person will recognize that using the teachings of the instant invention, the skilled person can identify sequences related to those described above, without limitation, in bacteria, in nucleic acid molecules from environmental samples, and in genome databases, where such sequences may be designated as hypothetical or they may have some other known function, and the like. It is contemplated that such related sequences are encompassed by the instant invention. The skilled person, upon reading this disclosure, will understand what is meant by the term "related sequences." As described in further detail below, sBin-IPs of the invention are components of a binary toxins and function together to confer activity against at least coleopteran insect pests.

[086] The present invention also relates to nucleic acids whose expression results in sBin-IPs of the invention, and to the making and using of the sBin-IPs to control insect pests. In certain non-limiting embodiments, the expression of the nucleic acids results in insecticidal proteins that can be used to control at least coleopteran insects such as western corn rootworm, northern corn rootworm and/or southern corn rootworm, particularly when expressed in a transgenic plant such as a transgenic corn plant.

[087] In some non-limiting embodiments, the invention encompasses a nucleic acid molecule comprising a nucleotide sequence that encodes a protein that is toxic to an insect pest, wherein the nucleotide sequence (a) encodes a protein comprising an amino acid sequence that has at least 80% to at least 99% sequence identity with any of SEQ ID NOs:1-6, or a toxin fragment thereof; or (b) has at least 80% to at least 99% sequence identity with any of SEQ ID NOs:7-12, or a toxin-encoding fragment thereof; or (c) is a synthetic sequence of (a) or (b) that has codons optimized for expression in a transgenic organism. In other embodiments, the insecticidal protein comprises, consists essentially of or consists of an amino acid sequence of any of SEQ ID NOs:1-6, or a toxic fragment thereof. In other embodiments, the nucleotide sequence comprises, consists essentially of or consists of any of SEQ ID NOs:7-12, or a toxin-encoding fragment thereof. In still other embodiments, the synthetic nucleotide sequence comprises, consists essentially of or consists of any of SEQ ID NOs:13-24, or a toxin-encoding fragment thereof.

[088] In some non-limiting embodiments, the invention encompasses a chimeric gene comprising a heterologous promoter operably linked to a nucleic acid molecule comprising, consisting essentially of or consisting of a nucleotide sequence that encodes a protein that is toxic to an insect pest, wherein the nucleotide sequence (a) encodes a protein comprising an amino acid sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or has 100% sequence identity with any of SEQ ID NOs:1-6, or a toxin fragment thereof; (b) has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or has 100% sequence identity with any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof; or (c) is a synthetic sequence of (a) or (b) that has codons optimized for expression in a transgenic organism. In other embodiments, the insecticidal protein comprises an amino acid sequence of any of SEQ ID NOs:1-6, or a toxic fragment thereof. In other embodiments, the nucleotide sequence comprises any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof. In some aspects of these embodiments, the chimeric gene is an expression cassette.

[089] In other non-limiting embodiments, the promoter comprised in a chimeric gene or an expression cassette of the invention is a plant expressible promoter. In aspects of these embodiments, the plant expressible promoter is selected from the group of promoters consisting of ubiquitin, cestrum yellow virus, corn TrpA, OsMADS 6, maize H3 histone, corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, maize mtl, pea small subunit RuBP carboxylase, rice actin, rice cyclophilin, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, potato patatin, lectin, CaMV 35S and S-E9 small subunit RuBP carboxylase promoter.

[090] In some non-limiting embodiments, the insecticidal protein encoded by a nucleic acid molecule of the invention or a chimeric gene of the invention or an expression cassette of the invention is active against a coleopteran insect pest. In some aspects of these embodiments, the coleopteran insect pest is in the Genus *Diabrotica*. In other aspects, the *Diabrotica* insect pest is *Diabrotica virgifera virgifera* (western corn rootworm; WCR), *Diabrotica barberi (*northern corn rootworm; NCR), and/or *Diabrotica undecimpunctata howardi* (southern corn rootworm; SCR) and/or other *Diabrotica* species including *Diabrotica virgifera zeae* (Mexican corn rootworm; MCR).

[091] In some non-limiting embodiments, a chimeric gene or expression cassette of the invention comprises a nucleotide sequence that encodes a sBin-IP of the invention, wherein the nucleotide sequence is codon optimized for expression in a transgenic organism. In some aspects of these embodiments, the transgenic organism is a bacteria or a plant.

[092] In other non-limiting embodiments, the transgenic bacteria is in the genus *Bacillus, Clostridium, Xenorhabdus, Photorhabdus, Pasteuria, Escherichia, Pseudomonas, Erwinia, Serratia, Klebsiella, Salmonella, Pasteurella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Sinorhizobium, Ensifer, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, Sphingomonas, Burkholderia, Candidatus* Glomeribacter, *Dyella, Herbaspirillum, Bradyrhizobium, Staphylococcus, Methylophilus, Variovorax, Streptococcus, Chitinophaga* or *Alcaligenes*. In other embodiments, the transgenic bacteria is *Escherichia coli*. In other embodiments, the nucleotide sequence comprises, consists essentially of or consists of any of SEQ ID NOs:13-18.

[093] In other non-limiting embodiments, the transgenic plant is a monocot plant or a dicot plant. In still other embodiments, the dicot plant is selected from the group consisting of a soybean, sunflower, tomato, cole crop, cotton, sugar beet and tobacco. In further aspects, the monocot plant is selected from the group consisting of barley, maize, oat, rice, sorghum, sugarcane and wheat. In some aspects, the transgenic plant is a maize plant. In other embodiments, the nucleotide sequence comprises

codons optimized for expression in maize. In still other embodiments, the nucleotide sequence comprises, consists essentially of or consists of any of SEQ ID NOs:19-24.

[094] In some non-limiting embodiments, the invention encompasses a protein, and optionally an isolated protein, that is toxic to an insect pest, i.e. an insecticidal protein, wherein the protein or isolated protein comprises, consists essentially of or consists of (a) an amino acid sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or has 100% sequence identity with any of SEQ ID NOs:1-6, or a toxin fragment thereof; (b) an amino acid sequence that comprises, consists essentially of or consists of any of SEQ ID NOs:1-6, or a toxin fragment thereof; (c) an amino acid sequence that is encoded by a nucleotide sequence that has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or has 100% sequence identity with any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof; (d) an amino acid sequence that is encoded by a nucleotide sequence comprising, consisting essentially of or consisting of any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof. Those skilled in the art will recognize that modifications can be made to the exemplified sBin-IPs encompassed by the invention. Such modifications and substantially identical nucleic acid or amino acid molecules are encompassed by the present invention.

[095] The invention also encompasses an engineered sBin Insecticidal Protein, which can be described as a mutant sBin-IP or a variant sBin-IP or a modified sBin-IP of the invention. In some embodiments, the modification can comprise a substitution and/or deletion of one or more amino acids in a naturally occurring sBin-IP sequence and/or insertion of one or more additional amino acids into a naturally occurring sBin-IP sequence. In other embodiments, the modification can comprise a substitution and/or deletion and/or insertion of one or more amino acids in an engineered sBin-IP. The substitution and/or insertion may be with a naturally occurring amino acid or a non-naturally occurring amino acid. In some non-limiting embodiments, the modification comprises, consists essentially of or consists of an substitution and/or insertion and/or deletion of one or more of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and/or valine amino acids at an amino acid position of a sBin-IP amino acid sequence. Such a substitution and/or insertion and/or deletion may be accomplished by changing codons in a nucleotide sequence

that encodes a sBin-IP resulting in the modified *sBin-IP* nucleotide sequence encoding the engineered sBin-IP, which is a mutant sBin-IP or a variant sBin-IP or a modified sBin-IP of the invention.

[096] In some non-limiting embodiments, the sBin-IP is modified by substitution and/or insertion of (a) one or more amino acids with an aliphatic hydrophobic side chain (*e.g.*, alanine, isoleucine, methionine and/or valine; in embodiments, the amino acid is not an alanine); (b) one or more amino acids with an aromatic hydrophobic side chain (*e.g.*, phenylalanine, tryptophan and/or tyrosine); (c) one or more amino acids with a polar neutral side chain (*e.g.*, asparagine, cysteine, glutamine, serine and/or threonine); (d) one or more amino acids with an acidic side chain (*e.g.*, aspartic acid and/or glutamic acid); one or more amino acids with a basic side chain (*e.g.*, arginine, histidine and/or lysine); (e) one or more glycine residues; (f) one or more proline residues; or (g) any combination of (a) to (f).

[097] In other embodiments, an amino acid is substituted and/or deleted and/or inserted in any of the amino acid sequences of sBin-IPs of the invention, particularly any of SEQ ID NOs:1-6. In other embodiments, amino acids acid are substituted in SEQ ID NO:1. In still other embodiments, the amino acids that are substituted in SEQ ID NO:1 are at positions 52, 102, 151 and/or 297. In other embodiments, the amino acid at position 52 is substituted with a A, the amino acid at position 102 is substituted with a T, the amino acid at position 151 is substituted with a A or K, or the amino acid at position 297 is substituted with a S or T.

[098] In additional embodiments, the invention provides a chimeric sBin-IP protein that includes a protein fusion tag which is linked to a full sBin-IP sequence or a portion of a sBin-IP sequence, e.g. a cytotoxin domain. The protein fusion tag can be linked at an N-terminus (e.g., at amino acid 1 or 2 of a sBin-IP sequence) or, alternatively, the protein fusion tag can be linked at a C-terminus of the sBin-IP sequence. The protein fusion tag can be a poly-histidine, poly-arginine, haloalkane dehalogenase, streptavidin-binding, glutathione s-transferase (GST), maltose-binding protein (MBP), thioredoxin, small ubiquitin-like modifier (SUMO), N-utilization substance A (NusA), protein disulfide isomerase I (DsbA), Mistic, Ketosteroid isomerase (KSI), or TrpE, c-myc, hemaglutinin antigen (HA), FLAG, 1D4, calmodulin-binding peptide, chitin-binding domain, cellulose-binding domain, S-tag, or Softag3 protein fusion tag. These can be used in methods of producing, isolating, or purifying any sBin-IP toxin of the invention. The invention also provides a recombinant polynucleotide, e.g., a construct, encoding the fusion tag which is linked to the sBin-IP toxin of the invention.

[099] In some non-limiting embodiments, the invention encompasses an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin, wherein the first component is a peptide selected from the group of peptides consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5, and the second component is a peptide selected from the

group of peptides consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, and wherein said insecticidal toxin is active against at least a *Diabrotica* pest insect.

[0100] In other embodiments, the first component of the insecticidal composition comprises SEQ ID NO:1 and the second component comprises SEQ ID NO:2, or SEQ ID NO:4 or SEQ ID NO:6; or the first component comprises SEQ ID NO:3 and the second component comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6; or the first component comprises SEQ ID NO:5 and the second component comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6; or the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component comprises SEQ ID NO:2; or the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component comprises SEQ ID NO:4; or the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component comprises SEQ ID NO:6. In other embodiments of the insecticidal composition, the first component comprises SEQ ID NO:1 and the second component comprises SEQ ID NO:2. In still other embodiments of the insecticidal composition, the first component comprises SEQ ID NO:3 and the second component comprises SEQ ID NO:4. In further embodiments of the insecticidal composition, the first component comprises SEQ ID NO:5 and the second component comprises SEQ ID NO:6.

[0101] In other embodiments, the insecticidal composition of is active against a Diabrotica pest. In still other embodiments, the *Diabrotica* pest is selected from the group of *Diabrotica* species consisting of *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi* and *Diabrotica zeae*.

[0102] In other embodiments, the insecticidal composition of the invention further comprises a second pesticidal agent. In other embodiments, the second pesticidal agent is a biological agent or a chemical agent. In other embodiments, the biological agent is or is derived from a *Bacillus thuringiensis* insecticidal protein, a *Bacillus cereus* insecticidal protein, a *Xenorhabdus* spp. insecticidal protein, a *Photorhabdus* spp. insecticidal protein, a *Brevibacillus laterosporous* insecticidal protein, a *Lysinibacillus sphearicus* insecticidal protein, a *Chromobacterium* spp. insecticidal protein, a *Yersinia entomophaga* insecticidal protein, a *Paenibacillus popiliae* insecticidal protein, or a *Clostridium* spp. insecticidal protein. In other embodiments, the biological agent is or is derived from a dsRNA, a Cry protein, a Vip protein, a patatin, a protease, a protease inhibitor, a urease, an alpha-amylase inhibitor, a pore-forming protein, a lectin, an engineered antibody or antibody fragment, or a chitinase. In still other embodiments the chemical agent is a carbamate, a pyrethroid, an organophosphate, a neonicotinoid, an organochloride, a nereistoxin, or a combination thereof; or (d) the chemical agent comprises an active ingredient selected from the group consisting of carbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin,

deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, and a combination thereof.

[0103] In some non-limiting embodiments, the invention encompasses a transgenic plant that produces an insecticidal composition of the invention. In other embodiments the transgenic plant is a transgenic maize plant that produces a binary toxin of the invention.

[0104] In some embodiments, the invention encompasses an isolated and purified antibody which specifically binds to a sBin-IP peptide selected from the group of peptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof, or an epitope therein, said antibody produced from the immune system of a vertebrate animal in response to the exposure of all or an antigenic part of said peptide to the animal's immune system.

[0105] In some embodiments, the invention encompasses a method for detecting the presence of a peptide in a sample comprising obtaining a/solution suspected of containing said peptide, probing said solution with the antibody of claim 29, and detecting the binding of said antibody to said peptide; wherein said peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof.

[0106] In some embodiments, the invention encompasses a kit for detecting the presence of the peptide in a sample comprising, in suitable container means, an antibody that binds to said peptide, reagents necessary for mixing the peptide and antibody in a solution at least a first immunodetection reagent providing said antibody along with control antibody, control antigen and the reagents and instructions necessary for detecting said binding; wherein said peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof.

[0107] In some embodiments, the sBin-IPs of the invention, including variant sBin-IPs of the invention, are active against a coleopteran insect pest. Insects in the order Coleoptera include but are not limited to any coleopteran insect now known or later identified including those in suborders Archostemata, Myxophaga, Adephaga and Polyphaga, and any combination thereof.

[0108] In some non-limiting embodiments, the binary toxins of the invention are active against *Diabrotica* spp. *Diabrotica* is a genus of beetles of the order Coleoptera commonly referred to as "corn rootworm" or "cucumber beetle." Exemplary *Diabrotica* species include without limitation *Diabrotica longicornis barberi* (northern corn rootworm), *D. virgifera virgifera* (western corn rootworm), *D. undecimpunctata howardii* (southern corn rootworm), *D. balteata* (banded cucumber

beetle), *D. undecimpunctata undecimpunctata* (western spotted cucumber beetle), *D. significata* (3-spotted leaf beetle), *D. speciosa* (chrysanthemum beetle), *D. virgifera zeae* (Mexican corn rootworm), *D. beniensis, D. cristata, D. curviplustalata, D. dissimilis, D. elegantula, D. emorsitans, D. graminea, D. hispanloe, D. lemniscata, D. linsleyi, D. milleri, D. nummularis, D. occlusal, D. porrecea, D. scutellata, D. tibialis, D. trifasciata* and *D. viridula*; and any combination thereof.

[0109] Other non-limiting examples of coleopteran insect pests according to the invention include *Leptinotarsa* spp. such as *L. decemlineata* (Colorado potato beetle); *Chrysomela* spp. such as *C. scripta* (cottonwood leaf beetle); *Hypothenemus* spp. such as *H. hampei* (coffee berry borer); *Sitophilus* spp. such as *S. zeamais* (maize weevil); *Epitrix* spp. such as *E. hirtipennis (*tobacco flea beetle) and *E. cucumeris* (potato flea beetle); *Phyllotreta* spp. such as *P. cruciferae* (crucifer flea beetle) and *P. pusilla* (western black flea beetle); *Anthonomus* spp. such as *A. eugenii* (pepper weevil); *Hemicrepidus* spp. such as *H. memnonius* (wireworms); *Melanotus* spp. such as *M. communis* (wireworm); *Ceutorhychus* spp. such as *C. assimilis* (cabbage seedpod weevil); *Phyllotreta* spp. such as *P. cruciferae* (crucifer flea beetle); *Aeolus* spp. such as *A. mellillus* (wireworm); *Aeolus* spp. such as *A. mancus* (wheat wireworm); *Horistonotus* spp. such as *H. uhlerii* (sand wireworm); *Sphenophorus* spp. such as *S. maidis* (maize billbug), *S. zeae* (timothy billbug), *S. parvulus* (bluegrass billbug), and *S. callosus* (southern corn billbug); *Phyllophaga* spp. (White grubs); *Chaetocnema* spp. such as *C. pulicaria* (corn flea beetle); *Popillia* spp. such as *P. japonica* (Japanese beetle); *Epilachna* spp. such as *E. varivestis* (Mexican bean beetle); *Cerotoma* spp. such as *C. trifurcate* (Bean leaf beetle); *Epicauta* spp. such as *E. pestifera and E. lemniscata* (Blister beetles); and any combination of the foregoing.

[0110] The binary toxins of the invention or the sBin-IPs of the invention may also be active against insects in the order Lepidoptera. Such lepidopteran insects include, without limitation any insect now known or later identified that is classified as a lepidopteran insect. Exemplary lepidopteran insects include, but are not limited to, *Ostrinia* spp. such as *O. nubilalis* (European corn borer); *Plutella* spp. such as *P. xylostella* (diamondback moth); *Spodoptera* spp. such as *S. frugiperda* (fall armyworm), *S. ornithogalli* (yellowstriped armyworm), *S. praefica* (western yellowstriped armyworm), *S. eridania* (southern armyworm) and *S. exigua* (beet armyworm); *Agrotis* spp. such as *A. ipsilon* (black cutworm), *A. segetum* (common cutworm), *A. gladiaria* (claybacked cutworm), and *A. orthogonia* (pale western cutworm); *Striacosta* spp. such as *S. albicosta* (western bean cutworm); *Helicoverpa* spp. such as *H. zea* (corn earworm), *H. punctigera* (native budworm), *S. littoralis* (Egyptian cotton leafworm) and *H. armigera* (cotton bollworm); *Heliothis* spp. such as *H. virescens* (tobacco budworm); *Diatraea* spp. such as *D. grandiosella* (southwestern corn borer) and *D. saccharalis* (sugarcane borer); *Trichoplusia* spp. such as *T. ni* (cabbage looper); *Sesamia* spp. such as *S.*

nonagroides (Mediterranean corn borer); *Pectinophora* spp. such as *P. gossypiella* (pink bollworm); *Cochylis* spp. such as *C. hospes* (banded sunflower moth); *Manduca* spp. such as *M. sexta* (tobacco hornworm) and *M. quinquemaculata* (tomato hornworm); *Elasmopalpus* spp. such as *E. lignosellus* (lesser cornstalk borer); *Pseudoplusia* spp. such as *P. includens* (soybean looper); *Anticarsia* spp. such as *A. gemmatalis* (velvetbean caterpillar); *Plathypena* spp. such as *P. scabra* (green cloverworm); *Pieris* spp. such as *P. brassicae* (cabbage butterfly), *Papaipema* spp. such as *P. nebris* (stalk borer); *Pseudaletia* spp. such as *P. unipuncta* (common armyworm); *Peridroma* spp. such as *P. saucia* (variegated cutworm); *Keiferia* spp. such as *K. lycopersicella* (tomato pinworm); *Artogeia* spp. such as *A. rapae* (imported cabbageworm); *Phthorimaea* spp. such as *P. operculella* (potato tuberworm); *Crymodes* spp. such as *C. devastator* (glassy cutworm); *Feltia* spp. such as *F. ducens* (dingy cutworm); and any combination of the foregoing.

[0111] The binary toxins of the invention of the sBin-IPs of the invention may also be active against Hemipteran, Dipteran, *Lygus* spp., and/or other piercing and sucking insects, for example of the order Orthoptera or Thysanoptera. Insects in the order Diptera include but are not limited to any dipteran insect now known or later identified including but not limited to *Liriomyza* spp. such as *L. trifolii* (leafminer) and *L. sativae* (vegetable leafminer); *Scrobipalpula* spp. such as *S. absoluta* (tomato leafminer); *Delia* spp. such as *D. platura* (seedcorn maggot), *D. brassicae* (cabbage maggot) and *D. radicum* (cabbage root fly); *Psilia* spp. such as *P. rosae* (carrot rust fly); *Tetanops* spp. such as *T. myopaeformis* (sugarbeet root maggot); and any combination of the foregoing.

[0112] Insects in the order Orthoptera include but are not limited to any orthopteran insect now known or later identified including but not limited to *Melanoplus* spp. such as *M. differentialis* (Differential grasshopper), *M. femurrubrum* (Redlegged grasshopper), *M. bivittatus* (Twostriped grasshopper); and any combination thereof.

[0113] Insects in the order Thysanoptera include but are not limited to any thysanopteran insect now known or later identified including but not limited to *Frankliniella* spp. such as *F. occidentalis* (western flower thrips) and *F. fusca* (tobacco thrips); and *Thrips* spp. such as *T. tabaci* (onion thrips), *T. palmi* (melon thrips); and any combination of the foregoing.

[0114] The binary toxins of the invention or the sBin-IPs of the invention may also be active against nematodes. The term "nematode" as used herein encompasses any organism that is now known or later identified that is classified in the animal kingdom, phylum Nematoda, including without limitation nematodes within class Adenophorea (including for example, orders Enoplida, Isolaimida, Mononchida, Dorylaimida, Trichocephalida, Mermithida, Muspiceida, Araeolaimida, Chromadorida, Desmoscolecida, Desmodorida and Monhysterida) and/or class Secernentea (including, for example,

orders Rhabdita, Strongylida, Ascaridida, Spirurida, Camallanida, Diplogasterida, Tylenchida and Aphelenchida).

[0115] Nematodes include but are not limited to parasitic nematodes such as root-knot nematodes, cyst nematodes and/or lesion nematodes. Exemplary genera of nematodes according to the present invention include but are not limited to, *Meloidogyne* (root-knot nematodes), *Heterodera* (cyst nematodes), *Globodera* (cyst nematodes), *Radopholus* (burrowing nematodes), *Rotylenchulus* (reniform nematodes), *Pratylenchus* (lesion nematodes), *Aphelenchoides* (foliar nematodes), *Helicotylenchus* (spiral nematodes), *Hoplolaimus* (lance nematodes), *Paratrichodorus* (stubby-root nematodes), *Longidorus*, *Nacobbus* (false root-knot nematodes), *Subanguina*, *Belonlaimus* (sting nematodes), *Criconemella*, *Criconemoides* (ring nematodes), *Ditylenchus*, *Dolichodorus*, *Hemicriconemoides*, *Hemicycliophora*, *Hirschmaniella*, *Hypsoperine*, *Macroposthonia*, *Melinius*, *Punctodera*, *Quinisulcius*, *Scutellonema*, *Xiphinema* (dagger nematodes), *Tylenchorhynchus* (stunt nematodes), *Tylenchulus, Bursaphelenchus* (round worms), and any combination thereof.

[0116] Exemplary plant parasitic nematodes according to the present invention include, but are not limited to, *Belonolaimus gracilis*, *Belonolaimus longicaudatus*, *Bursaphelenchus xylophilus* (pine wood nematode), *Criconemoides ornata*, *Ditylenchus destructor* (potato rot nematode), *Ditylenchus dipsaci* (stem and bulb nematode), *Globodera pallida* (potato cyst nematode), *Globodera rostochiensis* (golden nematode), *Heterodera glycines* (soybean cyst nematode), *Heterodera schachtii* (sugar beet cyst nematode); *Heterodera zeae* (corn cyst nematode), *Heterodera avenae* (cereal cyst nematode), *Heterodera carotae*, *Heterodera trifolii*, *Hoplolaimus columbus, Hoplolaimus galeatus, Hoplolaimus magnistylus*, *Longidorus breviannulatus, Meloidogyne arenaria, Meloidogyne chitwoodi, Meloidogyne hapla, Meloidogyne incognita, Meloidogyne javanica*, *Mesocriconema xenoplax*, *Nacobbus aberrans*, *Naccobus dorsalis, Paratrichodorus christiei*, *Paratrichodorus minor*, *Pratylenchus brachyurus, Pratylenchus crenatus, Pratylenchus hexincisus, Pratylenchus neglectus*, *Pratylenchus penetrans*, *Pratylenchus projectus, Pratylenchus scribneri*, *Pratylenchus tenuicaudatus*, *Pratylenchus thornei*, *Pratylenchus zeae*, *Punctodera chaccoensis, Quinisulcius acutus, Radopholus similis*, *Rotylenchulus reniformis*, *Tylenchorhynchus dubius, Tylenchulus semipenetrans* (citrus nematode), *Siphinema americanum, X. Mediterraneum*, and any combination of the foregoing.

[0117] The invention also encompasses recombinant vectors and/or recombinant constructs, which may also be referred to as vectors or constructs, comprising the expression cassettes and/or the nucleic acid molecules of invention. In such vectors, the nucleic acids are preferably in expression cassettes comprising regulatory elements for expression of the nucleotide molecules in a host cell capable of expressing the nucleotide molecules. Such regulatory elements usually comprise promoter and termination signals and preferably also comprise elements allowing efficient translation of

polypeptides encoded by the nucleic acids of the present invention. Vectors comprising the nucleic acids are capable of replication in particular host cells, preferably as extrachromosomal molecules, and are therefore used to amplify the nucleic acids of this invention in the host cells.

[0118] The invention also encompasses a host cell that comprises a recombinant vector, an expression cassette or a nucleic acid molecule of the invention. In other embodiments, such vectors are viral vectors and are used for replication of the nucleotide sequences in particular host cells, e.g. insect cells or plant cells. Recombinant vectors are also used for transformation of the nucleic acid molecules of this invention into host cells, whereby the nucleic acid molecules are stably integrated into the DNA of a transgenic host. In some embodiments, the host cell is a bacterial cell or a plant cell. In some aspects of these embodiments, the bacterial cell is in the Genus *Bacillus, Clostridium, Xenorhabdus, Photorhabdus, Pasteuria, Escherichia, Pseudomonas, Erwinia, Serratia, Klebsiella, Salmonella, Pasteurella, Xanthomonas, Streptomyces, Rhizobium, Sinorhizobium, Ensifer, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, Sphingomonas, Burkholderia, Candidatus* Glomeribacter, *Dyella, Herbaspirillum, Bradyrhizobium, Staphylococcus, Methylophilus, Variovorax, Streptococcus, Chitinophaga* or *Alcaligenes*. In other aspects of these embodiments, host cells for such recombinant vectors are endophytes or epiphytes. In some other aspects of these embodiments, the host cell is plant cell, for example a dicot plant cell or monocot plant cell. In other aspects, the dicot plant cell is selected from the group consisting of a soybean cell, sunflower cell, tomato cell, cole crop cell, cotton cell, sugar beet cell and tobacco cell. In still other aspects, the monocot plant cell is selected from the group consisting of a barley cell, maize cell, oat cell, rice cell, sorghum cell, sugar cane cell and wheat cell.

[0119] In some non-limiting embodiments of the invention, at least one of the nucleic acid molecules of the invention is inserted into an appropriate expression cassette, comprising a promoter and termination signal. Expression of the nucleic acid may be constitutive, or an inducible promoter responding to various types of stimuli to initiate transcription may be used. In another embodiment, the cell in which the insecticidal protein of the invention is expressed is a microorganism, such as a virus, bacteria, or a fungus. In yet another embodiment, a virus, such as a baculovirus, contains a nucleic acid of the invention in its genome and expresses large amounts of the corresponding insecticidal protein after infection of appropriate eukaryotic cells that are suitable for virus replication and expression of the nucleic acid. The insecticidal protein thus produced is used as an insecticidal agent. Alternatively, baculoviruses engineered to include the nucleic acid are used to infect insects *in vivo* and kill them either by expression of the insecticidal toxin or by a combination of viral infection and expression of the insecticidal toxin. In a further embodiment, the present invention also

encompasses a method for producing a polypeptide with insecticidal activity, comprising culturing the host cell under conditions in which the nucleic acid molecule encoding the polypeptide is expressed.

[0120] Bacterial cells are also hosts for the expression of the nucleic acids of the invention. In one embodiment, non-pathogenic symbiotic bacteria, which are able to live and replicate within plant tissues, so-called endophytes, or non- pathogenic symbiotic bacteria, which are capable of colonizing the phyllosphere or the rhizosphere, so-called epiphytes, are used. Such bacteria include bacteria of the genera *Agrobacterium, Alcaligenes, Azospirillum, Azotobacter, Bacillus, Clavibacter, Enterobacter, Erwinia, Flavobacter, Klebsiella, Pseudomonas, Rhizobium, Sinorhizobium, Ensifer, Serratia, Streptomyces, Sphingomonas, Burkholderia, Candidatus* Glomeribacter, *Dyella, Herbaspirillum, Bradyrhizobium, Staphylococcus, Methylophilus, Variovorax, Streptococcus, Chitinophaga* and *Xanthomonas*. Symbiotic fungi, such as *Trichoderma* and *Gliocladium* are also possible hosts for expression of the inventive nucleic acids for the same purpose.

[0121] Techniques for these genetic manipulations are specific for the different available hosts and are known in the art. For example, the expression vectors pKK223-3 and pKK223-2 can be used to express heterologous genes in *E. coli*, either in transcriptional or translational fusion, behind the tac or trc promoter. For the expression of operons encoding multiple ORFs, the simplest procedure is to insert the operon into a vector such as pKK223- 3 in transcriptional fusion, allowing the cognate ribosome binding site of the heterologous genes to be used. Techniques for overexpression in gram-positive species such as *Bacillus* are also known in the art and can be used in the context of this invention (Quax et al. In:Industrial Microorganisms:Basic and Applied Molecular Genetics, Eds. Baltz *et al*., American Society for Microbiology, Washington (1993)). Alternate systems for overexpression rely for example, on yeast vectors and include the use of Pichia, Saccharomyces and Kluyveromyces (Sreekrishna, In:Industrial microorganisms:basic and applied molecular genetics, Baltz, Hegeman, and Skatrud eds., American Society for Microbiology, Washington (1993); Dequin & Barre, Biotechnology L2:173- 177 (1994); van den Berg *et al*., Biotechnology 8:135-139 (1990)).

[0122] In yet other embodiments, the invention encompasses a method of controlling insect pests, comprising delivering to the insect pests an effective insect-controlling amount of an insecticidal protein of the invention. In some aspects of these embodiments, the insecticidal protein is delivered through a transgenic plant or by topical application of an insecticidal composition comprising the insecticidal protein. In other aspects, the transgenic plant or the insecticidal composition comprises a second insecticidal agent different from the sBin-IP of the invention. In still other aspects, the second insecticidal agent is a protein, a dsRNA or a chemical. In still other aspects, the protein is selected from the group consisting of a Cry protein, a VIP toxin, a patatin, a protease, a protease inhibitor, a

urease, an alpha-amylase inhibitor, a pore-forming protein, a lectin, an engineered antibody or antibody fragment, or a chitinase; or the chemical is a carbamate, a pyrethroid, an organophosphate, a friprole, a neonicotinoid, an organochloride, a nereistoxin, or a combination thereof; or the chemical comprises an active ingredient selected from the group consisting of carbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, and a combination thereof.

[0123] In some embodiments of the invention, at least one of the sBin-IP toxins of the invention is expressed in a higher organism such as a plant. Transgenic plants expressing effective insect-controlling amounts of the insecticidal protein protect themselves from insect pest damage. When the insect pest starts feeding on such a transgenic plant, it also ingests the expressed insecticidal protein. This may deter the insect from further biting into the plant tissue and/or may even harm or kill the insect. A nucleic acid molecule of the present invention is inserted into an expression cassette, which may then be stably integrated in the genome of the plant. In other embodiments, the nucleic acid molecule is included in a non- pathogenic self- replicating virus. Plants transformed in accordance with the present invention may be monocotyledonous or dicotyledonous and include, but are not limited to, corn, wheat, oat, turfgrass, pasture grass, flax, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugar beet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, Arabidopsis, and woody plants such as coniferous and deciduous trees.

[0124] In some embodiments, the invention encompasses a method of producing a protein that is toxic to insect pests, i.e. an insecticidal protein, comprising: (a) obtaining a host cell comprising a gene, which itself comprises an expression cassette and/or a nucleic acid molecule of the invention; and (b) growing the transgenic host cell or a transgenic host comprising the host cell under conditions in which the host cell produces the protein that is toxic to insect pests.

[0125] In other embodiments, the invention encompasses a method of producing a transgenic plant or plant part having enhanced insect resistance compared to a control plant or plant part, comprising: (a) introducing into a plant or plant part a chimeric gene or expression cassette or vector comprising a nucleic acid molecule encoding an insecticidal protein of the invention, wherein the insecticidal protein is expressed in the plant or plant part, thereby producing a plant or plant part with enhanced

insect-resistance. In other embodiments, the chimeric gene, expression cassette or vector may encode a sBin-IP toxin of the invention comprising, consisting essentially of or consisting of an amino acid sequence that is at least 80%, at least 85%, at least 90%, at least consisting 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is 100% identical or similar to any of SEQ ID NOs:1-6. "Enhanced" insect resistance may be measured as any toxic effect the transgenic plant has on the insect pest that feeds on the transgenic plant. Enhanced insect resistance may be greater than 0%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 125%, at least 150%, at least 200%, at least 300%, at least 400%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or at least 1000% greater insecticidal activity compared to a control plant that does not express the insecticidal protein. A plant or plant part having enhance insect resistance as compared to a control plant or plant part may be produced by methods of plant transformation, plant tissue culture, or breeding. The plant or plant part may be produced by methods of sexual or asexual propagation. Any suitable control plant or plant part can be used, for example a plant of the same or similar genetic background grown in the same environment. In embodiments, the control plant or plant part is of the same genetic background and is growing in the same environment as the described plant, but it does not comprise a molecule of the invention, while the described plant does comprise a nucleic acid molecule of the invention.

[0126] In other embodiments, the invention encompasses a method of enhancing insect resistance in a plant or plant part as compared to a control plant or plant part, comprising expressing in the plant or plant part a nucleic acid molecule or an expression cassette of the invention, wherein expression of the heterologous nucleic acid of the expression cassette results in enhanced insect resistance in a plant or plant part as compared to a control plant or plant part. In some embodiments, the expression cassette or nucleic acid molecule comprises a promoter operably linked to a heterologous nucleic acid molecule comprising a nucleotide sequence that comprises, consists essentially of or consists of (a) a nucleotide sequence of any of SEQ ID NOs:7-24; (b) a nucleotide sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is 100% identical to the nucleotide sequence of any one of SEQ ID NOs:7-24; (c) a nucleotide sequence that encodes a protein, wherein the amino acid sequence of the protein comprises, consists essentially of or consists of any of SEQ ID NOs:1-6; (d) a nucleotide sequence that encodes a protein, wherein the amino acid sequence of the protein is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is 100% identical to the amino acid

sequence of any of SEQ ID NOs:1-6; (e) a nucleotide sequence of any of (a) to (d) above, that is codon optimized for expression in a transgenic host organism; or (f) a nucleotide sequence that is complementary to the nucleotide sequence of any one of (a) to (e) above. The nucleic acid molecule or expression cassette may be introduced into the plant. In some embodiments, the nucleic acid molecule or expression cassette may be introduced into a plant part and a plant comprising the nucleic acid molecule or expression cassette may be produced from the plant part.

[0127] In some embodiments, the invention encompasses a method of producing a plant having enhanced insect resistance as compared to a control plant, comprising detecting, in a plant part, a heterologous nucleic acid comprising a nucleic acid molecule or an expression cassette of the invention and producing a plant from the plant part, thereby producing a plant having enhanced insect resistance as compared to a control plant. In a further embodiment, the invention encompasses a method of identifying a plant or plant part having enhanced insect resistance as compared to a control plant or plant part, comprising detecting, in the plant or plant part, a nucleic acid molecule or an expression cassette of the invention, thereby identifying a plant or plant part having enhanced insect resistance. In a further embodiment, the expression cassette or a diagnostic fragment thereof is detected in an amplification product from a nucleic acid sample from the plant or plant part. The diagnostic fragment may be a nucleic acid molecule at least 10 contiguous nucleotides long which is unique to the expression cassette of the invention.

[0128] In other embodiments, the invention encompasses a method of producing a plant having enhanced insect resistance as compared to a control plant or plant part, comprising crossing a first parent plant with a second parent plant, wherein at least the first parent plant comprises within its genome a heterologous nucleic acid that comprises a nucleic acid molecule or an expression cassette of the invention and producing a progeny generation, wherein the progeny generation comprises at least one plant that possesses the heterologous nucleic acid within its genome and that exhibits enhanced insect resistance as compared to a control plant.

[0129] In some aspects of the above described embodiments, the methods of the invention confer enhanced insect resistance in a plant or plant part against a coleopteran insect pest. Insect control of coleopteran insect pests are demonstrated in the Examples. In further aspects, the methods of the invention confer enhanced insect resistance in a plant or plant part against *Diabrotica* species, including *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi, Diabrotica virgifera zeae,* and/or *Diabrotica speciosa,* and/or related species. In further embodiments, the methods of the invention confer enhanced insect resistance in a plant or plant part against *Diabrotica virgifera virgifera, Diabrotica barberi,* and/or *Diabrotica undecimpunctata howardi.*

[0130] In some embodiments, invention encompasses a transgenic plant comprising a heterologous nucleic acid molecule or an expression cassette of the invention, which when transcribed and translated confers enhanced insect resistance to the transgenic plant. In some aspects of these embodiments, the heterologous nucleic acid molecule or expression cassette comprises a nucleotide sequence that has at least 80%, at least 85%, at least 90%, at least 91% at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% at least 99%, or 100% identical to any of SEQ ID NOs:7-24. In other embodiments, the transgenic plant is a dicotyledonous plant or a monocotyledonous plant. In further aspects, the transgenic plant is alfalfa, apple, apricot, artichoke, arugula, asparagus, avocado, banana, beans, beet, blackberry, blueberry, broccoli, brussel sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, cilantro, citrus, clementine, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom, nut, okra, onion, orange, an ornamental plant, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, turf, a vine, watermelon, yams, or zucchini. In still other aspects, the transgenic plant is millet, switchgrass, maize, sorghum, wheat, oat, turf grass, pasture grass, flax, rice, sugarcane, oilseed rape, or barley. In other embodiments, the transgenic plant is a transgenic maize (corn) plant comprising a *sBin-IP* coding sequence with codons optimized for expression in maize, for example any of SEQ ID NOs:19-24.

[0131] In some embodiments, the invention encompasses nucleic acid molecules encoding insecticidal proteins of the invention that are modified and optimized for expression in transgenic plants. Although in many cases genes from microbial organisms can be expressed in plants at high levels without modification, low expression in transgenic plants may result from microbial nucleic acids having codons that are not preferred in plants. It is known in the art that all organisms have specific preferences for codon usage, and the codons of the nucleic acids described in this invention can be changed to conform with plant preferences, while maintaining the amino acids encoded thereby, or making certain amino acid changes to the encoded insecticidal protein. Furthermore, high expression in plants is best achieved from coding sequences that have at least about 35% GC content, preferably more than about 45%, more preferably more than about 50%, and most preferably more than about 60%. Microbial nucleic acids that have low GC contents may express poorly in plants due to the existence of ATTTA motifs that may destabilize messages, and AATAAA motifs that may cause inappropriate polyadenylation. In embodiments, sequences can be modified to account for the specific

codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al*. Nucl. Acids Res. 17:477-498 (1989)). In addition, the nucleic acids are screened for the existence of illegitimate splice sites that may cause message truncation. All changes required to be made within the nucleic acids such as those described above can be made using well known techniques of site directed mutagenesis, PCR, and synthetic gene construction, for example, using the methods described in the published patent applications EP 0 385 962, EP 0 359 472, and WO 93/07278.

[0132] In some embodiments of the invention a coding sequence for an insecticidal protein of the invention is made according to the procedure disclosed in U.S. Patent 5,625,136, herein incorporated by reference. In this procedure, maize preferred codons, i.e., the single codon that most frequently encodes that amino acid in maize, are used. The maize preferred codon for a particular amino acid might be derived, for example, from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is found in Murray et al., Nucleic Acids Research 17:477-498 (1989), the disclosure of which is incorporated herein by reference. In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

[0133] For more efficient initiation of translation, sequences adjacent to the initiating methionine may be modified. For example, they can be modified by the inclusion of sequences known to be effective in plants. Joshi has suggested an appropriate consensus for plants (NAR 15:6643-6653 (1987)) and Clontech suggests a further consensus translation initiator (1993/1994 catalog, page 210). These consensus sequences are suitable for use with the nucleic acids of this invention. In embodiments, the sequences are incorporated into constructions comprising the nucleic acids, up to and including the ATG (whilst leaving the second amino acid unmodified), or alternatively up to and including the GTC subsequent to the ATG (with the possibility of modifying the second amino acid of the transgene).

[0134] Expression of the nucleic acids in transgenic plants is driven by promoters that function in plants. The choice of promoter will vary depending on the temporal and spatial requirements for expression, and also depending on the target species. Thus, expression of the nucleic acids of this invention in leaves, in stalks or stems, in ears, in inflorescences (e.g. spikes, panicles, cobs, etc.), in roots, and/or seedlings is preferred. In many cases, however, protection against more than one type of insect pest is sought, and thus expression in multiple tissues is desirable. Although many promoters from dicotyledons have been shown to be operational in monocotyledons and vice versa, ideally dicotyledonous promoters are selected for expression in dicotyledons, and monocotyledonous promoters for expression in monocotyledons. However, there is no restriction to the provenance of

selected promoters; it is sufficient that they are operational in driving the expression of the nucleic acids in the desired cell.

[0135] In some embodiments, promoters are used that are expressed constitutively including the actin or ubiquitin or cmp promoters or the CaMV35S and 19S promoters. The nucleic acids of this invention can also be expressed under the regulation of promoters that are chemically regulated. Preferred technology for chemical induction of gene expression is detailed in the published application EP 0 332 104 (to Ciba-Geigy) and U.S. Patent 5,614,395. A preferred promoter for chemical induction is the tobacco PR-1a promoter.

[0136] In other embodiments, a category of promoters which is wound inducible can be used. Numerous promoters have been described which are expressed at wound sites and also at the sites of phytopathogen infection. Ideally, such a promoter should only be active locally at the sites of infection, and in this way the insecticidal proteins of the invention only accumulate in cells that need to synthesize the proteins to kill the invading insect pest. Preferred promoters of this kind include those described by Stanford *et al.* Mol. Gen. Genet. 215:200-208 (1989), Xu *et al.* Plant Molec. Biol. 22:573-588 (1993), Logemann *et al.* Plant Cell 1:151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22:783-792 (1993), Firek *et al.* Plant Molec. Biol. 22:129-142 (1993), and Warner *et al.* Plant J. 3:191-201 (1993).

[0137] Tissue-specific or tissue-preferential promoters useful for the expression of genes encoding insecticidal proteins of the invention in plants, particularly corn, are those which direct expression in root, pith, leaf or pollen, particularly root. Such promoters, e.g. those isolated from PEPC or trpA, are disclosed in U.S. Pat. No. 5,625,136, or MTL, disclosed in U.S. Pat. No. 5,466,785. Both U. S. patents are herein incorporated by reference in their entirety.

[0138] In addition, promoters functional in plastids can be used. Non-limiting examples of such promoters include the bacteriophage T3 gene 9 5' UTR and other promoters disclosed in U.S. Patent No. 7,579,516. Other promoters useful with the invention include but are not limited to the S-E9 small subunit RuBP carboxylase promoter and the Kunitz trypsin inhibitor gene promoter (Kti3).

[0139] In some embodiments of the invention, inducible promoters can be used. Thus, for example, chemical-regulated promoters can be used to modulate the expression of nucleotide sequences of the invention in a plant through the application of an exogenous chemical regulator. Regulation of the expression of nucleotide sequences of the invention via promoters that are chemically regulated enables the polypeptides of the invention to be synthesized only when the crop plants are treated with the inducing chemicals. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of a chemical induces expression of a nucleotide sequence of the

invention, or a chemical-repressible promoter, where application of the chemical represses expression of a nucleotide sequence of the invention.

[0140] Chemical inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1 a promoter, which is activated by salicylic acid (e.g., the PR1a system), steroid steroid-responsive promoters (see, e.g., the glucocorticoid-inducible promoter in Schena et al. (1991) Proc. Natl. Acad. Sci. USA 88, 10421-10425 and McNellis et al. (1998) Plant J. 14, 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, e.g., Gatz et al. (1991) Mol. Gen. Genet. 227, 229-237, and U.S. Patent Numbers 5,814,618 and 5,789,156, Lac repressor system promoters, copper-inducible system promoters, salicylate-inducible system promoters (e.g., the PR1a system), glucocorticoid-inducible promoters (Aoyama et al. (1997) Plant J. 11:605-612), and ecdysone-inducible system promoters.

[0141] Other non-limiting examples of inducible promoters include ABA- and turgor-inducible promoters, the auxin-binding protein gene promoter (Schwob et al. (1993) Plant J. 4:423-432), the UDP glucose flavonoid glycosyl-transferase promoter (Ralston et al. (1988) Genetics 119:185-197), the MPI proteinase inhibitor promoter (Cordero et al. (1994) Plant J. 6:141-150), and the glyceraldehyde-3-phosphate dehydrogenase promoter (Kohler et al. (1995) Plant Mol. Biol. 29:1293-1298; Martinez et al. (1989) J. Mol. Biol. 208:551-565; and Quigley et al. (1989) J. Mol. Evol. 29:412-421). Also included are the benzene sulphonamide-inducible (US Patent No. 5,364,780) and alcohol-inducible (Int'l Patent Application Publication Nos. WO 97/06269 and WO 97/06268) systems and glutathione S-transferase promoters. Likewise, one can use any of the inducible promoters described in Gatz (1996) Current Opinion Biotechnol. 7:168-172 and Gatz (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:89-108. Other chemically inducible promoters useful for directing the expression of the nucleotide sequences of this invention in plants are disclosed in US Patent 5,614,395 herein incorporated by reference in its entirety. Chemical induction of gene expression is also detailed in the published application EP 0 332 104 (to Ciba- Geigy) and U.S. Patent 5,614,395. In some embodiments, a promoter for chemical induction can be the tobacco PR-1a promoter.

[0142] In further embodiments, the nucleotide sequences of the invention can be operably associated with a promoter that is wound inducible or inducible by pest or pathogen infection (e.g., a insect or nematode plant pest). Numerous promoters have been described which are expressed at wound sites and/or at the sites of pest attack (e.g., insect/nematode feeding) or phytopathogen infection. Ideally, such a promoter should be active only locally at or adjacent to the sites of attack, and in this way

expression of the nucleotide sequences of the invention will be focused in the cells that are being invaded or fed upon. Such promoters include, but are not limited to, those described by Stanford et al., Mol. Gen. Genet. 215:200-208 (1989), Xu et al. Plant Molec. Biol. 22:573-588 (1993), Logemann et al. Plant Cell 1:151-158 (1989), Rohrmeier and Lehle, Plant Molec. Biol. 22:783-792 (1993), Firek et al. Plant Molec. Biol. 22:129-142 (1993), Warner et al. Plant J. 3:191-201 (1993), U.S. Patent No. 5,750,386, U.S. Patent No. 5,955, 646, U.S. Patent No. 6,262,344, U.S. Patent No. 6,395,963, U.S. Patent No. 6,703,541, U.S. Patent No. 7,078,589, U.S. Patent No. 7,196,247, U.S. Patent No. 7,223,901, and U.S. Patent Application Publication 2010043102.

[0143] In some embodiments of the invention, a "minimal promoter" or "basal promoter" is used. A minimal promoter is capable of recruiting and binding RNA polymerase II complex and its accessory proteins to permit transcriptional initiation and elongation. In some embodiments, a minimal promoter is constructed to comprise only the nucleotides/nucleotide sequences from a selected promoter that are required for binding of the transcription factors and transcription of a nucleotide sequence of interest that is operably associated with the minimal promoter including but not limited to TATA box sequences. In other embodiments, the minimal promoter lacks cis sequences that recruit and bind transcription factors that modulate (e.g., enhance, repress, confer tissue specificity, confer inducibility or repressibility) transcription. A minimal promoter is generally placed upstream (i.e., 5') of a nucleotide sequence to be expressed. Thus, nucleotides/nucleotide sequences from any promoter useable with the present invention can be selected for use as a minimal promoter.

[0144] Numerous other sequences can be incorporated into expression cassettes described in this invention. These include sequences that have been shown to enhance expression such as intron sequences (e.g. from Adhl and bronzel) and viral leader sequences (e.g. from TMV, MCMV and AMV).

[0145] It may be preferable to target expression of the nucleic acids of the present invention to different cellular localizations in the plant. In some cases, localization in the cytosol may be desirable, whereas in other cases, localization in some subcellular organelle may be preferred. Subcellular localization of transgene-encoded enzymes is undertaken using techniques well known in the art. Typically, the DNA encoding the target peptide from a known organelle-targeted gene product is manipulated and fused upstream of the nucleic acid. Many such target sequences are known for the chloroplast and their functioning in heterologous constructions has been shown. The expression of the nucleic acids of the present invention is also targeted to the endoplasmic reticulum or to the vacuoles of the host cells. Techniques to achieve this are well known in the art.

[0146] Vectors suitable for plant transformation are described elsewhere in this specification. For Agrobacterium-mediated transformation, binary vectors or vectors carrying at least one T-DNA

border sequence are suitable, whereas for direct gene transfer any vector is suitable and linear DNA containing only the construction of interest may be preferred. In the case of direct gene transfer, transformation with a single DNA species or co-transformation can be used (Schocher *et al.* Biotechnology 4:1093- 1096 (1986)). For both direct gene transfer and Agrobacterium-mediated transfer, transformation is usually (but not necessarily) undertaken with a selectable marker that may provide resistance to an antibiotic (kanamycin, hygromycin or methotrexate) or a herbicide (basta). Plant transformation vectors comprising the nucleic acid molecules of the present invention may also comprise genes (e.g. phosphomannose isomerase; PMI) which provide for positive selection of the transgenic plants as disclosed in U.S. Patents 5,767,378 and 5,994,629, herein incorporated by reference. The choice of selectable marker is not, however, critical to the invention.

[0147] In some embodiments, the nucleic acid can be transformed into the nuclear genome. In another embodiment, a nucleic acid of the present invention is directly transformed into the plastid genome. A major advantage of plastid transformation is that plastids are generally capable of expressing bacterial genes without substantial codon optimization, and plastids are capable of expressing multiple open reading frames under control of a single promoter. Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, and 5,545,818, in PCT application no. WO 95/16783, and in McBride *et al.* (1994) Proc. Nati. Acad. Sci. USA 91, 7301-7305. The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, e.g., using biolistics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Nati. Acad. Sci. USA 87, 8526-8530; Staub, J. M., and Maliga, P. (1992) Plant Cell 4, 39-45). This resulted in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub, J.M., and Maliga, P. (1993) EMBO J. 12, 601-606). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial aadA gene encoding the spectinomycin-detoxifying enzyme aminoglycoside- 3'- adenyl transferase (Svab, Z., and Maliga, P. (1993) Proc. Natl. Acad. Sci. USA 90, 913-917). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga *Chlamydomonas reinhardtii* (Goldschmidt- Clermont, M.

(1991) Nucl. Acids Res. 19:4083-4089). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention. Typically, approximately 15-20 cell division cycles following transformation are required to reach a homoplastidic state. Plastid expression, in which genes are inserted by homologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear- expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment, a nucleic acid of the present invention is inserted into a plastid-targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplastic for plastid genomes containing a nucleic acid of the present invention are obtained, and are preferentially capable of high expression of the nucleic acid.

[0148] In yet other embodiments, a transgenic plant of the invention may comprise a heterologous nucleic acid molecule which encodes for at least one additional desired trait. The additional trait may be encoded on the same heterologous nucleic acid molecule as a nucleic acid molecule of the invention, or it may be encoded on a second heterologous nucleic acid molecule. The additional desired trait may confer insect resistance to a second insect pest, insect resistance to the same insect pest, abiotic stress tolerance, male sterility, herbicide resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode resistance, modified fatty acid metabolism, modified carbohydrate metabolism, improved nutritional value, improved performance in an industrial process or altered reproductive capability. The additional desired trait may also induce production within the plant of a commercially valuable enzyme or metabolite.

[0149] In some embodiments, the desired additional trait is a second pesticidal agent. The second pesticidal agent may be active on any plant pest, including insects, nematodes, fungi, viruses or bacteria. Examples of insect plant pests include and are not limited to *Nilaparvata* spp. (e.g. *N. lugens (brown planthopper)); Laodelphax* spp. (e.g. *L. striatellus (*small brown planthopper)); *Nephotettix* spp. (e.g. *N. virescens* or *N. cincticeps* (green leafhopper), or *N.nigropictus* (rice leafhopper)); *Sogatella* spp. (e.g. *S. furcifera* (white-backed planthopper)); *Blissus* spp. (e.g. *B. leucopterus leucopterus* (chinch bug)); *Scotinophora* spp. (e.g. *S. vermidulate* (rice blackbug)); *Acrosternum* spp. (e.g. *A. hilare* (green stink bug)); *Parnara* spp. (e.g. *P. guttata* (rice skipper)); *Chilo* spp. (e.g. *C. suppressalis* (rice striped stem borer), *C. auricilius* (gold-fringed stem borer), or *C. polychrysus* (dark-headed stem borer)); *Chilotraea* spp. (e.g. *C. polychrysa* (rice stalk borer)); *Sesamia* spp. (e.g. *S. inferens* (pink rice borer)); *Tryporyza* spp. (e.g. *T. innotata* (white rice borer), or *T. incertulas* (yellow rice borer)); *Cnaphalocrocis* spp. (e.g. *C. medinalis* (rice leafroller)); *Agromyza* spp. (e.g. *A. oryzae* (leafminer), or *A. parvicornis* (corn blot leafminer)); *Diatraea* spp. (e.g. *D. saccharalis* (sugarcane borer), *or D. grandiosella* (southwestern corn borer)); *Narnaga* spp. (e.g. *N.*

aenescens (green rice caterpillar)); *Xanthodes* spp. (e.g. *X. transversa* (green caterpillar)); *Spodoptera* spp. (e.g. *S. frugiperda* (fall armyworm), *S. exigua* (beet armyworm), *S. littoralis* (climbing cutworm) or *S. praefica* (western yellowstriped armyworm)); *Mythimna* spp. (e.g. *Mythmna (Pseudaletia) seperata* (armyworm)); *Helicoverpa* spp. (e.g. *H. zea* (corn earworm)); *Colaspis* spp. (e.g. *C. brunnea* (grape colaspis)); *Lissorhoptrus* spp. (e.g. *L. oryzophilus* (rice water weevil)); *Echinocnemus* spp. (e.g. *E. squamos* (rice plant weevil)); *Diclodispa* spp. (e.g. *D. armigera* (rice hispa)); *Oulema* spp. (e.g. *O. oryzae* (leaf beetle); *Sitophilus* spp. (e.g. *S. oryzae* (rice weevil)); *Pachydiplosis* spp. (e.g. *P. oryzae* (rice gall midge)); *Hydrellia* spp. (e.g. *H. griseola* (small rice leafminer), or *H. sasakii* (rice stem maggot)); *Chlorops* spp. (e.g. *C. oryzae* (stem maggot)); *Diabrotica* spp. (e.g. *D. virgifera virgifera* (western corn rootworm), *D. barberi* (northern corn rootworm), *D. undecimpunctata* howardi (southern corn rootworm), *D. virgifera zeae* (Mexican corn rootworm); *D. balteata* (banded cucumber beetle)); *Ostrinia* spp. (e.g. *O. nubilalis* (European corn borer)); *Agrotis* spp. (e.g. *A.ipsilon* (black cutworm)); *Elasmopalpus* spp. (e.g. *E. lignosellus* (lesser cornstalk borer)); *Melanotus* spp. (wireworms); *Cyclocephala* spp. (e.g. *C. borealis* (northern masked chafer), or *C. immaculata* (southern masked chafer)); *Popillia* spp. (e.g. *P. japonica* (Japanese beetle)); *Chaetocnema* spp. (e.g. *C. pulicaria* (corn flea beetle)); *Sphenophorus* spp. (e.g. *S. maidis* (maize billbug)); *Rhopalosiphum* spp. (e.g. *R. maidis* (corn leaf aphid)); *Anuraphis* spp. (e.g. *A. maidiradicis* (corn root aphid)); *Melanoplus* spp. (e.g. *M. femurrubrum* (redlegged grasshopper) *M. differentialis* (differential grasshopper) or *M. sanguinipes* (migratory grasshopper)); *Hylemya* spp. (e.g. *H. platura* (seedcorn maggot)); *Anaphothrips* spp. (e.g. *A. obscrurus* (grass thrips)); *Solenopsis* spp. (e.g. *S. milesta* (thief ant)); *or* spp. (e.g. *T. urticae* (twospotted spider mite), *T. cinnabarinus* (carmine spider mite); *Helicoverpa spp. (e.g. H. zea* (cotton bollworm), or *H. armigera* (American bollworm)); *Pectinophora* spp. (e.g. *P. gossypiella* (pink bollworm)); *Earias* spp. (e.g. *E. vittella* (spotted bollworm)); *Heliothis* spp. (e.g. *H. virescens* (tobacco budworm)); *Anthonomus* spp. (e.g. *A. grandis* (boll weevil)); *Pseudatomoscelis* spp. (e.g. *P. seriatus* (cotton fleahopper)); *Trialeurodes* spp. (e.g. *T. abutiloneus* (banded-winged whitefly) *T. vaporariorum* (greenhouse whitefly)); *Bemisia* spp. (e.g. *B. argentifoli*i (silverleaf whitefly)); *Aphis* spp. (e.g. *A. gossypii* (cotton aphid)); *Lygus* spp. (e.g. *L. lineolaris* (tarnished plant bug) or *L. hesperus* (western tarnished plant bug)); *Euschistus* spp. (e.g. *E. conspersus* (consperse stink bug)); *Chlorochroa* spp. (e.g. *C. sayi* (Say stinkbug)); *Nezara* spp. (e.g. *N. viridula* (southern green stinkbug)); *Thrips* spp. (e.g. *T. tabaci* (onion thrips)); *Frankliniella* spp. (e.g. *F. fusca* (tobacco thrips), or *F. occidentalis* (western flower thrips)); *Leptinotarsa* spp. (e.g. *L. decemlineata* (Colorado potato beetle), *L. juncta* (false potato beetle), or *L. texana* (Texan false potato beetle)); *Lema* spp. (e.g. *L. trilineata* (three-lined potato beetle)); *Epitrix* spp. (e.g. *E. cucumeris* (potato flea beetle), *E. hirtipennis* (flea beetle), or *E. tuberis* (tuber flea beetle)); *Epicauta*

spp. (e.g. *E. vittata* (striped blister beetle)); *Phaedon* spp. (e.g. *P. cochleariae* (mustard leaf beetle)); *Epilachna* spp. (e.g. *E. varivetis* (mexican bean beetle)); *Acheta* spp. (e.g. *A. domesticus* (house cricket)); *Empoasca* spp. (e.g. *E. fabae* (potato leafhopper)); *Myzus* spp. (e.g. *M. persicae* (green peach aphid)); *Paratrioza* spp. (e.g. *P. cockerelli* (psyllid)); *Conoderus* spp. (e.g. *C. falli* (southern potato wireworm), or *C. vespertinus* (tobacco wireworm)); *Phthorimaea* spp. (e.g. *P. operculella* (potato tuberworm)); *Macrosiphum spp. (e.g. M. euphorbiae* (potato aphid)); *Thyanta spp. (e.g. T. pallidovirens* (redshouldered stinkbug)); *Phthorimaea spp. (e.g. P. operculella* (potato tuberworm)); *Helicoverpa spp. (e.g. H. zea* (tomato fruitworm); *Keiferia spp. (e.g. K. lycopersicella* (tomato pinworm)); *Limonius* spp. (wireworms); *Manduca spp. (e.g. M. sexta* (tobacco hornworm), or *M. quinquemaculata* (tomato hornworm)); *Liriomyza spp. (e.g. L. sativae*, *L. trifolli* or *L. huidobrensis* (leafminer)); *Drosophilla* spp. (e.g. *D. melanogaster, D. yakuba, D. pseudoobscura* or *D. simulans*); *Carabus* spp. (e.g. *C. granulatus*); *Chironomus* spp. (e.g. *C. tentanus*); *Ctenocephalides* spp. (e.g. *C. felis* (cat flea)); *Diaprepes* spp. (e.g. *D. abbreviatus* (root weevil)); *Ips* spp. (e.g. *I. pini* (pine engraver)); *Tribolium* spp. (e.g. *T. castaneum* (red floor beetle)); *Glossina* spp. (e.g. *G. morsitans* (tsetse fly)); *Anopheles* spp. (e.g. *A. gambiae* (malaria mosquito)); *Helicoverpa* spp. (e.g. *H. armigera* (African Bollworm)); *Acyrthosiphon* spp. (e.g. *A. pisum* (pea aphid)); *Apis* spp. (e.g. *A. melifera* (honey bee)); *Homalodisca* spp. (e.g. H. *coagulate* (glassy-winged sharpshooter)); Aedes spp. (e.g. Ae. aegypti *(yellow fever mosquito)); Bombyx spp. (e.g. B. mori (silkworm)); Locusta spp. (e.g. L. migratoria (*migratory locust)); *Boophilus* spp. (e.g. *B. microplus* (cattle tick)); *Acanthoscurria* spp. (e.g. *A. gomesiana* (red-haired chololate bird eater)); *Diploptera* spp. (e.g. *D. punctata* (pacific beetle cockroach)); *Heliconius* spp. (e.g. *H. erato* (red passion flower butterfly) or *H. melpomene* (postman butterfly)); *Curculio* spp. (e.g. *C. glandium* (acorn weevil)); *Plutella* spp. (e.g. *P. xylostella* (diamondback moth)); *Amblyomma* spp. (e.g. *A. variegatum* (cattle tick)); *Anteraea* spp. (e.g. *A. yamamai* (silkmoth)); and *Armigeres* spp. (e.g. *A. subalbatus*).

[0150] The binary toxins of the invention can be used in combination with other pesticidal agents to increase pest target range. Furthermore, the use of the binary toxins of the invention in combination with a second insecticidal agent which has a different mode of action or targets a different receptor in the insect gut has particular utility for the prevention and/or management of insect resistance. In some embodiments, a binary toxin of the invention is combined with a second insecticidal protein selected from the group consisting of Cry1A, Cry1Ab, Cry1Ac, Cry1A.105, Cry1Ae, Cry1B, Cry1C, Cry1C variants, Cry1D, Cry1E, Cry1F, Cry1A/F chimeras, Cry1G, Cry1H, Cry1I, Cry1J, Cry1K, Cry1L, Cry2A, Cry2Ab, Cry2Ae, Cry3, Cry3A variants, Cry3B, Cry4B, Cry6, Cry7, Cry8, Cry9, Cry15, Cry34, Cry35, Cry43A, Cry43B, Cry46A, Cry51Aa1, PtIP-96, PtIP-83, PHI-4, MP467, MP81, PS149B1, DIG-3, DIG-5, DIG-10, DIG-11, DIG-17, DIG-657, IRDIG28688.1, IRDIG28688.1,

IRDIG28684.1, IRDIG28682.1, IRDIG28680.1, IRDIG28674.1, IRDIG28672.1, IRDIG27642, IRDIG28688.1, IRDIG28686.1, IRDIG28684.1, IRDIG28682.1, IRDIG28680.1, IRDIG28674.1, IRDIG28672.1, IRDIG27642, IRDIG28678.2, IRDIG28678.1, IRDIG31125.1, IRDIG28696.1, IRDIG29781.1, IRDIG29779.1, IRDIG30844.1, IRDIG30850.1, IRDIG30852.1, IRDIG30854.1, IRDIG30856.1, IRDIG30858.1, IRDIG30862.1, IRDIG30860.1, IRDIG30848.1 RETIRE2021, VIP3A, VIP3B, VIP3Ab, the binary VIP1 and VIP2, or other vegetative insecticidal protein, mCry3A, eCry3.1Ab, AXMI-001, AXMI-002, AXMI-030, AXMI-035, AXMI-036, AXMI-045, AXMI52, AXMI58, AXMI88, AXMI97, AXMI102, AXMI112, AXMI113, AXMI115, AXMI117, AXMI100, AXMI-115, AXMI-113, and AXMI-005, AXMI134, AXMI-150, AXMI171, AXMI-184, AXMI196, AXMI204, AXMI207, AXMI209, AXMI205, AXMI218, AXMI220, AXMI221z, AXMI222z, AXMI223z, AXMI224z and AXMI225z, AXMI238, AXMI270, AXMI279, AXMI345, AXMI-R1 and variants thereof, IP3 and variants thereof, ET29, ET33, ET34, ET35, ET66, ET70, TIC400, TIC407, TIC417, TIC431, TIC800, TIC807, TIC834, TIC836, TIC844, TIC853, TIC860 or variant thereof, TIC867 or variant thereof, TIC868 or variant thereof, TIC869, TIC900 or related protein, TIC901, TIC1100, TIC1201, TIC1362, TIC1414, TIC1415, TIC1422, TIC1497, TIC1498, TIC1885, TIC1886, TIC1922, TIC1925, TIC1974, TIC2032, TIC2120, TIC2160, TIC3131, TIC3244, TIC6757, TIC7243, TIC7472, and a TIC7473 protein, or hybrid proteins or chimeras made from any of the preceding insecticidal proteins. The second insecticidal agent can also be an agent selected from the group comprising an α amylase, a peroxidase, a cholesterol oxidase, a patatin, a protease, a protease inhibitor, a urease, an alpha-amylase inhibitor, a pore-forming protein, a chitinase, a lectin, an engineered antibody or antibody fragment, a *Bacillus cereus* insecticidal protein, a *Xenorhabdus* spp. (such as *X. nematophila* or *X. bovienii*) insecticidal protein, a *Photorhabdus* spp. (such as *P. luminescens* or *P. asymobiotica*) insecticidal protein, a *Brevibacillus* spp. (such as *B. laterosporous*) insecticidal protein, a *Lysinibacillus* spp. (such as *L. sphearicus*) insecticidal protein, a *Chromobacterium* spp. (such as *C. subtsugae* or *C. piscinae*) insecticidal protein, a *Yersinia* spp. (such as *Y. entomophaga*) insecticidal protein, a *Paenibacillus* spp. (such as *P. propylaea*) insecticidal protein, a *Clostridium* spp. (such as *C. bifermentans*) insecticidal protein, and a lignin. In other embodiments, the second agent may be at least one insecticidal protein derived from an insecticidal toxin complex (Tc) from *Photorhabdus, Xenorhabus, Serratia,* or *Yersinia*. In still other embodiments, the second insecticidal protein may be a binary toxin derived from an insecticidal bacteria, such as ISP1A and ISP2A from *B. laterosporous* or BinA and BinB from *L. sphaericus*. The combination of a sBin-IP of the invention and a second pesticidal agent may be through expression of both in a transgenic plant. In some embodiments the transgenic plant is a transgenic

corn plant. In other embodiments, the combination in the transgenic corn plant is a sBin-IP of the invention and mCry3A and/or eCry3.1Ab and/or Cry3Bb1 and/or Cry34/Cry35.

[0151] In some embodiments, the transgenic plant of the invention may comprise at least a second pesticidal agent which is non-proteinaceous. In preferred embodiments, the second pesticidal agent is an interfering RNA molecule. An interfering RNA typically comprises at least a RNA fragment against a target gene, a spacer sequence, and a second RNA fragment which is complementary to the first, so that a double-stranded RNA structure can be formed. RNA interference (RNAi) occurs when an organism recognizes double-stranded RNA (dsRNA) molecules and hydrolyzes them. The resulting hydrolysis products are small RNA fragments of about 19–24 nucleotides in length, called small interfering RNAs (siRNAs). The siRNAs then diffuse or are carried throughout the organism, including across cellular membranes, where they hybridize to mRNAs (or other RNAs) and cause hydrolysis of the RNA. Interfering RNAs are recognized by the RNA interference silencing complex (RISC) into which an effector strand (or "guide strand") of the RNA is loaded. This guide strand acts as a template for the recognition and destruction of the duplex sequences. This process is repeated each time the siRNA hybridizes to its complementary-RNA target, effectively preventing those mRNAs from being translated, and thus "silencing" the expression of specific genes from which the mRNAs were transcribed. Interfering RNAs are known in the art to be useful for insect control (see, for example, publication WO2013/192256, incorporated by reference herein). An interfering RNA designed for use in insect control produces a non-naturally occurring double-stranded RNA, which takes advantage of the native RNAi pathways in the insect to trigger down-regulation of target genes that may lead to the cessation of feeding and/or growth and may result in the death of the insect pest. The interfering RNA molecule may confer insect resistance against the same target pest as the protein of the invention or may target a different pest. The targeted insect plant pest may feed by chewing, sucking, or piercing. Interfering RNAs are known in the art to be useful for insect control. In other embodiments, the interfering RNA may confer resistance against a non-insect plant pest, such as a nematode pest or a virus pest.

[0152] The co-expression of more than one pesticidal agent in the same transgenic plant can be achieved by making a single recombinant vector comprising coding sequences of more than one pesticidal agent in a so-called molecular stack and genetically engineering a plant to contain and express all the pesticidal agents in the transgenic plant. Such molecular stacks may be also be made by using mini-chromosomes as described, for example in US Patent 7,235,716. Alternatively, a transgenic plant comprising one nucleic acid encoding a first pesticidal agent can be re-transformed with a different nucleic acid encoding a second pesticidal agent and so forth. Alternatively, a plant, Parent 1, can be genetically engineered for the expression of genes of the present invention. A second plant, Parent 2,

can be genetically engineered for the expression of a second pesticidal agent. By crossing Parent 1 with Parent 2, progeny plants are obtained which express all the genes introduced into Parents 1 and 2.

[0153] Transgenic plants or seed comprising an insecticidal protein of the invention can also be treated with an insecticide or insecticidal seed coating as described in U. S. Patent Nos. 5,849,320 and 5,876,739, herein incorporated by reference. Where both the insecticide or insecticidal seed coating and the transgenic plant or seed of the invention are active against the same target insect, for example a Coleopteran pest or a *Diabrotica* target pest, the combination is useful (i) in a method for further enhancing activity of the composition of the invention against the target insect, and (ii) in a method for preventing development of resistance to the composition of the invention by providing yet another mechanism of action against the target insect. Thus, the invention provides a method of enhancing control of a Diabrotica insect population comprising providing a transgenic plant or seed of the invention and applying to the plant or the seed an insecticide or insecticidal seed coating to a transgenic plant or seed of the invention.

[0154] Even where the insecticidal seed coating is active against a different insect, the insecticidal seed coating is useful to expand the range of insect control, for example by adding an insecticidal seed coating that has activity against lepidopteran insects to a transgenic seed of the invention, which, in some embodiments, has activity against coleopteran and some lepidopteran insects, the coated transgenic seed produced controls both lepidopteran and coleopteran insect pests.

[0155] Examples of such insecticides and/or insecticidal seed coatings include, without limitation, a carbamate, a pyrethroid, an organophosphate, a neonicotinoid, an organochloride, a neurotoxin, or a combination thereof. In another embodiment, the insecticide or insecticidal seed coating are selected from the group consisting of carbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, and a combination thereof. Commercial products containing such insecticides and insecticidal seed coatings include, without limitation, Furadan® (carbofuran), Lanate® (methomyl, metomil, mesomile), Sevin® (carbaryl), Talstar® (bifenthrin), Force® (tefluthrin), Ammo® (cypermethrin), Cymbush®(cypermethrin), Delta Gold® (deltamethrin), Karate® (lambda-cyhalothrin), Ambush® (permethrin), Pounce® (permethrin), Brigade® (bifenthrin), Capture® (bifenthrin), ProShield® (tefluthrin), Warrior® (lambda-cyhalothrin), Dursban® (chlorphyrifos), Fortress® (chlorethoxyfos), Mocap® (ethoprop), Thimet® (phorate), AAstar® (phorate, flucythinate), Rampart® (phorate), Counter® (terbufos), Cygon® (dimethoate), Dicapthon, Regent® (fipronil), Cruiser® (thiamethoxam), Gaucho®

(imidacloprid), Prescribe® (imidacloprid), Poncho® (clothianidin) and Aztec® (cyfluthrin, tebupirimphos).

[0156] In some embodiments, the invention also encompasses a composition comprising an effective insect-controlling amount of an insecticidal protein of the invention. In further embodiments, the composition comprises a suitable agricultural carrier and a binary toxin of the invention. The agricultural carrier may include adjuvants, mixers, enhancers, etc. beneficial for application of an active ingredient, such as a protein of the invention, including a protein comprising, consisting essentially of or consisting of an amino acid sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to of any of SEQ ID NO:1-6. Suitable carriers should not be phytotoxic to valuable crops, particularly at the concentrations employed in applying the compositions in the presence of crops and should not react chemically with the compounds of the active ingredient herein, namely a polypeptide of the invention, or other composition ingredients. Such mixtures can be designed for application directly to crops or can be concentrates or formulations which are normally diluted with additional carriers and adjuvants before application. They may include inert or active components and can be solids, such as, for example, dusts, powders, granules, water dispersible granules, or wettable powders, or liquids, for example, emulsifiable concentrates, solutions, emulsions or suspensions. Suitable agricultural carriers may include liquid carriers, for example water, toluene, xylene, petroleum naphtha, crop oil, acetone, methyl ethyl ketone, cyclohexanone, trichloroethylene, perchloroethylene, ethyl acetate, amyl acetate, butyl acetate, propylene glycol monomethyl ether and diethylene glycol monomethyl ether, methanol, ethanol, isopropanol, amyl alcohol, ethylene glycol, propylene glycol, glycerine, and the like. Water is generally the carrier of choice for the dilution of concentrates. Suitable solid carriers may include talc, pyrophyllite clay, silica, clay, kieselguhr, chalk, diatomaceous earth, lime, calcium carbonate, bentonire clay, Fuller's earth, cotton seed hulls, wheat flour, soybean flour, pumice, wood flour, walnut shell flour, lignin, and the like. In other embodiments, a protein of the invention may be encapsulated in a synthetic matrix such as a polymer and applied to the surface of a host such as a plant. Ingestion of the host cells by an insect permits delivery of the insect control agents to the insect and results in a toxic effect in the insect pest.

[0157] In further embodiments, a composition of the invention may be a powder, dust, pellet, granule, spray, emulsion, colloid, or solution. A composition of the invention may be prepared by desiccation, lyophilization, homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of bacterial cells. A composition of the invention may comprise at least 1%, about 5%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or at least 99% by weight a

polypeptide of the invention. A composition of the invention may comprise at least a second pesticidal agent, which may be insecticidal, nematicidal, fungicidal, or bactericidal. At least a second pesticidal agent may be insecticidal to the same insect as a polypeptide of the invention or to a different insect. The second pesticidal agent may be a polypeptide. The pesticidal agent may be an interfering RNA. The second pesticidal agent may be a microorganism, such as a bacteria, which comprises a nucleic acid molecule that encodes for a pesticidal agent and/or contains a pesticidal agent such as a polypeptide or interfering RNA. The microorganism may be attenuated, heat-inactivated, or lyophilized. The microorganism may be dead or unable to reproduce. The second pesticidal agent may be an insecticide, for example arbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, or a combination thereof, or a commercial product containing such insecticides and insecticidal seed coatings as described above.

[0158] A composition of the invention, for example a composition comprising a protein of the invention and an agriculturally acceptable carrier, may be used in conventional agricultural methods. An agriculturally acceptable carrier is a formulation useful for applying a composition comprising a polypeptide of the invention to a plant or seed. For example, the compositions of the invention may be mixed with water and/or fertilizers and may be applied preemergence and/or postemergence to a desired locus by any means, such as airplane spray tanks, irrigation equipment, direct injection spray equipment, knapsack spray tanks, cattle dipping vats, farm equipment used in ground spraying (e.g., boom sprayers, hand sprayers), and the like. The desired locus may be soil, plants, and the like.

[0159] A composition of the invention may be applied to a seed or plant propagule in any physiological state, at any time between harvest of the seed and sowing of the seed; during or after sowing; and/or after sprouting. It is preferred that the seed or plant propagule be in a sufficiently durable state that it incurs no or minimal damage, including physical damage or biological damage, during the treatment process. A formulation may be applied to the seeds or plant propagules using conventional coating techniques and machines, such as fluidized bed techniques, the roller mill method, rotostatic seed treaters, and drum coaters.

[0160] In some embodiments, the invention also comprises a method for controlling a coleopteran pest population comprising contacting the pest population with an effective insect-controlling amount of a binary toxin of the invention, where the binary toxin comprises, consist essentially of or consists of at least two amino acid sequences that have at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least

99%,or is 100% identical to any one of SEQ ID NO:1-6. Contacting includes members of the pest population feeding on or ingesting the insecticidal protein. The insecticidal protein may be incorporated into insect diet food or may be expressed in or present on plant tissue which the insect population then ingests. In further embodiments, controlling the coleopteran pest population includes killing the insects by contacting the insects with an effective insect-controlling amount of an insecticidal protein of the invention.

[0161] The present invention also comprises a method for increasing yield in a plant comprising growing in a field a plant, or a seed thereof, having stably incorporated into its genome a nucleic acid molecule of an expression cassette of the invention, and wherein said field is infested with a pest against which said polypeptide has insecticidal activity.

[0162] Once a desired nucleic acid has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques.

[0163] In some embodiments, the invention encompasses a method of providing a corn grower with a means of controlling a *Diabrotica* insect pest population in a corn crop comprising (a) selling or providing to the grower transgenic corn seed that comprises a nucleic acid molecule, an expression cassette, a vector or a chimeric gene of the invention; and (b) advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a *Diabrotica* pest population.

[0164] In some embodiments, the invention also encompasses a method of identifying an insecticidal protein comprising, consisting essentially of or consisting of a nucleotide sequence having has at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or having 100% sequence identity with any of SEQ ID NOs:1-6, or a toxin fragment thereof, the method comprising the steps of: (a) producing a primer pair that will amplify a polynucleotide of any of SEQ ID NOs:7-12 from a nucleic acid sample, or a complement thereof, (b) amplifying an orthologous polynucleotide from the nucleic acid sample, (c) identifying a nucleotide sequence of an orthologous polynucleotide, (d) producing a protein encoded by the orthologous polynucleotide, and (e) determining that the protein of step (d) has insecticidal activity against an insect pest.

EXAMPLES

[0165] Embodiments of this invention can be better understood by reference to the following examples. The foregoing and following description of embodiments of the invention and the various

embodiments are not intended to limit the claims but are rather illustrative thereof. Therefore, it will be understood that the claims are not limited to the specific details of these examples. It will be appreciated by those skilled in the art that other embodiments of the invention may be practiced without departing from the spirit and the scope of the disclosure, the scope of which is defined by the appended claims. Art recognized recombinant DNA and molecular cloning techniques may be found in, for example, J. Sambrook, *et al.*, Molecular Cloning: *A Laboratory Manual, 3d Ed.,* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (2001); by T.J. Silhavy, M.L. Berman, and L.W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. *et al., Current Protocols in Molecular Biology*, New York, John Wiley and Sons Inc., (1988), Reiter, *et al.*, *Methods in Arabidopsis Research*, World Scientific Press (1992), and Schultz *et al.*, *Plant Molecular Biology Manual*, Kluwer Academic Publishers (1998).

Example 1: Identification of genomic sequences in bacteria that encode insecticidal proteins.

[0166] Based on proprietary algorithms, candidate nucleotide sequences encoding proteins, described in the art as hypothetical proteins, were identified in the genomes of gram-negative bacteria in *Segetibacter*, *Dyadobacter*, and related genera, which belong to the Orders Shingobacteriales. Four candidate sequences were chosen for expression and testing against insect pests. The four candidate nucleotide sequences were identified in the genomes of a strain of *Segetibacter koreensis* (SEQ ID NO:1 and SEQ ID NO:2) and a strain of *Dyadobacter sp.* SG02 (SEQ ID NO:3 and SEQ ID NO:4). *Escherichia coli* (*E. coli*) codon-optimized versions of each candidate coding sequence was produced, SEQ ID NOs:13-16, respectively, and individually introduced into a pET29a bacterial expression vector, designated pET29a-13, pET29a-14, pET29a-15 and pET29a-16, respectively, to generate protein. Each pET29a expression vector was transformed into *E. coli* BL21*(DE3) and a lysate was made from isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cultures with protein production at about 18°C overnight. Lysates were tested for insecticidal activity against western corn rootworm (WCR) in a diet-incorporation bioassay experiment. Briefly, *E. coli* lysates were mixed with an equal volume of heated artificial insect diet (Bioserv, Inc., Frenchtown, NJ) in 1.5 mL centrifuge tubes and then applied to small petri-dishes. After the diet-sample mixture cooled and solidified, WCR larvae were added to each plate. The plates were sealed and maintained at ambient laboratory conditions with regard to temperature, lighting and relative humidity. Buffer without lysate, lysates from *E. coli* BL21*(DE3) cultures harboring the empty pET29a vector, and artificial insect diet alone were used as negative controls. Percent mortality and growth inhibition observations, designated as s = small larvae, m= medium larvae and l= large larvae, were taken at 4 and 6 days post-infestation.

[0167] Results, shown in Table 1, demonstrate that lysates from *E. coli* cultures expressing proteins encoded in the genomes of bacteria in the Orders Shingobacteriales surprisingly have insecticidal activity against *Diabrotica* insect pests, particularly when combined together. These sBin Insecticidal Protein (sBin-IP) combinations were determined to be binary toxins. The binary toxin that comprises the *Segetibacter* proteins was designated the Seg_kor binary toxin and comprises sBin1Aa (SEQ ID NO:1) and sBin2Aa (SEQ ID NO:2). The binary toxin that comprises the *Dyadobacter* proteins was designated the Dyad-SG02 binary toxin and comprises sBin1Ba (SEQ ID NO:3) and sBin2Ab (SEQ ID NO:4). The sBin1 proteins are approximately 32-33 kDa and based on a sequence comparison search, are ETX-MTX2 orthologs. The sBin2 proteins are approximately 12 kDa and do not have any similarity to known protein families. However, they may function as small *Beta*-sheets.

Table 1. Insecticidal activity of lysates comprising sBin proteins against WCR.

Treatment	**% Mortality**	
	Day 3	**Day 6**
BL21*/sBin1Aa	8	17
BL21*/sBin2Aa	0	0
BL21*/sBin1Aa + BL21*/sBin2Aa (1:1)	50	100
BL21*/sBin1Ba	8	33
BL21*/sBin2Ab	17	17
BL21*/sBin1Ba + BL21*/sBin2Ab (1:1)	100	100
BL21*/pET29a-empty	0	8

[0168] The Dyad_SG02 binary toxin comprising the sBin1Ba and sBin2Ab protein components was tested at different concentrations against western corn rootworm and northern corn rootworm using bioassays methods described above. Results, shown in Table 2, demonstrate that the sBin-IPs are active against both *Diabrotica* species.

Table 2. Insecticidal activity of lysates comprising sBin1Ba and sBin2Ab against corn rootworm.

Treatment	**Percent Mortality**			
	Western corn rootworm		**Northern corn rootworm**	
	Day 3	**Day 6**	**Day 3**	**Day 6**
BL21*/sBin1Ba + BL21*/sBin2Ab (1:1@250 µl)	92	100	21	83
BL21*/sBin1Ba + BL21*/sBin2Ab (1:1@100µl)	75	83	NT	NT
BL21*/sBin1Ba + BL21*/sBin2Ab (1:1@40µl)	33	58	NT	NT
BL21*/pET29a-empty	17	8	21	33

Example 2. Purified sBin-IP proteins are active against *Diabrotica*.

[0169] The Dyad-SG02 binary toxin was further characterized for its insecticidal properties. Two liters of *E.coli* BL21* (DE3) cells harboring either a pET vector encoding sBin1Ba or a pET vector encoding sBin2Ab were grown at 37 °C in LB media. IPTG (1 mM) was added to the cultures when the O.D. reached 0.8-1.0 and then the cultures were moved to 18 °C for 18 hours. The cell pellet was harvested and re-suspended in 20 mM Tris, pH 8.5 with 10% glycerol. The cells were lysed using a French pressure cell and the lysate was then spun at 100k x *g* in an ultracentrifuge. The supernatant was collected and then filtered before loading onto a HiPrepQ anion-exchange column that was pre-equilibrated in 20 mM Tris, pH 8.5 with 10% glycerol. The HiPrepQ column bound sBin proteins effectively; each protein was eluted from the column using a linear NaCl gradient. The high-salt buffer consisted of 20 mM Tris, pH 8.5, 0.5 M NaCl with 10% glycerol. The purest fractions were pooled and then concentrated to approximately 2 ml. The protein was loaded onto a Sephadex 200 gel filtration column that had been pre-equilibrated in 1X PBS. Fractions from the Sephadex 200 column were analyzed for purity by SDS-PAGE. The purest fractions were pooled and then concentrated to about 7 mg/ml, prior to storage at -80 °C. The pure proteins were then tested against WCR larvae in the diet-incorporation method essentially as described in Example 1. As shown in Table 3, neither of the components of the Dyad-SG02 binary toxin, sBin1Ba and sBin2Ab, are active alone, but cause 100% mortality when combined. At the concentrations tested against WCR and NCR, the sBin proteins were not active against southern corn rootworm.

Table 3. Insecticidal activity of sBin1Ba and sBin2Ab against corn rootworm.

Treatment	**Percent Mortality**	
	Day 3	**Day 6**
sBin1Ba (200 µg/ml)	8	17
BL21*/sBin2Ab (200 µg/ml)	4	12
sBin1Ba + sBin2Aa (1:1@200µg/ml)	54	100
BL21*/pET29a-empty	4	8

Example 3. Identification of genomic sequences in *Paracoccus* sp. encoding insecticidal proteins.

[0170] Based on proprietary algorithms, candidate nucleotide sequences encoding proteins, described in the art as hypothetical proteins, were identified in the genomes of gram-negative bacteria in *Paracoccus*, and related genera, which belong to the Orders Rhodobacterales. Two candidate sequences were identified in a strain of *Paracoccus pantotrophus* (SEQ ID NO:5 and SEQ ID NO:6). *Escherichia coli* (*E. coli*) codon-optimized versions of each candidate coding sequence was produced,

SEQ ID NOs:17 and SEQ ID NO:18, respectively, and individually introduced into a pET29a bacterial expression vector, designated pET29a-17 and pET29a-18, respectively, to generate protein. Each pET29a expression vector was transformed into *E. coli* BL21*(DE3) and a lysate was made from isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cultures with protein production at about 18°C overnight. Lysates were tested for insecticidal activity against western corn rootworm (WCR) in a diet-incorporation bioassay experiment. Briefly, *E. coli* lysates were mixed with an equal volume of heated artificial insect diet (Bioserv, Inc., Frenchtown, NJ) in 1.5 mL centrifuge tubes and then applied to small petri-dishes. After the diet-sample mixture cooled and solidified, 12 WCR larvae were added to each plate. The plates were sealed and maintained at ambient laboratory conditions with regard to temperature, lighting and relative humidity. Buffer without lysate, lysates from *E. coli* BL21*(DE3) cultures harboring the empty pET29a vector, and artificial insect diet alone were used as negative controls. Percent mortality and growth inhibition observations, designated as s = small larvae, m= medium larvae and l= large larvae, were taken at 4- and 6-days post-infestation.

[0171] Results, shown in Table 4, demonstrate that lysates from *E. coli* cultures expressing proteins encoded in the genomes of bacteria in the Orders Rhodobacterales surprisingly have insecticidal activity against *Diabrotica* insect pests, particularly against western and northern corn rootworm. These sBin Insecticidal Protein (sBin-IP) combinations were designated binary toxins. The binary toxin that comprises the *Segetibacter* proteins was designated the Seg_kor binary toxin and comprises sBin1Aa (SEQ ID NO:1) and sBin2Aa (SEQ ID NO:2). The binary toxin that comprises the Dyadobacter proteins was designated the Dyad-SG02 binary toxin and comprises sBin1Ba (SEQ ID NO:3) and sBin2Ab (SEQ ID NO:4). At the concentrations tested against WCR and NCR, the sBin proteins were not active against southern corn rootworm.

Table 4. Insecticidal activity of lysates comprising sBin proteins against WCR.

Treatment	% Mortality	
	Day 3	Day 6
BL21*/sBin1Ca @250µl	58	92
BL21*/sBin1Ca + BL21*/sBin2Ba (1:1@250µl)	100	100
BL21*/sBin1Ca @100µl	58	83
BL21*/sBin1Ca + BL21*/sBin2Ba (1:1@100µl)	67	92
BL21*/sBin1Ca @50µl	17	83
BL21*/sBin1Ca + BL21*/sBin2Ba (1:1@50µl)	67	92
BL21*/sBin1Ca @20µl	0	8
BL21*/sBin1Ca + BL21*/sBin2Ba (1:1@20µl)	25	67
BL21*/pET29a-empty	0	0

[0172] Unlike the binary toxins described above, the ETX-MTX2-like protein in the Parac_panto binary toxin, sBin1Ca, has some insecticidal activity by itself. However, the insecticidal activity of the Parac_panto binary toxin, sBin1Ca + sBin2Ba, is higher than either of the two components alone. Therefore, maximum insecticidal activity requires both components, sBin1Ca + sBin2Ba.

Example 4. Characterization of sBin protein function.

[0173] This example describes replacing an sBin2 protein from one binary toxin of the invention with a sBin2 protein from a different binary toxin of the invention. The smaller protein component of the Seg-kor binary toxin, sBin2Aa, was tested in combination with the larger protein component of the Dyad_SG02 binary toxin, sBin1Ba, to determine if a binary toxin comprising heterologous proteins would still be functional against corn rootworm. The resulting heterologous binary toxin was tested in bioassays as described above at two different concentrations

The results, shown in Table 5, demonstrate that the components in the different binary toxins of the invention are cross-functional.

Table 5. Insecticidal activity of sBin mixtures against WCR

Treatment	Percent Mortality	
	Day 3	Day 6
BL21*/sBin1Ba + BL21*/sBin2Ab (1:1@250 µl)	4	67
BL21*/sBin1Ba + BL21*/sBin2Aa (1:1@100µl)	30	91
BL21*/pET29a-empty	0	17

Example 5. Sequence relationship of sBin-IPs.

[0174] An alignment of the sBin-IPs active against *Diabrotica* and a sequence identity comparison is shown in Table 6 and Table 7. The sBin1 components (Table 6) have low identity over the full length of their sequences. The sBin2 components from the Seg_kor binary toxin and the Dyad_SG02 binary toxin have 84% identity, whereas the sBin2 component of the Parac_panto binary toxin has only about 56-59% identity to the sBin2 components from the other binary toxins (Table 7).

Table 6. Alignment and percent identity comparison of sBin1 proteins.

	% Identity Across Entire Length		
	sBin1Aa (SEQ ID NO:1)	sBin1Ba (SEQ ID NO:3)	sBin1Ca (SEQ ID NO:5)
sBin1Aa (SEQ ID NO:1)	-	53	49
sBin1Ba (SEQ ID NO:3)	53	-	45
sBin1Ca (SEQ ID NO:5)	49	45	-

```
SEQ ID NO:1       1 mslqyldkrligaiivdawqnhfntemdpadgqwftngpgnngglygilt
SEQ ID NO:3       1 ...kf...qr........f.eql.......n.................s.v
SEQ ID NO:5       1 .t.....tlgl...........le..k...g....a.........f.k..

SEQ ID NO:1      51 dtlaselkflpdqqvftmnkiaaatstadnrngltphqtvsltyeyqnst
SEQ ID NO:3      51 .a...s.v...nel.l.ph.l..d.aii........kssit.s.sttet.
SEQ ID NO:5      51 .......vldvpa.t.svyqs....giv........e...g.sctf.dtv

SEQ ID NO:1     101 tvthsttntitvgtgveikssaeflgtgaevtvsfnteysysywteerses
SEQ ID NO:3     101 .t..tvs.alk..i..d..a..k.f.s.vdi.tkis.d.t...sdav.ka
SEQ ID NO:5     101 .t...vskavkt..t.s..gtidakvvkk.fgi..ta...h...datav.

SEQ ID NO:1     151 vsetktfgqevst-dipsglvyqvtlladkanirvpfyadiiltgqsvan
SEQ ID NO:3     151 a....q.s.t.pv-ev.t.r....v.tc..tdlna.y...vt...t.t..
SEQ ID NO:5     151 k..srs.svs.pvrnv.a.r.w..v.m.n.kelsm.yr.....k.st...

SEQ ID NO:1     200 faspvngqktwaidagtlcewinqygsagnesyrymkdasnpkqgfirle
SEQ ID NO:3     200 ..nn...kn..vl..........rs....g..hm.lr.pqvtg..l..mr
SEQ ID NO:5     201 .l..ir..ri.qa..........rh....d..ws.gr.pad.t..r.s.l

SEQ ID NO:1     250 gnltatqtlnftaltsditdsftataqpalmre--ln-nselekleskai
SEQ ID NO:3     250 .s..ssi.a..vvn.y....tyn..gka.iendhlfa-a...as.n..lv
SEQ ID NO:5     251 .t.k.vh.v...vr.l.v.e..rpdgdgg.vla--t.ag..apvvdevlv

SEQ ID NO:1     297 kkvalg-
SEQ ID NO:3     299 sekvi.k
SEQ ID NO:5     299 tel.aa-
```

A "." under an amino acid indicates the same amino acid

Table 7. Alignment and percent identity comparison of sBin2 proteins.

	% Identity Across Entire Length		
	sBin2Aa (SEQ ID NO:2)	sBin2Ab (SEQ ID NO:4)	sBin2Ba (SEQ ID NO:6)
sBin2Aa (SEQ ID NO:2)	-	84	56
sBin2Ab (SEQ ID NO:4)	84	-	59
sBin2Ba (SEQ ID NO:6)	56	59	-

```
SEQ ID NO:2      1 mplqqigkmslknsggfvariqfsyldengekkltgqsgdvllgqtktld
SEQ ID NO:4      1 ....k..............v......d............it..f...y.
SEQ ID NO:6      1 .s..kv.nf..h.g......mk.a.i.de.q..s.ret..i.......ak

SEQ ID NO:2     51 pgemgvpdgsmtymyvsvvwgrdneatraflyqkgnvstahylisgttln
SEQ ID NO:4     51 ...........v..h.f....t....k.....e.....v...n.......
SEQ ID NO:6     51 le.fdi...alv.lh.d....k....a...t.er..tc..a.t.t....s

SEQ ID NO:2    101 ndlglieis-
SEQ ID NO:4    101 .....sd..-
SEQ ID NO:6    101 .t....dvnc
```

Example 6. Insecticidal Activity of sBin toxins against Cry-resistant WCR.

[0175] To determine if sBin toxin activity is through a mode-of-action different from Cry3-related proteins, SUMO labelled sBin1Ba and sBin2Ab were purified as described above and were tested for efficacy against a strain of WCR that is resistant to a Cry3Bb protein, a strain that is resistant to a modified Cry3A (mCry3A) protein (mCry3A-R) and a strain that is resistant to an eCry3.1Ab protein (eCry3.1Ab-R). Diet-incorporation assays were performed essentially as described above, and mortality and growth inhibition observations, where s=small larvae, m=medium larvae and l=large larvae, were taken on days 4 and/or 6 post-infestation. The negative control consisted of 1xPBS. A wild-type WCR strain that is not resistant to Cry proteins was used as a positive control in the. As shown in Table 8, binary toxins of the invention have insecticidal activity against Cry-resistant WCR strains indicating that these proteins have a unique mode of action compared to Cry proteins from *Bacillus thuringiensis*. Thus, combinations of the binary toxins of the invention, for example Dyad-SG02 binary toxin comprising sBin1Ba + sBin2Ab, and Cry proteins would be effective in mitigating the development of resistance to either Cry proteins or to the binary toxins.

Table 8. Activity of binary toxins against Cry-resistant WCR.

Protein Concentration	**Cry3Bb-R** **% Mort.**	**eCry3.1Ab-R** **% Mort.**	**mCry3A-R** **% Mort.**
1X PBS (Neg. Control)	4	8	12
sBin1Ba + sBin2Ab (200 µg/mL)	60	80	63
sBin1Ba (100 µg/mL) + sBin2Ab (200 µg/mL)	60	NT	42

Example 7. Transformation of Maize with sBin-IP coding sequences.

[0176] A nucleotide sequence that encodes a sBin1Aa, sBin2Aa, sBin1Ba, sBin2Ab, sBin1Ca and sBin2Ba of the invention, or variants thereof, e.g. any of SEQ ID NOs:1-6, or a maize-optimized nucleotide sequence, e.g. any of SEQ ID NOs:19-24, which can be generated, for example, as described in US Patent No. 6,051,760, is transformed into corn for control of corn rootworm.

[0177] Two or three plant expression cassettes are constructed to introduce sBin-IP coding sequencse into maize. A first cassette comprises a plant expressible promoter operably linked to an *sBin1* coding sequence, for example SEQ ID NO:19, 21 or 23, which is operably linked to a terminator functional in maize. The second cassette comprises a plant expressible promoter operably linked a sBin2 coding sequence, for example SEQ ID NO: 20, 22 or 24, which is operably linked to a terminator functional in maize. The third cassette comprises a plant expressible promoter operably linked to a *pmi* coding sequence that encodes the selectable marker phosphomannose isomerase (PMI), which is operably linked to a terminator functional in maize. Optionally, a first expression can be constructed operably linking a plant expressible promoter to a nucleotide sequence that comprises a sBin1 coding sequence and a sBin2 coding sequence that are functionally fused, which is operably linked to a terminator functional in maize. Then the second expression cassette would comprise the selectable marker. A recombinant plant transformation binary vector comprising the two or three expression cassettes is generated for maize transformation experiments.

[0178] The binary vector is transformed into *Agrobacterium tumefaciens* using standard molecular biology techniques. To prepare the Agrobacteria for transformation, cells are cultured in liquid YPC media at 28°C and 220 rpm overnight.

[0179] *Agrobacterium* transformation of immature maize embryos is performed essentially as described in Negrotto *et al*., 2000, Plant Cell Reports 19: 798-803. For this example, all media constituents are essentially as described in Negrotto *et al*., *supra*. However, various media constituents known in the art may be substituted.

[0180] Briefly, *Agrobacterium* strain LBA4404 (pSB1) containing the binary vector plant transformation vector is grown on YEP (yeast extract (5 g/L), peptone (10g/L), NaCl (5g/L), 15g/l agar, pH 6.8) solid medium for 2 – 4 days at 28°C. Approximately 0.8X 10^9 *Agrobacterium* are suspended in LS-inf media supplemented with 100 μM As (Negrotto *et al., supra*). Bacteria are pre-induced in this medium for 30-60 minutes.

[0181] Immature embryos from a suitable genotype are excised from 8 – 12 day old ears into liquid LS-inf + 100 μM As. Embryos are rinsed once with fresh infection medium. *Agrobacterium* solution is then added and embryos are vortexed for 30 seconds and allowed to settle with the bacteria for 5 minutes. The embryos are then transferred scutellum side up to LSAs medium and cultured in the dark for two to three days. Subsequently, between 20 and 25 embryos per petri plate are transferred

to LSDc medium supplemented with cefotaxime (250 mg/l) and silver nitrate (1.6 mg/l) and cultured in the dark for 28°C for 10 days.

[0182] Immature embryos, producing embryogenic callus are transferred to LSD1M0.5S medium. The cultures are selected on this medium for about 6 weeks with a subculture step at about 3 weeks. Surviving calli are transferred to Reg1 medium supplemented with mannose. Following culturing in the light (16 hour light/ 8 hour dark regiment), green tissues are then transferred to Reg2 medium without growth regulators and incubated for about 1-2 weeks. Plantlets are transferred to Magenta GA-7 boxes (Magenta Corp, Chicago Ill.) containing Reg3 medium and grown in the light.

[0183] Following transformation, selection, and regeneration, plants are assayed for the presence of the *pmi* gene and the sBin maize codon-optimized coding sequences using TaqMan® analysis. Plants are also tested for the presence of the vector backbone. Plants negative for the vector backbone and comprising one copy of the transgene from the binary vector are transferred to the greenhouse and tested for insecticidal activity against WCR.

Example 8. Binary toxins of the invention in combination with second insecticidal agent.

[0184] The components of a binary toxin of the invention as described above are purified as in Example 2. A Cry protein or a dsRNA against an essential target and known to have insecticidal activity is prepared. In non-limiting examples, the dsRNA may target a gene encoding vacuolar ATP synthase, beta-tubulin, 26S proteosome subunit p28 protein, EF1α 48D, troponin I, tetraspanin, clathrin heavy chain, gamma-coatomer, beta-coatomer, and/or juvenile hormone epoxide hydrolase (PCT Patent Application Nos. PCT/US17/044825; PCT/US17/044831; PCT/US17/044832; U.S. Patent No. 7,812,219; each herein incorporated by reference). The Cry protein and/or dsRNA and purified binary toxin components are tested for efficacy against WCR in a diet-incorporation assay, performed essentially as described in Example 1.

Example 9. Genome editing in plant cells in situ to generate modified sBin-IPs.

[0185] The following Example illustrates the use of genome editing of a plant cell genome *in situ* to incorporate the mutations into a coding sequence for a native sBin-IP, for example sBin1Aa (SEQ ID NO:1).

[0186] Targeted genome modification, also known as genome editing, is useful for introducing mutations in specific DNA sequences. These genome editing technologies, which include zinc finger nucleases (ZNFs), transcriptionactivator-like effector nucleases (TALENS), meganucleases and clustered

regularly interspaced short palindromic repeats (CRISPR) have been successfully applied to over 50 different organisms including crop plants. *See*, *e.g.*, Belhaj, K., et al., Plant Methods 9, 39 (2013); Jiang, W., et al., Nucleic Acids Res, 41, e188 (2013)). The CRISPR/Cas system for genome editing is based on transient expression of Cas9 nuclease and an engineered single guide RNA (sgRNA) that specifies the targeted polynucleotide sequence.

[0187] Cas9 is a large monomeric DNA nuclease guided to a DNA target sequence with the aid of a complex of two 20-nucleotide (nt) non-coding RNAs: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), which are functionally available as single synthetic RNA chimera. The Cas9 protein contains two nuclease domains homologous to RuvC and HNH nucleases. The HNH nuclease domain cleaves the complementary DNA strand, whereas the RuvC-like domain cleaves the non-complementary strand and, as a result, a blunt cut is introduced in the target DNA.

[0188] When the Cas9 and the sgRNA are transiently expressed in living maize cells, double strand breaks (DSBs) in the specific targeted DNA is created in the transgenic maize cell. Mutation at the break site is introduced through the non-homologous end joining and homology-directed DNA repair pathways.

[0189] Specific mutations are introduced into a coding sequence for the native sBin1Aa component protein (SEQ ID NO:1) through the use of recombinant plasmids expressing the Cas9 nuclease and the sgRNA target that is maize codon optimized for the *sBin1Aa* sequence, for example SEQ ID NO:19, in the transgenic maize. Implementation of the method is by an agroinfiltration method with *Agrobacterium tumefaciens* carrying the binary plasmid harboring the specified target sequence of interest. After the sgRNA binds to the target *sBin1Aa* coding sequence, the Cas9 nuclease makes specific cuts into the coding sequence and introduces the desired mutation(s) during DNA repair. Thus, a now mutated *sBin1Aa* coding sequence will encode a modified variant sBin1Aa protein, for example, where a mutation at T52 residue is changed to A, the V102 residue is changed to T, the V151 residue is changed to A or K, or the K297 residue is changed to S or T, or any combination thereof.

[0190] Plant cells comprising the genome edited *sBin-IP* coding sequences are screened by PCR and sequencing. Calli that harbor genome edited mutations in the *sBin-IP* or modified *sBin-IP* coding sequences are induced to regenerate plants for phenotype evaluation for insecticidal activity of the expressed sBin-IPs against western corn rootworm (*Diabrotica virgifera*), Northern Corn Rootworm (*Diabrotica barberi*), Southern Corn Rootworm (*Diabrotica undecimpunctata howardi*) and/or Mexican Corn Rootworm (*Diabrotica virgifera zeae*).

Example 10. Testing sBin-IPs for insecticidal activity against lepidopteran pests.

[0191] The Dyad_SG02 binary toxin was tested against lepidopteran insects. A pET-6His-SUMO construct comprising *sBin1Ba* (SEQ ID NO:15) and one comprising *sBin-IP1Aa* (SEQ ID NO:16) was made to express tagged proteins. The constructs were transformed into *E. coli* BL21*(DE3) for protein production. Lysates from bacterial cultures expressing the sBin proteins were tested for bioactivity on a panel of lepidopteran insect pests that included corn earworm (CEW), fall armyworm (FAW), black cutworm (BCW) and European corn borer (ECB) using diet-overlay bioassays. A positive-control consisted of larvae exposed to *E.coli* BL21* lysates expressing a Cry protein known to have activity against all four species. A buffer only and lysates from BL21* (DE3) bacterial cultures harboring an empty pET29 vector were used as negative controls. Mortality was assessed at day 7. Results of the bioassay indicated that tagged-sBin-IP1Aa is active against WCR but was not active against any of the lepidopteran pests tested.

[0192] It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof of the description will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

[0193] All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art that this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A nucleic acid molecule comprising a nucleotide sequence that encodes a protein that is toxic to an insect pest, wherein the nucleotide sequence (a) encodes a protein comprising an amino acid sequence that has at least 80% to at least 99% sequence identity with any of SEQ ID NOs:1-6, or a toxin fragment thereof; or (b) has at least 80% to at least 99% sequence identity with any of SEQ ID NOs:7-12, or a toxin-encoding fragment thereof; or (c) is a synthetic sequence of (a) or (b) that has codons optimized for expression in a transgenic organism.

2. The nucleic acid molecule of claim 1, wherein the insecticidal protein comprises an amino acid sequence of any of SEQ ID NOs:1-6, or a toxic fragment thereof.

3. The nucleic acid molecule of claim 1, wherein the nucleotide sequence comprises any of SEQ ID NOs:7-12, or a toxin-encoding fragment thereof.

4. The nucleic acid molecule of claim 1, wherein the synthetic nucleotide sequence comprises any of SEQ ID NOs:13-24, or a toxin-encoding fragment thereof.

5. A chimeric gene comprising a heterologous promoter operably linked to the nucleic acid molecule of any one of claims 1-4.

6. The chimeric gene of claim 5, wherein the heterologous promoter is a plant expressible promoter.

7. The chimeric gene of claim 6, wherein the plant expressible promoter is selected from the group of promoters consisting of ubiquitin, cestrum yellow virus, corn TrpA, OsMADS 6, maize H3 histone, corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, maize mtl, pea small subunit RuBP carboxylase, rice actin, rice cyclophilin, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, potato patatin, lectin, CaMV 35S and S-E9 small subunit RuBP carboxylase promoter.

8. The chimeric gene of claim 5, wherein the insect pest is a coleopteran insect pest.

9. The chimeric gene of claim 8, wherein the coleopteran insect pest is a *Diabrotica* insect pest.

10. The chimeric gene of claim 9, wherein the *Diabrotica* insect pest is selected from the group consisting of *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi* and *Diabrotica zeae*.

11. The chimeric gene of claim 5, wherein the transgenic organism is a bacteria or a plant.

12. The chimeric gene of claim 11, wherein the transgenic bacteria is selected from the goup of genera consisting of *Bacillus, Clostridium, Xenorhabdus, Photorhabdus, Pasteuria, Escherichia, Pseudomonas, Erwinia, Serratia, Klebsiella, Salmonella, Pasteurella, Xanthomonas, Streptomyces, Rhizobium, Sinorhizobium, Ensifer, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc,* and *Alcaligenes*.

13. The chimeric gene of claim 11, wherein the plant is a maize plant.

14. A protein that is toxic to an insect pest, wherein the protein comprises (a) an amino acid sequence that has at least 80% to at least 99% sequence identity with an amino acid sequence of any of SEQ ID NOs:1-6, or a toxin fragment thereof; or (b) an amino acid sequence that comprises any of SEQ ID NOs:1-6, or a toxin fragment thereof; or (c) an amino acid sequence that is encoded by a nucleotide sequence that has at least 80% to at least 99% sequence identity with a nucleotide sequence of any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof; or (d) an amino acid sequence that is encoded by a nucleotide sequence comprising any of SEQ ID NOs:7-24, or a toxin-encoding fragment thereof.

15. The protein of claim 14, wherein the insect pest is a coleopteran insect pest.

16. The protein of claim 15, wherein the coleopteran insect pest is a *Diabrotica* insect pest.

17. The protein of claim 16, wherein the *Diabrotica* insect pest is selected from the group consisting of *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi* and *Diabrotica zeae*.

18. A recombinant vector comprising the chimeric gene of claim 5.

19. A host cell comprising the recombinant vector of claim 18, wherein the host cell is a bacterial cell or plant cell.

20. The transgenic bacterial cell of claim 19, wherein the bacterial cell is in the genus *Bacillus, Clostridium, Xenorhabdus, Photorhabdus, Pasteuria, Escherichia, Pseudomonas, Erwinia, Serratia, Klebsiella, Salmonella, Pasteurella, Xanthomonas, Streptomyces, Rhizobium, Sinorhizobium, Ensifer, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc,* or *Alcaligenes*.

21. The transgenic *Bacillus* cell of claim 20, wherein the *Bacillus* cell is a *Bacillus thuringiensis* cell.

22. The transgenic plant cell of claim 19, wherein the plant cell is a dicot plant cell or a monocot plant cell.

23. The transgenic plant cell of claim 22, wherein (a) the dicot plant cell is selected from the group consisting of a soybean cell, sunflower cell, tomato cell, cole crop cell, cotton cell, sugar beet cell and tobacco cell; or (b) the monocot plant cell is selected from the group consisting of a barley cell, maize cell, oat cell, rice cell, sorghum cell, sugar cane cell and wheat cell.

24. A transgenic plant or plant part comprising the transgenic plant cell of claim 23.

25. The transgenic pant or plant part of claim 24 that is a transgenic maize plant or plant part.

26. An insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin, wherein the first component is a peptide comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5, and the second component is a peptide comprising a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, and wherein said insecticidal toxin is active against at least a *Diabrotica* pest insect.

27. The insecticidal composition of claim 26, wherein (a) the first component comprises SEQ ID NO:1 and the second component comprises SEQ ID NO:2, or SEQ ID NO:4 or SEQ ID NO:6; or (b) the first component comprises SEQ ID NO:3 and the second component comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6; or (c) the first component comprises SEQ ID NO:5 and the second component comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6; or (d) the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component comprises SEQ ID NO:2; or (e) the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component

comprises SEQ ID NO:4; or (f) the first component comprises SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and the second component comprises SEQ ID NO:6

28. The insecticidal composition of claim 27, wherein the first component comprises SEQ ID NO:1 and the second component comprises SEQ ID NO:2.

29. The insecticidal composition of claim 27, wherein the first component comprises SEQ ID NO:3 and the second component comprises SEQ ID NO:4.

30. The insecticidal composition of claim 27, wherein the first component comprises SEQ ID NO:5 and the second component comprises SEQ ID NO:6.

31. The insecticidal composition of claim 26, wherein the Diabrotica pest is selected from the group of Diabrotica species consisting of *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi* and *Diabrotica zeae*.

32. The composition of any one of claims 26-31, wherein the composition further comprises a second pesticidal agent.

33. The composition of claim 32, wherein the second pesticidal agent is a biological agent or a chemical agent.

34. The composition of claim 33, wherein (a) the biological agent is or is derived from a *Bacillus thuringiensis* insecticidal protein, a *Bacillus cereus* insecticidal protein, a *Xenorhabdus* spp. insecticidal protein, a *Photorhabdus* spp. insecticidal protein, a *Brevibacillus laterosporous* insecticidal protein, a *Lysinibacillus sphearicus* insecticidal protein, a *Chromobacterium* spp. insecticidal protein, a *Yersinia entomophaga* insecticidal protein, a *Paenibacillus popiliae* insecticidal protein, or a *Clostridium* spp. insecticidal protein; (b) the biological agent is or is derived from a dsRNA, a Cry protein, a Vip protein, a patatin, a protease, a protease inhibitor, a urease, an alpha-amylase inhibitor, a pore-forming protein, a lectin, an engineered antibody or antibody fragment, or a chitinase; (c) the chemical agent is a carbamate, a pyrethroid, an organophosphate, a neonicotinoid, an organochloride, a nereistoxin, or a combination thereof; or (d) the chemical agent comprises an active ingredient selected from the group consisting of carbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-

parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, and a combination thereof.

35. A transgenic plant that produces the insecticidal composition of any of claims 26-31.

36. The transgenic plant of claim 35, wherein said plant is a maize plant.

37. A nucleic acid molecule that encodes the first component and the second component of any one of claims 26-31.

38. The nucleic acid molecule of claim 37, wherein the nucleic acid molecule comprises a heterologous promoter operably linked to the first component and the second component; or a first heterologous promoter operably linked to the first component and a second heterologous promoter operably linked to the second component.

39. A transgenic plant comprising the nucleic acid molecule of claim 37 or 38.

40. The transgenic plant of claim 39, wherein the plant is a maize plant.

41. A method for producing an insecticidal protein, comprising culturing the host cell of claim 19 or an organism comprising the host cell under conditions in which the host cell produces the insecticidal protein.

42. A method of producing a transgenic plant or plant part having enhanced insect resistance compared to a control plant or plant part, comprising: (a) introducing into a plant or plant part the chimeric gene of claim 5 or the nucleic acid molecule of claim 38, wherein the insecticidal protein is expressed in the plant or plant part, thereby producing a plant or plant part with enhanced insect-resistance.

43. The method of claim 42, wherein the introducing step is achieved by (a) transforming the plant or plant part; or (b) crossing a first plant comprising the chimeric gene with a different second plant.

44. The method of claim 43, wherein the chimeric gene encodes an insecticidal protein having an amino acid sequence of any of SEQ ID NOs:1-6.

45. A method of controlling an insect pest comprising, delivering to the insect pest or an environment thereof an effective amount of the insecticidal protein of claim 14 or the insecticidal composition of claim 26.

46. The method of claim 45, wherein the insecticidal protein or insecticidal composition is delivered through a transgenic plant or by topical application of a composition comprising the insecticidal protein or insecticidal composition.

47. The method of claim 46, wherein the transgenic plant or the composition comprises a second insecticidal agent different from the insecticidal protein.

48. The method of claim 47, wherein the second insecticidal agent is a protein, a dsRNA or a chemical.

49. The method of claim 48, wherein (a) the protein is selected from the group consisting of a Cry protein, a Vip protein, a patatin, a protease, a protease inhibitor, a urease, an alpha-amylase inhibitor, a pore-forming protein, a lectin, an engineered antibody or antibody fragment, or a chitinase; (b) the chemical is a carbamate, a pyrethroid, an organophosphate, a friprole, a neonicotinoid, an organochloride, a nereistoxin, or a combination thereof; or (c) the chemical comprises an active ingredient selected from the group consisting of carbofuran, carbaryl, methomyl, bifenthrin, tefluthrin, permethrin, cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, chlorpyrifos, chlorethoxyfos, dimethoate, ethoprophos, malathion, methyl-parathion, phorate, terbufos, tebupirimiphos, fipronil, acetamiprid, imidacloprid, thiacloprid, thiamethoxam, endosulfan, bensultap, and a combination thereof.

50. The method of any one of claims 45-49, wherein the insect pest is a coleopteran insect pest.

51. The method of 50, wherein the coleopteran insect pest is a *Diabrotica* species.

52. The method of claim 51, wherein the Diabrotica species is selected from the group consisting of *Diabrotica virgifera virgifera, Diabrotica barberi, Diabrotica undecimpunctata howardi* and *Diabrotica zeae*.

53. A method of reducing resistance development in a *Diabrotica* insect population to an insecticidal protein of claim 14 or the insecticidal composition of claim 26, the method comprising expressing in a transgenic plant fed upon by the Diabrotica insect population the insecticidal protein or the insecticidal

composition and an interfering RNA molecule which inhibits expression of a target gene in a larval and adult Diabrotica insect, thereby reducing resistance development in the Diabrotica insect population compared to a Diabrotica insect population exposed only to the insecticidal protein.

54. A method of providing a corn grower with a means of controlling a *Diabrotica* insect pest population in a corn crop comprising (a) selling or providing to the grower transgenic corn seed that comprises a nucleic acid molecule of claim 1; and (b) advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a *Diabrotica* pest population.

55. A method of identifying an insecticidal protein comprising a nucleotide sequence having from at least 80% to at least 99% sequence identity to any of SEQ ID NOs:7-12, said method comprising the steps of: (a) producing a primer pair that will amplify a polynucleotide of any of SEQ ID NOs:7-12 from a nucleic acid sample, or a complement thereof, (b) amplifying an orthologous gene from the nucleic acid sample, (c) identifying a polynucleotide sequence of an orthologous gene, (d) producing a protein encoded by the orthologous gene, and (e) determining that the protein of step (d) has insecticidal activity against an insect pest.

56. An isolated and purified antibody which specifically binds to a peptide selected from the group of peptides consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof, or an epitope therein, said antibody produced from the immune system of a vertebrate animal in response to the exposure of all or an antigenic part of said peptide to the animal's immune system.

57. A method for detecting the presence of a peptide in a sample comprising obtaining a/solution suspected of containing said peptide, probing said solution with the antibody of claim 56, and detecting the binding of said antibody to said peptide; wherein said peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof.

58. A kit for detecting the presence of the peptide in a sample comprising, in suitable container means, an antibody that binds to said peptide, reagents necessary for mixing the peptide and antibody in a solution at least a first immunodetection reagent providing said antibody along with control antibody, control antigen and the reagents and instructions necessary for detecting said binding; wherein said peptide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, and immunologically detectable variants thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US20/64873

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/82; C07K 14/20; A01H 5/00; A01N 25/00; A01N 63/00 (2020.01)

CPC - C12N 15/82; C07K 14/20; A01H 5/00; A01N 25/00; A01N 63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	(THANABALU, T et al.) A Bacillus sphaericus gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. Gene. 17 April 1996, Vol. 170, No. 1; pages 85-89; abstract; Genbank supplement, page 1; DOI: 10.1016/0378-1119(95)00836-5	14 --- 1-2, 5/1-5/2, 6/5/1-6/5/2, 7/6/5/1-7/6/5/2, 8/5/1-8/5/2, 9/8/5/1-9/8/5/2, 10/9/8/5/1-10/9/8/5/2, 11/5/1-11/5/2, 12/11/5/1-12/11/5/2, 13/11/5/1-13/11/5/2, 14-17, 18/5/1-18/5/2, 19/18/5/1-19/18/5/2, 20/19/18/5/1-20/19/18/5/2, 21/20/19/18/5/1-21/20/19/18/5/2, 22/19/18/5/1-22/19/18/5/2, 23/22/19/18/5/1-23/22/19/18/5/2, 24/23/22/19/18/5/1-24/23/22/19/18/5/2,
-***-Continued on next page-***-	-***-Continued on next page-***-	-***-Continued on next page-***-

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 April 2021 (12.04.2021)	MAY 07 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
-***-Continued from previous page-***- Y — A	-***-Continued from previous page-***- (THANABALU, T et al.) A Bacillus sphaericus gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. Gene. 17 April 1996, Vol. 170, No. 1; pages 85-89; abstract; Genbank supplement, page 1; DOI: 10.1016/0378-1119(95)00836-5	-***-Continued from previous page-***- 25/24/23/22/19/18/5/1-25/24/23/22/19/18/5/1, 41/19/18/5/1-41/19/18/5/2, 45-49, 53-54 --- 3, 5/3, 6/5/3, 7/6/5/3, 8/5/3, 9/8/5/3, 10/9/8/5/3, 11/5/3, 12/11/5/3, 13/11/5/3, 18/5/3, 19/18/5/3, 20/19/18/5/3, 21/20/19/18/5/3, 22/19/18/5/3, 23/22/19/18/5/3, 24/23/22/19/18/5/3, 25/24/23/22/19/18/5/3, 41/19/18/5/3, 55
Y — A	WO 2019/211850 A1 (EVOGENE LTD.) 7 November 2019; abstract; page 5, lines 21-27; page 16, lines 16-24; page 22, line 11; page 38, lines 5-7; page 104, lines 27-32; page 125, line 4; page 188, line 28; page 204, lines 1-6	1-2, 5/1-5/2, 6/5/1-6/5/2, 7/6/5/1-7/6/5/2, 8/5/1-8/5/2, 9/8/5/1-9/8/5/2, 10/9/8/5/1-10/9/8/5/2, 11/5/1-11/5/2, 12/11/5/1-12/11/5/2, 13/11/5/1-13/11/5/2, 14-17, 18/5/1-18/5/2, 19/18/5/1-19/18/5/2, 20/19/18/5/1-20/19/18/5/2, 21/20/19/18/5/1-21/20/19/18/5/2, 22/19/18/5/1-22/19/18/5/2, 23/22/19/18/5/1-23/22/19/18/5/2, 24/23/22/19/18/5/1-24/23/22/19/18/5/2, 25/24/23/22/19/18/5/1-25/24/23/22/19/18/5/1, 41/19/18/5/1-41/19/18/5/2, 54 --- 3, 5/3, 6/5/3, 7/6/5/3, 8/5/3, 9/8/5/3, 10/9/8/5/3, 11/5/3, 12/11/5/3, 13/11/5/3, 18/5/3, 19/18/5/3, 20/19/18/5/3, 21/20/19/18/5/3, 22/19/18/5/3, 23/22/19/18/5/3, 24/23/22/19/18/5/3, 25/24/23/22/19/18/5/3, 41/19/18/5/3, 55
Y — A	US 2019/0330652 A1 (SYNGENTA PARTICIPATIONS AG) 31 October 2019; paragraphs [0126], [0129] [0138]	45-49, 53 --- 3, 5/3, 6/5/3, 7/6/5/3, 8/5/3, 9/8/5/3, 10/9/8/5/3, 11/5/3, 12/11/5/3, 13/11/5/3, 18/5/3, 19/18/5/3, 20/19/18/5/3, 21/20/19/18/5/3, 22/19/18/5/3, 23/22/19/18/5/3, 24/23/22/19/18/5/3, 25/24/23/22/19/18/5/3, 41/19/18/5/3, 55
Y	WO 2018/026774 A1 (SYNGENTA PARTICIPATIONS AG) 8 February 2018; paragraphs [0014], [0029]	54

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

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

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

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

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

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US20/64873

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 39, 40, 42-44, 50-52
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+, Claims 1-38, 41, 45-49, 53-55 and a nucleic acid encompassing SEQ ID NO: 7 (nucleic acid), encoding a polypeptide encompassing SEQ ID NO: 1 (polypeptide)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/64873

-***-Continued From Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-38, 41, 45-49, 53-55 and a nucleic acid encompassing SEQ ID NO: 7 (nucleic acid), encoding a polypeptide encompassing SEQ ID NO: 1 (polypeptide) are directed toward nucleic acid molecules encoding proteins, the protein so encoded, constructs and host cells comprising the nucleic acids and constructs, compositions comprising the polypeptides; and methods associated with any of the foregoing.

The nucleic acid molecules, encoded proteins, constructs, host cells, compositions, and methods will be searched to the extent they encompass a nucleic acid encompassing SEQ ID NO: 7 (first exemplary nucleic acid), encoding a peptide encompassing SEQ ID NO: 1 (first exemplary polypeptide). Applicant is invited to elect additional nucleic acid(s), and corresponding polypeptide(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable nucleobases, residues or substituents), where available as an option within at least one searchable claim, to be searched. Additional nucleic acid and/or corresponding polypeptide sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-3 (each in-part), 5-25 (each in-part), 41 (in-part), 45-49 (each in-part), and 53-55 (each in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 7 (nucleic acid), and SEQ ID NO: 1 (polypeptide). Applicants must specify the searchable claims that encompass any additionally elected nucleic acid and/or polypeptide sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 8 (nucleic acid), encoding SEQ ID NO: 2 (polypeptide).

Groups II+, Claims 56-58 and SEQ ID NO: 1 (target polypeptide) are directed toward antibodies which bind to polypeptides, and methods and kits associated therewith.

The antibodies can be searched to the extent they specifically bind a polypeptide encompassing SEQ ID NO: 1 (first exemplary target polypeptide). Applicant is invited to elect additional target polypeptide(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional target polypeptide sequence(s) can be searched upon the payment of additional fees. It is believed that claims 56-58 (each in-part) encompass this first named invention of Groups II+ and thus these claims can be searched with payment of a fee for the search of Groups II+, to the extent that they encompass SEQ ID NO: 1 (target polypeptide). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) can result in only the first claimed invention of groups II+ to be searched/examined. An exemplary election would be SEQ ID NO: 2 (target polypeptide).

The inventions listed as Groups I+ and II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include SEQ ID NO: 7, not present in any of Groups II+; the special technical features of Groups II+ include an antibody, not present in any of Groups I+.

Groups I+ and II+ share the technical features including: a peptide selected from the group of peptides consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6.

However, these shared technical features are previously disclosed by NCBI reference sequence WP_018616621 (hereinafter 'WP_018616621').

WP_018616621 discloses a peptide comprising SEQ ID NO: 2 (a hypothetical protein from Segetibacter koreensis (a peptide comprising SEQ ID NO: 2); page 1, wherein the sequence disclosed is 100% identical to Applicant's SEQ ID NO: 2).

No technical features are shared between the nucleic acid and/or polypeptide sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a nucleic acid molecule comprising a nucleotide sequence that encodes a protein that is toxic to an insect pest; a chimeric gene comprising a heterologous promoter operably linked to the nucleic acid molecule; a recombinant vector comprising the chimeric gene; a host cell comprising the recombinant vector, wherein the host cell is a bacterial cell or plant cell; a transgenic plant or plant part comprising the transgenic plant cell; a method for producing an insecticidal protein, comprising culturing the host cell or an organism comprising the host cell under conditions in which the host cell produces the insecticidal protein; a method of providing a corn grower with a means of controlling a Diabrotica insect pest population in a corn crop comprising (a) selling or providing to the grower transgenic corn seed that comprises the nucleic acid molecule; and (b) advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a Diabrotica pest population; a protein that is toxic to an insect pest, wherein the protein comprises an amino acid sequence; a method of controlling an insect pest comprising, delivering to the insect pest or an environment thereof an effective amount of the insecticidal protein; a method of reducing resistance development in a Diabrotica insect population to an insecticidal protein, the method comprising expressing in a transgenic plant fed upon by the Diabrotica insect population the insecticidal protein or the insecticidal composition and an interfering RNA molecule which inhibits expression of a target gene in a larval and adult Diabrotica insect, thereby reducing resistance development in the Diabrotica insect population compared to a Diabrotica insect population exposed only to the insecticidal protein; an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin, wherein the first component is a peptide, and the second component is a peptide, and wherein said insecticidal toxin is active against at least a Diabrotica pest insect; a transgenic plant that produces the insecticidal composition; a nucleic acid molecule that encodes the first component and the second component; and a method of identifying an insecticidal protein encoded by a nucleotide sequence, said method comprising the steps of: (a) producing a primer pair that will amplify a polynucleotide from a nucleic acid sample, or a complement thereof, (b) amplifying an orthologous gene from the nucleic acid sample, (c) identifying a polynucleotide sequence of an orthologous gene, (d) producing a protein encoded by the orthologous gene, and (e)

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determining that the protein of step (d) has insecticidal activity against an insect pest.

However, these shared technical features are previously disclosed by US 2019/0330652 A1 to Syngenta Participations AG (hereinafter 'Syngenta') in view of WO 2018/026774 A1 to Syngenta Participations AG (hereinafter 'Participations'), further in view of WO 2019/211850 A1 to Evogene, Ltd. (hereinafter 'Evogene'), and US 2014/0199760 A1 to Northey et al. (hereinafter 'Northey').

Syngenta discloses a nucleic acid molecule comprising a nucleotide sequence that encodes a protein that is toxic to an insect pest (a nucleic acid molecule comprising a nucleotide sequence that encodes a protein that is toxic to an insect pest; paragraph [0008]); a chimeric gene comprising a heterologous promoter operably linked to the nucleic acid molecule (a chimeric gene comprising a heterologous promoter operably linked to the nucleic acid molecule; paragraphs [0008], [0059]); a recombinant vector comprising the chimeric gene (a recombinant vector comprising the chimeric gene; paragraphs [0009], [0100]); a host cell comprising the recombinant vector (a host cell comprising the recombinant vector; paragraph [0100]), wherein the host cell is a bacterial cell or plant cell (wherein the host cell is a bacterial cell or plant cell; paragraph [0100]); a transgenic plant or plant part comprising the transgenic plant cell (a transgenic plant or plant part comprising the transgenic plant cell; paragraph [0100]); a method for producing an insecticidal protein, comprising culturing the host cell or an organism comprising the host cell under conditions in which the host cell produces the insecticidal protein (a method for producing an insecticidal protein, comprising culturing the host cell or an organism comprising the host cell under conditions in which the host cell produces the insecticidal protein; paragraph [0101]); a method of controlling a Diabrotica insect pest population (a method of controlling a Diabrotica insect pest population; paragraphs [0131], [0138]) in a corn crop (in a corn crop; paragraph [0004], wherein Diabrotica is a destructive corn pest) comprising providing transgenic corn seed that comprises the nucleic acid molecule (comprising providing transgenic corn seed that comprises the nucleic acid molecule; paragraph [0131]); a protein that is toxic to an insect pest, wherein the protein comprises an amino acid sequence (a protein that is toxic to an insect pest, wherein the protein comprises an amino acid sequence; paragraphs [0008], [0014]); a method of controlling an insect pest comprising, delivering to the insect pest or an environment thereof an effective amount of the insecticidal protein (a method of controlling an insect pest comprising, contacting a pest (delivering to the insect pest) or an environment thereof an effective amount of the insecticidal protein; paragraph [0138]); a method of reducing resistance development in a Diabrotica insect population to an insecticidal protein (a method of reducing resistance development in a Diabrotica insect population to an insecticidal protein; paragraphs [0007], [0126]); expressing in a transgenic plant fed upon by the Diabrotica insect population an insecticidal protein or an insecticidal composition and an interfering RNA molecule (expressing in a transgenic plant fed upon by the Diabrotica insect population an insecticidal protein or an insecticidal composition and an interfering RNA molecule; paragraph [0129]) which inhibits expression of a target gene in a larval and adult Diabrotica insect (which inhibits expression of a target gene in a larval and adult Diabrotica insect; paragraphs [0006], [0129]

Syngenta does not disclose a method of providing a corn grower with a means of controlling a Diabrotica insect pest population in a corn crop comprising (a) selling or providing to the grower transgenic corn seed that comprises the nucleic acid molecule; and (b) advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a Diabrotica pest population; a method of reducing resistance development in a Diabrotica insect population to an insecticidal protein, the method comprising expressing in a transgenic plant fed upon by the Diabrotica insect population the insecticidal protein or the insecticidal composition and an interfering RNA molecule which inhibits expression of a target gene in a larval and adult Diabrotica insect, thereby reducing resistance development in the Diabrotica insect population compared to a Diabrotica insect population exposed only to the insecticidal protein; an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin, wherein the first component is a peptide, and the second component is a peptide, and wherein said insecticidal toxin is active against at least a Diabrotica pest insect; a transgenic plant that produces the insecticidal composition; a nucleic acid molecule that encodes the first component and the second component; and a method of identifying an insecticidal protein encoded by a nucleotide sequence, said method comprising the steps of: (a) producing a primer pair that will amplify a polynucleotide from a nucleic acid sample, or a complement thereof, (b) amplifying an orthologous gene from the nucleic acid sample, (c) identifying a polynucleotide sequence of an orthologous gene, (d) producing a protein encoded by the orthologous gene, and (e) determining that the protein of step (d) has insecticidal activity against an insect pest.

Participations discloses a method of providing a corn grower with a means of controlling a Diabrotica insect pest population in a corn crop (a method of providing a corn grower with a means of controlling a Diabrotica insect pest population in a corn crop; paragraphs [0014], [0029]) comprising (a) selling or providing to the grower transgenic corn seed that comprises a nucleic acid molecule (selling or providing to the grower transgenic corn seed that comprises a nucleic acid molecule; paragraph [0029]); and (b) advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a Diabrotica pest population (advertising to the grower that the transgenic corn seed produce transgenic corn plants that control a Diabrotica pest population; paragraphs [0014], [0029]).

Evogene discloses an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin (a binary insecticidal system (an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin); page 10, lines 26-32), wherein the first component is a peptide, and the second component is a peptide (wherein the first component is a peptide, and the second component is a peptide; page 10, lines 26-32), and wherein said insecticidal toxin is active against at least a Diabrotica pest insect (wherein said insecticidal toxin is active against at least a Diabrotica pest insect; page 18, lines 9-11), and a transgenic plant that produces the insecticidal composition (a transgenic plant that produces the insecticidal composition; page 5, lines 16-20; page 10, lines 26-32).

Northey discloses wherein methods of identifying orthologs in other plant species are known in the art (wherein methods of identifying orthologs in other plant species are known in the art; paragraph [0047]), and include creating and screening a cDNA library from a plant species (include creating and screening a cDNA library from a plant species; paragraph [0047]), for sequences homologous to the original sequences from a different species (for sequences homologous to the original sequences from a different species; paragraph [0047]); wherein the orthologs retain the function of the protein (wherein the orthologs retain the function of the protein; paragraph [0047]).

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to have modified the disclosure of Syngenta to have provided a method of reducing resistance development in a Diabrotica insect population to an insecticidal protein, as disclosed by Syngenta, the method comprising expressing in a transgenic plant fed upon by the Diabrotica insect population the

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insecticidal protein and an interfering RNA molecule which inhibits expression of a target gene in a larval and adult Diabrotica insect, as disclosed by Syngenta, thereby reducing resistance development in the Diabrotica insect population compared to a Diabrotica insect population exposed only to the insecticidal protein, as Syngenta discloses wherein the second, RNA, agent has a different mode of action, which has utility for the prevention of insect resistance (the second, RNA, agent has a different mode of action, which has utility for the prevention of insect resistance; paragraphs [0126], [0129]), which, therefore, would have had the effect of reducing resistance development in the Diabrotica insect population compared to a Diabrotica insect population exposed only to the insecticidal protein. It further would have been obvious to a person of ordinary skill in the art at the time the invention was made to have modified the disclosure of Syngenta to have provided a method of providing a corn grower with a means of controlling a Diabrotica insect pest population, as disclosed by Participations, since advertising and selling to a grower the seeds would have been a common means of commerce to enable the grower to obtain the seed and grow crops therefrom. It also would have been obvious to a person of ordinary skill in the art at the time the invention was made to have modified the disclosure of Syngenta to have provided an insecticidal composition comprising a first component and a second component that function together as an insecticidal toxin, as disclosed by Evogene, in order to provide an insecticidal composition that functions via a different mechanism in order to better enable the combination of one or more insecticidal agents, increasing effectiveness of the combination. It additionally would have been obvious to a person of ordinary skill in the art at the time the invention was made to have modified the disclosure of Syngenta to have provided means of identifying an ortholog of one or more insecticidal proteins by creating a cDNA library, as disclosed by Northey by producing a primer pair that will amplify a polynucleotide from a nucleic acid sample, or a complement thereof, and amplifying an orthologous gene from the nucleic acid sample; then using sequence homology comparisons to identify a polynucleotide sequence of an orthologous gene, and subsequently verifying the insecticidal activity of the ortholog by producing a protein encoded by the orthologous gene, and determining that the protein has insecticidal activity against an insect pest, in order to enable the identification of orthologous proteins from different plant species with a similar pest inhibitory activity to the original insecticidal protein, but potentially greater compatibility with one or more different plant species that would enhance protein production or stability in said different plant species.

No technical features are shared between the polypeptide sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups II+ share the technical features including: an isolated and purified antibody which specifically binds to a peptide, said antibody produced from the immune system of a vertebrate animal in response to the exposure of all or an antigenic part of said peptide to the animal's immune system; a method for detecting the presence of a peptide in a sample comprising obtaining a solution suspected of containing said peptide, probing said solution with the antibody, and detecting the binding of said antibody to said peptide; and a kit for detecting the presence of the peptide in a sample comprising, in suitable container means, an antibody that binds to said peptide, reagents necessary for mixing the peptide and antibody in a solution at least a first immunodetection reagent providing said antibody along with control antibody, control antigen and the reagents and instructions necessary for detecting said binding.

However, these shared technical features are previously disclosed by WO 01/87940 A2 to Monsanto Technology LLC (hereinafter 'Monsanto').

Monsanto discloses an isolated and purified antibody which specifically binds to a peptide (an isolated and purified antibody which specifically binds to a peptide; page 4, lines 28-29, Claim 30), said antibody produced from the immune system of a vertebrate animal in response to the exposure of all or an antigenic part of said peptide to the animal's immune system (said antibody produced from the immune system of a vertebrate animal in response to the exposure of all or an antigenic part of said peptide to the animal's immune system; Claim 30); a method for detecting the presence of a peptide in a sample comprising obtaining a solution suspected of containing said peptide (a method for detecting the presence of a peptide in a sample comprising obtaining a sample (solution) suspected of containing said peptide; page 5, lines 4-8), probing said solution with the antibody (contacting the sample (probing said solution) with the antibody; page 5, lines 4-8), and detecting the binding of said antibody to said peptide (detecting the antibody-peptide complex (binding of said antibody to said peptide); page 5, lines 4-8); and a kit for detecting the presence of the peptide in a sample (a kit for detecting the presence of the peptide in a sample; page 5, lines 9-13, Claim 32) comprising, in suitable container means, an antibody that binds to said peptide (comprising, in suitable container means, an antibody that binds to said peptide; page 5, lines 9-13, Claim 32), reagents necessary for mixing the peptide and antibody in a solution at least a first immunodetection reagent (reagents necessary for mixing the peptide and antibody in a solution at least a first immunodetection reagent; page 5, lines 9-13, Claim 32) providing said antibody along with control antibody, control antigen and the reagents and instructions necessary for detecting said binding (providing said antibody along with control antibody, control antigen and the reagents and instructions necessary for detecting said binding; Claim 32).

Since none of the special technical features of the Groups I+ and II+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the WP_018616621, Syngenta, Evogene, Participations, Northey, and Monsanto references, unity of invention is lacking.