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(54) **Title**: COMPOSITIONS AND METHODS FOR INSECTICIDAL CONTROL OF STINKBUGS

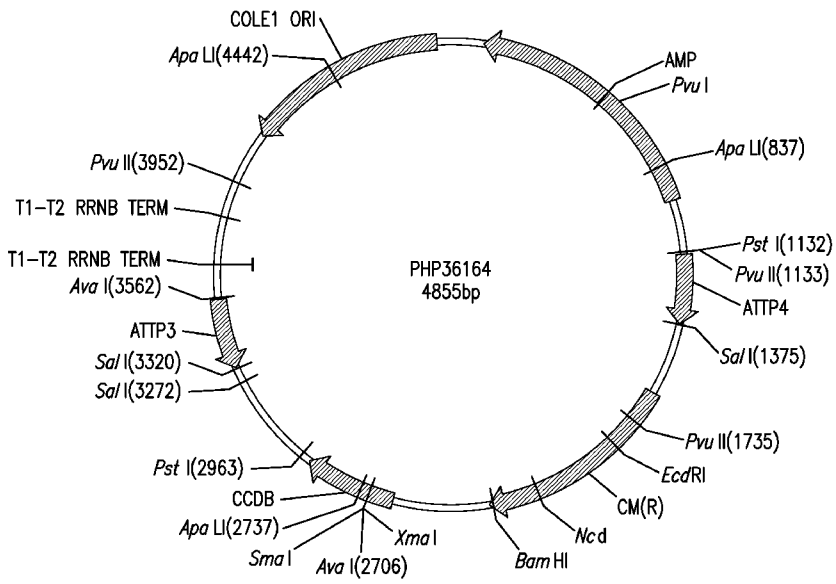


FIG. 1

(57) **Abstract**: Methods and compositions are provided which employ a silencing element that, when ingested by a pest, such as a *Pentatomidae* plant pest, decrease the expression of a target sequence in the pest. The present invention provides various target polynucleotides set forth in any one of SEQ ID NOS: 6-12, 18-40 or active variants and fragments thereof, wherein a decrease in expression of one or more the sequences in the target pest controls the pest (*i.e.*, has insecticidal activity). Plants, plant part, bacteria and other host cells comprising the silencing elements or an active variant or fragment thereof of the invention are also provided.

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COMPOSITIONS AND METHODS FOR INSECTICIDAL CONTROL OF STINKBUGS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This Application claims the benefit of U.S. Provisional Application No. 61/779,643, filed on March 13, 2013, which is incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] The Sequence Listing submitted March 13, 2014 as a text file named "36446_0006U1_2013_03_13_Sequences_as_Filed," created on March 7, 2014, and having a size of 133,534 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

FIELD OF THE INVENTION

[0003] The present invention relates generally to methods of molecular biology and gene silencing to control pests.

BACKGROUND OF THE INVENTION

[0004] Insect pests are a serious problem in agriculture. They destroy millions of acres of staple crops such as corn, soybeans, peas, and cotton. Yearly, these pests cause over $100 billion dollars in crop damage in the U.S. alone. In an ongoing seasonal battle, farmers must apply billions of gallons of synthetic pesticides to combat these pests. Other methods employed in the past delivered insecticidal activity by microorganisms or genes derived from microorganisms expressed in transgenic plants. For example, certain species of microorganisms of the genus *Bacillus* are known to possess pesticidal activity against a broad range of insect pests including *Lepidoptera, Diptera, Coleoptera, Hemiptera*, and others. In fact, microbial pesticides, particularly those obtained from *Bacillus* strains, have played an important role in agriculture as alternatives to chemical pest control. Agricultural scientists have developed crop plants with enhanced insect resistance by genetically engineering crop plants to produce insecticidal proteins from *Bacillus*. For example, corn and cotton plants genetically engineered to produce Cry toxins (see, e.g., Aronson (2002) *Cell Mol. Life Sci.* 59(3):417-425; Schnepf *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62(3):775-806) are now widely used in American agriculture and have provided the farmer with an alternative to traditional insect-control methods. However, these *Bt* insecticidal proteins only protect plants from a relatively narrow range of pests.

Moreover, these modes of insecticidal activity provided varying levels of specificity and, in some cases, caused significant environmental consequences.

[0005] Previous control of stinkbugs relied on broad spectrum insecticides. With the adoption of transgenic controls for major lepidopteran pests in several crops, these insecticides are no longer used and stinkbugs have become a major secondary pest. No successful use of transgenic control of stinkbugs has been described or adopted. This may be due in part to the extra oral digestion employed by stinkbugs where digestive enzymes are injected into the host plant prior to feeding. This makes it difficult to find proteins that survive long enough to manifest activity against these insects. RNAi may overcome that feeding behavior by relying on double stranded RNAs rather than proteins. Thus, there is an immediate need for alternative methods to control pests.

BRIEF SUMMARY OF THE INVENTION

[0006] Methods and compositions are provided which employ a silencing element that, when ingested by a pest, such as a *Pentatomidae* plant pest including for example, a *N. viridula* (southern green stinkbug), *Acrosternum hilare* (green stinkbug), *Piezodorus guildini* (redbanded stinkbug), Euschistus servus (brown stinkbug), and/or *Halymorpha halys* (brown marmorated stinkbug) plant pest, is capable of decreasing the expression of a target sequence in the pest. In specific embodiments, the decrease in expression of the target sequence controls the pest and thereby the methods and compositions are capable of limiting damage to a plant. The present invention provides various target polynucleotides as set forth in SEQ ID NOS: 6-12, 18-40, or active variants or fragments thereof, or complements thereof, wherein a decrease in expression of one or more the sequences in the target pest controls the pest (i.e., has insecticidal activity). Further provided are silencing elements, which when ingested by the pest, decrease the level of expression of one or more of the target polynucleotides. Plants, plant parts, plant cells, bacteria and other host cells comprising the silencing elements or an active variant or fragment thereof are also provided.

[0007] In another embodiment, a method for controlling a pest, such as a *Pentatomidae* plant pest, such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, Euschistus servus (brown stinkbug), and/or *Halymorpha halys* plant pest, is provided. The method comprises feeding to a pest a composition comprising a silencing element, wherein the silencing element, when ingested by the pest, reduces the level of a target sequence in the pest and thereby

controls the pest. Further provided are methods to protect a plant from a pest. Such methods comprise introducing into the plant or plant part, or alternatively onto the plant as part of a topical formulation, a silencing element of the invention. When the pest ingests a plant comprising the silencing element, the level of the target sequence is decreased in the pest and the pest is controlled.

[0008] In specific embodiments, the silencing element comprises at least 15, 20, or 22 consecutive nucleotides of any one or more of SEQ ID NOS: 6-12, 18-40. In specific embodiments, the pest that is controlled is a *Pentatomidae* plant pest, such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, Euschistus servus (brown stinkbug), and/or *Halymorpha halys* plant pest. Plants, plant parts, plant cells, bacteria and other host cells comprising the silencing element comprising at least 15, 20, or 22 consecutive nucleotides of any one or more of SEQ ID NOS: 6-12, 18-40 or an active variant or fragment thereof, or complements thereof, are also provided.

[0009] In another embodiment, a method for controlling a pest, such as a pest from *Pentatomidae* plant pest, such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, Euschistus servus (brown stinkbug), and/or *Halymorpha halys* (Hemiptera order) is provided. The method comprises feeding to a pest a composition comprising a silencing element comprising at least 15, 20, or 22 consecutive nucleotides of any one or more of SEQ ID NOS: 6-12, 18-40, wherein the silencing element, when ingested by the pest, reduces the level of a target sequence in the pest and thereby controls the pest. Further provided are methods to protect a plant from a pest. Such methods comprise introducing into the plant or plant part, or alternatively onto the plant as part of a topical formulation, a silencing element of the invention. When the pest ingests a plant expressing the silencing element, the level of the target sequence is decreased in the pest and the pest is controlled.

BRIEF DESCRIPTION OF THE FIGURES

[00010] Figure 1 is a map of plasmid PHP 36164.

[00011] Figure 2 is a map of plasmid PHP 59032.

[00012] Figure 3 is a map of plasmid PHP 62151.

DETAILED DESCRIPTION OF THE INVENTION

[00013] The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout. It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, genera, and reagents described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

[00014] Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

[00015] As used herein the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the protein" includes reference to one or more proteins and equivalents thereof known to those skilled in the art, and so forth. All technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs unless clearly indicated otherwise.

I. Overview

[00016] The present invention comprises methods and compositions employing one or more silencing elements that, when ingested by a pest, such as a Pentatomidae plant pest such as, for example, a N. viridula, Acrosternum hilare, Piezodorus guildini, and/or Halymorpha halys plant pest, is capable of decreasing the expression of a target sequence in the pest. In specific embodiments, the decrease in expression of the target sequence controls the pest and thereby the methods and compositions are capable of limiting damage to a plant or plant part.

The present invention provides target polynucleotides as set forth in SEQ ID NOS: 6-12, 18-40, or active variants and fragments thereof, or complements thereof. Silencing elements comprising sequences, complementary sequences, active fragments or variants of these target polynucleotides are provided which, when ingested by a pest, decrease the expression of one or more of the target sequences and thereby controls the pest (i.e., has insecticidal activity).

[00017] As used herein, by "controlling a pest" or "controls a pest" is intended any effect on a pest that results in limiting the damage that the pest causes. Controlling a pest includes, but is not limited to, killing the pest, inhibiting development of the pest, altering fertility or growth of the pest in such a manner that the pest provides less damage to the plant, decreasing the number of offspring produced, producing less fit pests, producing pests more susceptible to predator attack, or deterring the pests from eating the plant.

[00018] Reducing the level of expression of the target polynucleotide or the polypeptide encoded thereby, in the pest results in the suppression, control, and/or killing of the invading pathogenic organism. Reducing the level of expression of the target sequence of the pest will reduce the disease symptoms resulting from pathogen challenge by at least about 2% to at least about 6%, at least about 5% to about 50%, at least about 10% to about 60%, at least about 30% to about 70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the invention can be utilized to control pests, particularly, *Pentatomidae* plant pests such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pest.

[00019] Assays measuring the control of a pest are commonly known in the art, as are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. See, for example, Thomma *et al.* (1998) *Plant Biology* 95:15107-15111, herein incorporated by reference. See, also Baum *et al.* (2007) *Nature Biotech* 11:1322-1326 and WO 2007/035650 which proved both whole plant feeding assays and corn root feeding assays. Both of these references are herein incorporated by reference in their entirety. See, also the examples below.

[00020] The invention comprises compositions and methods for protecting plants from a plant pest, such as *Pentatomidae* plant pests such as, for example, a *N. viridula*, *Acrosternum*

hilare, *Piezodorus guildini*, and/or *Halymorpha halys* plant pests or inducing resistance in a plant to a plant pest, such as *Pentatomidae* plant pests such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pests. As used herein "*Pentatomidae* plant pest" is used to refer to any member of the *Pentatomidae* family. Accordingly, the compositions and methods are also useful in protecting plants against any *Pentatomidae* plant pest including representative genera and species such as, but not limited to, *Acrocorisellus (A. serraticollis), Acrosternum (A. adelpha, A. hilare, A. herbidum, A. scutellatum), Agonoscelis (A. nubila), Alcaeorrhynchus (A. grandis, A. phymatophorus), Amaurochrous (A. brevitylus), Apateticus (A. anatarius, A. bracteatus, A. cynicus, A. lineolatus, A. marginiventris), Apoecilus, Arma (A. custos), Arvelius, Bagrada, Bagrada hilaris, Banasa (B. calva, B. dimiata, B. grisea, B. induta, B. sordida), Brochymena (B. affinis, B. cariosa, B. haedula, B. hoppingi, B. sulcata), Carbula (C. obtusangula, C. sinica), Chinavia, Chlorochroa (C. belfragii, C. kanei, C. norlandi, C. senilis, C. viridicata), Chlorocoris (C. distinctus, C. flaviviridis, C. hebetatus, C. subrugosus, C. tau), Codophila (C. remota, C. sulcata, C. varius), Coenus (C. delius, C. inermis, C. tarsalis), Cosmopepla (C. bimaculata, C. binotata, C. carnifex, C. decorata, C. intergressus), Dalpada (D. oculata), Dendrocoris (D. arizonesis, D. fruticicola, D. humeralis, D. parapini, D. reticulatus), Dolycoris (D. baccarum* (sloe bug)*), Dybowskyia (D. reticulata), Edessa, Erthesina (E. fullo), Eurydema (E. dominulus, E. gebleri* (shield bug)*, E. pulchra, E. rugosa), Euschistus (E. biformis, E. integer, E. quadrator, E. servus, E. tristigma), Euthyrhynchus (E. floridanus, E. macronemis), Gonopsis (G. coccinea), Graphosoma (G. lineatum* (stinkbug)*, G. rubrolineatum), Halyomorpha (H. halys* (brown marmorated stinkbug)*), Halys (H. sindillus, H. sulcatus), Holcostethus (H. abbreviatus, H. fulvipes, H. limbolarius, H. piceus, H. sphacelatus), Homalogonia (H. obtusa), Hymenarcys (H. aequalis, H. crassa, H. nervosa, H. perpuncata, H. reticulata), Lelia (L. decempunctata), Lineostethus, Loxa (L. flavicollis, L. viridis), Mecidea (M. indicia, M. major, M. minor), Megarrhamphus (M. hastatus), Menecles (M. insertus, M. portacrus), Mormidea (M. cubrosa, M. lugens, M. pama, M. pictiventris, M. ypsilon), Moromorpha (M. tetra), Murgantia (M. angularis, M. tessellata, M. varicolor, M. violascens), Neottiglossa (N. californica, N. cavifrons, N. coronaciliata, N. sulcifrons, N. undata), Nezara (N. smaragdulus, N. viridula* (southern green stinkbug)*), Oebalus (O. grisescens, O. insularis, O. mexicanus, O. pugnax, O. typhoeus), Oechalia (O. schellenbergii* (spined predatory shield bug)*), Okeanos (O. quelpartensis), Oplomus (O. catena, O. dichrous, O. tripustulatus), Palomena (P. prasina* (green shield bug)*), Parabrochymena, Pentatoma (P. angulata, P. illuminata, P. japonica, P. kunmingensis, P. metallifera, P. parataibaiensis, P. rufipes, P. semiannulata, P. viridicornuta), Perillus (P. bioculatus, P. confluens, P. strigipes),*

Picromerus (P. griseus), Piezodorus (P. degeeri, P. guildinii, P. lituratus (gorse shield bug)*), Pinthaeus (P. humeralis), Plautia (P. crossota, P. stali* (brown-winged green bug)*), Podisus (P. maculiventris), Priassus (P. testaceus), Prionosoma, Proxys (P. albopunctulatus, P. punctulatus, P. victor), Rhaphigaster (R. nebulosa), Scotinophara (S. horvathi), Stiretrus (S. anchorago, S. fimbriatus), Thyanta (T. accerra, T. calceata, T. casta, T. perditor, T. pseudocasta), Trichopepla (T. aurora, T. dubia, T. pilipes, T. semivittata, T. vandykei), Tylospilus,* and *Zicrona.* Other order and species for which the present invention is intended include Hemiptera, Kudzu bug, *Megacopta cribraria* (fa. *Plataspidae*) and Sunn pest, *Eurygaster integriceps* (fa. *Scutelleridae*).

II. Target Sequences

[00021] As used herein, a "target sequence" or "target polynucleotide" comprises any sequence in the pest that one desires to reduce the level of expression. In specific embodiments, decreasing the level of the target sequence in the pest controls the pest. For instance, the target sequence may be essential for growth and development. While the target sequence can be expressed in any tissue of the pest, in specific embodiments, the sequences targeted for suppression in the pest are expressed in cells of the gut tissue of the pest, cells in the midgut of the pest, and cells lining the gut lumen or the midgut. Such target sequences can be involved in, for example, gut cell metabolism, growth or differentiation. Non-limiting examples of target sequences of the invention include a polynucleotide set forth in SEQ ID NOS: 6-12, 18-40, active fragments or variants thereof, or complements thereof. As exemplified elsewhere herein, decreasing the level of expression of one or more of these target sequences in a *Pentatomidae* plant pest such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pest controls the pest.

III. Silencing Elements

[00022] By "silencing element" is intended a polynucleotide which when ingested by a pest, is capable of reducing or eliminating the level or expression of a target polynucleotide or the polypeptide encoded thereby. The silencing element employed can reduce or eliminate the expression level of the target sequence by influencing the level of the target RNA transcript or, alternatively, by influencing translation and thereby affecting the level of the encoded polypeptide. Methods to assay for functional silencing elements that are capable of reducing or eliminating the level of a sequence of interest are disclosed elsewhere herein. A single polynucleotide employed in the methods of the invention can comprise one or more silencing

elements to the same or different target polynucleotides. The silencing element can be produced *in vivo* (i.e., in a host cell such as a plant or microorganism) or *in vitro*.

[00023] In other embodiments, while the silencing element controls pests, preferably the silencing element has no effect on the normal plant or plant part.

[00024] As discussed in further detail below, silencing elements can include, but are not limited to, a sense suppression element, an antisense suppression element, a double stranded RNA, a siRNA, an amiRNA, a miRNA, or a hairpin suppression element. Silencing elements of the present invention may comprise a chimera where two or more sequences of the present invention or active fragments or variants, or complements thereof, are found in the same RNA molecule. Further, a sequence of the present invention or active fragment or variant, or complement thereof, may be present as more than one copy in a DNA construct, silencing element, DNA molecule or RNA molecule. Non-limiting examples of silencing elements that can be employed to decrease expression of these target Pentatomidae plant pest sequences such as, for example, a N. viridula, Acrosternum hilare, Piezodorus guildini, and/or Halymorpha halys plant pest sequences comprise, or alternatively consist of, fragments and variants of the sense or antisense sequences set forth in SEQ ID NOS: 6-12, 18-40 or one or more variants or fragments thereof. The silencing element can further comprise additional sequences that advantageously effect transcription and/or the stability of a resulting transcript.

[00025] By "reduces" or "reducing" the expression level of a polynucleotide or a polypeptide encoded thereby is intended to mean, the polynucleotide or polypeptide level of the target sequence is statistically lower than the polynucleotide level or polypeptide level of the same target sequence in an appropriate control pest which is not exposed to (i.e., has not ingested) the silencing element. In particular embodiments of the invention, reducing the polynucleotide level and/or the polypeptide level of the target sequence in a pest according to the invention results in less than 95%, less than 90%, less than 80%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% of the polynucleotide level, or the level of the polypeptide encoded thereby, of the same target sequence in an appropriate control pest. Methods to assay for the level of the RNA transcript, the level of the encoded polypeptide, or the activity of the polynucleotide or polypeptide are discussed elsewhere herein.

a. Sense Suppression Elements

[00026] As used herein, a "sense suppression element" comprises a polynucleotide designed to express an RNA molecule corresponding to at least a part of a target messenger RNA in the "sense" orientation. Expression of the RNA molecule comprising the sense suppression element reduces or eliminates the level of the target polynucleotide or the polypeptide encoded thereby. The polynucleotide comprising the sense suppression element may correspond to all or part of the sequence of the target polynucleotide, all or part of the 5' and/or 3' untranslated region of the target polynucleotide, all or part of the coding sequence of the target polynucleotide, or all or part of both the coding sequence and the untranslated regions of the target polynucleotide.

[00027] Typically, a sense suppression element has substantial sequence identity to the target polynucleotide, typically greater than about 65% sequence identity, greater than about 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference. The sense suppression element can be any length so long as it allows for the suppression of the targeted sequence. The sense suppression element can be, for example, 15, 16, 17, 18 19, 20, 22, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 900, 1000, 1100, 1200, 1300 nucleotides or longer of the target polynucleotides set forth in any of SEQ ID NOS: 6-12, 18-40. In other embodiments, the sense suppression element can be, for example, about 15-25, 25-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1050, 1050-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800 nucleotides or longer of the target polynucleotides set forth in any of SEQ ID NOS: 6-12, 18-40.

b. Antisense Suppression Elements

[00028] As used herein, an "antisense suppression element" comprises a polynucleotide which is designed to express an RNA molecule complementary to all or part of a target messenger RNA. Expression of the antisense RNA suppression element reduces or eliminates the level of the target polynucleotide. The polynucleotide for use in antisense suppression may correspond to all or part of the complement of the sequence encoding the target polynucleotide, all or part of the complement of the 5' and/or 3' untranslated region of the target polynucleotide, all or part of the complement of the coding sequence of the target polynucleotide, or all or part

of the complement of both the coding sequence and the untranslated regions of the target polynucleotide. In addition, the antisense suppression element may be fully complementary (i.e., 100% identical to the complement of the target sequence) or partially complementary (i.e., less than 100% identical to the complement of the target sequence) to the target polynucleotide. In specific embodiments, the antisense suppression element comprises at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence complementarity to the target polynucleotide. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, U.S. Patent No. 5,942,657. Furthermore, the antisense suppression element can be complementary to a portion of the target polynucleotide. Generally, sequences of at least 15, 20, 22, 25, 50, 100, 200, 300, 400, 450 nucleotides or greater of the sequence set forth in any of SEQ ID NOS: 6-12, 18-40 may be used. Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu *et al* (2002) *Plant Physiol.* 129:1732-1743 and U.S. Patent Nos. 5,759,829 and 5,942,657, each of which is herein incorporated by reference.

c. Double Stranded RNA Suppression Element

[00029] A "double stranded RNA silencing element" or "dsRNA" comprises at least one transcript that is capable of forming a dsRNA either before or after ingestion by a pest. Thus, a "dsRNA silencing element" includes a dsRNA, a transcript or polyribonucleotide capable of forming a dsRNA or more than one transcript or polyribonucleotide capable of forming a dsRNA. "Double stranded RNA" or "dsRNA" refers to a polyribonucleotide structure formed either by a single self-complementary RNA molecule or a polyribonucleotide structure formed by the expression of least two distinct RNA strands. The dsRNA molecule(s) employed in the methods and compositions of the invention mediate the reduction of expression of a target sequence, for example, by mediating RNA interference ("RNAi") or gene silencing in a sequence-specific manner. In the context of the present invention, the dsRNA is capable of reducing or eliminating the level of expression of a target polynucleotide or the polypeptide encoded thereby in a pest.

[00030] The dsRNA can reduce or eliminate the expression level of the target sequence by influencing the level of the target RNA transcript, by influencing translation and thereby affecting the level of the encoded polypeptide, or by influencing expression at the pre-transcriptional level (i.e., via the modulation of chromatin structure, methylation pattern, etc., to alter gene expression). See, for example, Verdel et al. (2004) Science 303:672-676; Pal-Bhadra

et al. (2004) Science 303:669-672; Allshire (2002) Science 297:1818-1819; Volpe et al. (2002) Science 297:1833-1837; Jenuwein (2002) Science 297:2215-2218; and Hall et al. (2002) Science 297:2232-2237. Methods to assay for functional dsRNA that are capable of reducing or eliminating the level of a sequence of interest are disclosed elsewhere herein. Accordingly, as used herein, the term "dsRNA" is meant to encompass other terms used to describe nucleic acid molecules that are capable of mediating RNA interference or gene silencing, including, for example, short-interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), hairpin RNA, short hairpin RNA (shRNA), post-transcriptional gene silencing RNA (ptgsRNA), and others.

[00031] In specific embodiments, at least one strand of the duplex or double-stranded region of the dsRNA shares sufficient sequence identity or sequence complementarity to the target polynucleotide to allow for the dsRNA to reduce the level of expression of the target sequence. As used herein, the strand that is complementary to the target polynucleotide is the "antisense strand" and the strand homologous to the target polynucleotide is the "sense strand."

[00032] In another embodiment, the dsRNA comprises a hairpin RNA. A hairpin RNA comprises an RNA molecule that is capable of folding back onto itself to form a double stranded structure. Multiple structures can be employed as hairpin elements. In specific embodiments, the dsRNA suppression element comprises a hairpin element which comprises in the following order, a first segment, a second segment, and a third segment, where the first and the third segment share sufficient complementarity to allow the transcribed RNA to form a double-stranded stem-loop structure.

[00033] The "second segment" of the hairpin comprises a "loop" or a "loop region." These terms are used synonymously herein and are to be construed broadly to comprise any nucleotide sequence that confers enough flexibility to allow self-pairing to occur between complementary regions of a polynucleotide (i.e., segments 1 and 3 which form the stem of the hairpin). For example, in some embodiments, the loop region may be substantially single stranded and act as a spacer between the self-complementary regions of the hairpin stem-loop. In some embodiments, the loop region can comprise a random or nonsense nucleotide sequence and thus not share sequence identity to a target polynucleotide. In other embodiments, the loop region comprises a sense or an antisense RNA sequence or fragment thereof that shares identity to a target polynucleotide. See, for example, International Patent Publication No. WO 02/00904, herein incorporated by reference. In specific embodiments, the loop region can be optimized to

be as short as possible while still providing enough intramolecular flexibility to allow the formation of the base-paired stem region. Accordingly, the loop sequence is generally less than 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 25, 20, 15, 10 nucleotides or less.

[00034] The "first" and the "third" segment of the hairpin RNA molecule comprise the base-paired stem of the hairpin structure. The first and the third segments are inverted repeats of one another and share sufficient complementarity to allow the formation of the base-paired stem region. In specific embodiments, the first and the third segments are fully complementary to one another. Alternatively, the first and the third segment may be partially complementary to each other so long as they are capable of hybridizing to one another to form a base-paired stem region. The amount of complementarity between the first and the third segment can be calculated as a percentage of the entire segment. Thus, the first and the third segment of the hairpin RNA generally share at least 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to and including 100% complementarity.

[00035] The first and the third segment are at least about 1000, 500, 400, 300, 200, 100, 50, 40, 30, 25, 22, 20, 19, 18, 17, 16, 15 or 10 nucleotides in length. In specific embodiments, the length of the first and/or the third segment is about 10-100 nucleotides, about 10 to about 75 nucleotides, about 10 to about 50 nucleotides, about 10 to about 40 nucleotides, about 10 to about 35 nucleotides, about 10 to about 30 nucleotides, about 10 to about 25 nucleotides, about 10 to about 19 nucleotides, about 50 nucleotides to about 100 nucleotides, about 100 nucleotides to about 150 nucleotides, about 150 nucleotides to about 200 nucleotides, about 200 nucleotides to about 250 nucleotides, about 250 nucleotides to about 300 nucleotides, about 300 nucleotides to about 350 nucleotides, about 350 nucleotides to about 400 nucleotides, about 400 nucleotides to about 500 nucleotides, about 600 nucleotides, about 700 nucleotides, about 800 nucleotides, about 900 nucleotides, about 1000 nucleotides, about 1100 nucleotides, about 1200 nucleotides, 1300 nucleotides, 1400 nucleotides, 1500 nucleotides, 1600 nucleotides, 1700 nucleotides, 1800 nucleotides, 1900 nucleotides, 2000 nucleotides or longer. In other embodiments, the length of the first and/or the third segment comprises at least 10-19 nucleotides; 19-35 nucleotides; 30-45 nucleotides; 40-50 nucleotides; 50-100 nucleotides; 100-300 nucleotides; about 500 -700 nucleotides; about 700-900 nucleotides; about 900-1100 nucleotides; about 1300 -1500 nucleotides; about 1500 - 1700 nucleotides; about 1700 - 1900 nucleotides; about 1900 - 2100 nucleotides; about 2100 - 2300 nucleotides; or about 2300 - 2500 nucleotides. See, for example, International Publication No. WO 0200904. In specific embodiments, the first and the third segment comprise at least 19 nucleotides having at least 85% complementary to the first

segment. In still other embodiments, the first and the third segments which form the stem-loop structure of the hairpin comprises 3' or 5' overhang regions having unpaired nucleotide residues.

[00036] Hairpin molecules or double-stranded RNA molecules of the present invention may have more than one sequence of the present invention or active fragments or variants, or complements thereof, found in the same portion of the RNA molecule. For example, in a chimeric hairpin structure, the first segment of a hairpin molecule comprises two polynucleotide sections, each with a different sequence of the present invention. For example, reading from one terminus of the hairpin, the first segment is composed of sequences from two separate genes (A followed by B). This first segment is followed by the second segment, the loop portion of the hairpin. The loop segment is followed by the third segment, where the complementary strands of the sequences in the first segment are found (B* followed by A*) In forming the stem-loop, hairpin structure, the stem contains SeqA-A* at the distal end of the stem and SeqB-B* proximal to the loop region.

[00037] In specific embodiments, the sequences used in the first, the second, and/or the third segments comprise domains that are designed to have sufficient sequence identity to a target polynucleotide of interest and thereby have the ability to decrease the level of expression of the target polynucleotide. The specificity of the inhibitory RNA transcripts is therefore generally conferred by these domains of the silencing element. Thus, in some embodiments of the invention, the first, second and/or third segment of the silencing element comprise a domain having at least 10, at least 15, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 500, at least 1000, or more than 1000 nucleotides that share sufficient sequence identity to the target polynucleotide to allow for a decrease in expression levels of the target polynucleotide when expressed in an appropriate cell. In other embodiments, the domain is between about 15 to 50 nucleotides, about 19-35 nucleotides, about 25-50 nucleotides, about 19 to 75 nucleotides, about 40-90 nucleotides, about 15-100 nucleotides, 10-100 nucleotides, about 10 to about 75 nucleotides, about 10 to about 50 nucleotides, about 10 to about 40 nucleotides, about 10 to about 35 nucleotides, about 10 to about 30 nucleotides, about 10 to about 25 nucleotides, about 10 to about 19 nucleotides, about 50 nucleotides to about 100 nucleotides, about 100 nucleotides to about 150 nucleotides, about 150 nucleotides to about 200 nucleotides, about 200 nucleotides to about 250 nucleotides, about 250 nucleotides to about 300 nucleotides, about 300 nucleotides to about 350 nucleotides, about 350 nucleotides to about 400 nucleotides, about 400 nucleotide to about 500 nucleotides or longer. In other embodiments, the length of the first and/or the third

segment comprises at least 10-19 nucleotides, 19-35 nucleotides, 30-45 nucleotides, 40-50 nucleotides, 50-100 nucleotides, or about 100-300 nucleotides.

[00038] In specific embodiments, the domain of the first, the second, and/or the third segment has 100% sequence identity to the target polynucleotide. In other embodiments, the domain of the first, the second and/or the third segment having homology to the target polypeptide have at least 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to a region of the target polynucleotide. The sequence identity of the domains of the first, the second and/or the third segments to the target polynucleotide need only be sufficient to decrease expression of the target polynucleotide of interest. See, for example, Chuang and Meyerowitz (2000) *Proc. Natl. Acad. Sci. USA* 97:4985-4990; Stoutjesdijk *et al.* (2002) *Plant Physiol.* 129:1723-1731; Waterhouse and Helliwell (2003) *Nat. Rev. Genet.* 4:29-38; Pandolfini *et al. BMC Biotechnology* 3:7, and U.S. Patent Publication No. 20030175965; each of which is herein incorporated by reference. A transient assay for the efficiency of hpRNA constructs to silence gene expression *in vivo* has been described by Panstruga *et al.* (2003) *Mol. Biol. Rep.* 30:135-140, herein incorporated by reference.

[00039] The amount of complementarity shared between the first, second, and/or third segment and the target polynucleotide or the amount of complementarity shared between the first segment and the third segment (i.e., the stem of the hairpin structure) may vary depending on the organism in which gene expression is to be controlled. Some organisms or cell types may require exact pairing or 100% identity, while other organisms or cell types may tolerate some mismatching. In some cells, for example, a single nucleotide mismatch in the targeting sequence abrogates the ability to suppress gene expression. In these cells, the suppression cassettes of the invention can be used to target the suppression of mutant genes, for example, oncogenes whose transcripts comprise point mutations and therefore they can be specifically targeted using the methods and compositions of the invention without altering the expression of the remaining wild-type allele.

[00040] Any region of the target polynucleotide can be used to design the domain of the silencing element that shares sufficient sequence identity to allow expression of the hairpin transcript to decrease the level of the target polynucleotide. For instance, the domain can be designed to share sequence identity to the 5' untranslated region of the target polynucleotide(s), the 3' untranslated region of the target polynucleotide(s), exonic regions of the target polynucleotide(s), intronic regions of the target polynucleotide(s), and any combination thereof.

In specific embodiments, a domain of the silencing element shares sufficient homology to at least about 15, 16, 17, 18, 19, 20, 22, 25 or 30 consecutive nucleotides from about nucleotides 1-50, 25-75, 75-125, 50-100, 125-175, 175-225, 100-150, 150-200, 200-250, 225-275, 275-325, 250-300, 325-375, 375-425, 300-350, 350-400, 425-475, 400-450, 475-525, 450-500, 525-575, 575-625, 550-600, 625-675, 675-725, 600-650, 625-675, 675-725, 650-700, 725-825, 825-875, 750-800, 875-925, 925-975, 850-900, 925-975, 975-1025, 950-1000, 1000-1050, 1025-1075, 1075-1125, 1050-1100, 1125-1175, 1100-1200, 1175-1225, 1225-1275, 1200-1300, 1325-1375, 1375-1425, 1300-1400, 1425-1475, 1475-1525, 1400-1500, 1525-1575, 1575-1625, 1625-1675, 1675-1725, 1725-1775, 1775-1825, 1825-1875, 1875-1925, 1925-1975, 1975-2025, 2025-2075, 2075-2125, 2125-2175, 2175-2225, 1500-1600, 1600-1700, 1700-1800, 1800-1900, 1900-2000 of the target sequence. In some instances to optimize the siRNA sequences employed in the hairpin, the synthetic oligodeoxyribonucleotide/RNase H method can be used to determine sites on the target mRNA that are in a conformation that is susceptible to RNA silencing. See, for example, Vickers et al. (2003) J. Biol. Chem 278:7108-7118 and Yang et al. (2002) Proc. Natl. Acad. Sci. USA 99:9442-9447, herein incorporated by reference. These studies indicate that there is a significant correlation between the RNase-H-sensitive sites and sites that promote efficient siRNA-directed mRNA degradation.

[00041] The hairpin silencing element may also be designed such that the sense sequence or the antisense sequence do not correspond to a target polynucleotide. In this embodiment, the sense and antisense sequence flank a loop sequence that comprises a nucleotide sequence corresponding to all or part of the target polynucleotide. Thus, it is the loop region that determines the specificity of the RNA interference. See, for example, WO 02/00904, herein incorporated by reference.

[00042] In addition, transcriptional gene silencing (TGS) may be accomplished through use of a hairpin suppression element where the inverted repeat of the hairpin shares sequence identity with the promoter region of a target polynucleotide to be silenced. See, for example, Aufsatz *et al.* (2002) *PNAS* 99 (Suppl. 4):16499-16506 and Mette *et al.* (2000) *EMBO J* 19(19):5194-5201.

d. MicroRNA (miRNA) Silencing Element

[00043] In other embodiments, the silencing element can comprise a microRNA (miRNA). "MicroRNAs" or "miRNAs" are regulatory agents comprising about 19 to about 24

ribonucleotides in length, which are highly efficient at inhibiting the expression of target polynucleotides. See, for example Javier *et al.* (2003) *Nature* 425: 257-263, herein incorporated by reference. For miRNA interference, the silencing element can be designed to express a dsRNA molecule that forms a partially base-paired structure containing a 19, 20, 21, 22, 23, 24 or 25 nucleotide sequence that is complementary to the target polynucleotide of interest. The miRNA can be synthetically made, or transcribed as a longer RNA which is subsequently cleaved to produce the active miRNA. The miRNA can be an "artificial miRNA" or "amiRNA" which comprises a miRNA sequence that is synthetically designed to silence a target sequence.

[00044] When expressing an miRNA, the final (mature) miRNA is present in a duplex in a precursor backbone structure, the two strands being referred to as the miRNA (the strand that will eventually base pair with the target) and miRNA* (star sequence). It has been demonstrated that miRNAs can be transgenically expressed and target genes of interest efficiently silenced (Highly specific gene silencing by artificial microRNAs in Arabidopsis Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D. Plant Cell. 2006 May; 18(5):1121-33. Epub 2006 Mar 10 & Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH. Nat Biotechnol. 2006 Nov; 24(11):1420-8. Epub 2006 Oct 22. Erratum in: Nat Biotechnol. 2007 Feb; 25(2):254.)

[00045] The silencing element for miRNA interference comprises a miRNA primary sequence. The miRNA primary sequence comprises a DNA sequence having the miRNA and star sequences separated by a loop as well as additional sequences flanking this region that are important for processing. When expressed as an RNA, the structure of the primary miRNA is such as to allow for the formation of a hairpin RNA structure that can be processed into a mature miRNA. In some embodiments, the miRNA backbone comprises a genomic or cDNA miRNA precursor sequence, wherein said sequence comprises a native primary in which a heterologous (artificial) mature miRNA and star sequence are inserted.

[00046] As used herein, a "star sequence" is the sequence within a miRNA precursor backbone that is complementary to the miRNA and forms a duplex with the miRNA to form the stem structure of a hairpin RNA. In some embodiments, the star sequence can comprise less than 100% complementarity to the miRNA sequence. Alternatively, the star sequence can comprise at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80% or lower sequence complementarity to the miRNA sequence as long as the star sequence has sufficient complementarity to the miRNA

sequence to form a double stranded structure. In still further embodiments, the star sequence comprises a sequence having 1, 2, 3, 4, 5 or more mismatches with the miRNA sequence and still has sufficient complementarity to form a double stranded structure with the miRNA sequence resulting in production of miRNA and suppression of the target sequence.

[00047] The miRNA precursor backbones can be from any plant. In some embodiments, the miRNA precursor backbone is from a monocot. In other embodiments, the miRNA precursor backbone is from a dicot. In further embodiments, the backbone is from maize or soybean. MicroRNA precursor backbones have been described previously. For example, US20090155910A1 (WO 2009/079532) discloses the following soybean miRNA precursor backbones: 156c, 159, 166b, 168c, 396b and 398b, and US20090155909A1 (WO 2009/079548) discloses the following maize miRNA precursor backbones: 159c, 164h, 168a, 169r, and 396h. Each of these references is incorporated by reference in their entirety.

[00048] Thus, the primary miRNA can be altered to allow for efficient insertion of heterologous miRNA and star sequences within the miRNA precursor backbone. In such instances, the miRNA segment and the star segment of the miRNA precursor backbone are replaced with the heterologous miRNA and the heterologous star sequences, designed to target any sequence of interest, using a PCR technique and cloned into an expression construct. It is recognized that there could be alterations to the position at which the artificial miRNA and star sequences are inserted into the backbone. Detailed methods for inserting the miRNA and star sequence into the miRNA precursor backbone are described in, for example, US Patent Applications 20090155909A1 and US20090155910A1, herein incorporated by reference in their entirety.

[00049] When designing a miRNA sequence and star sequence, various design choices can be made. See, for example, Schwab R, *et al.* (2005) *Dev Cell* 8: 517-27. In non-limiting embodiments, the miRNA sequences disclosed herein can have a "U" at the 5'-end, a "C" or "G" at the 19th nucleotide position, and an "A" or "U" at the 10th nucleotide position. In other embodiments, the miRNA design is such that the miRNA have a high free delta-G as calculated using the ZipFold algorithm (Markham, N. R. & Zuker, M. (2005) *Nucleic Acids Res.* 33: W577-W581.) Optionally, a one base pair change can be added within the 5' portion of the miRNA so that the sequence differs from the target sequence by one nucleotide.

[00050] The methods and compositions of the invention employ silencing elements that when transcribed "form" a dsRNA molecule. Accordingly, the heterologous polynucleotide being expressed need not form the dsRNA by itself, but can interact with other sequences in the plant cell or in the pest gut after ingestion to allow the formation of the dsRNA. For example, a chimeric polynucleotide that can selectively silence the target polynucleotide can be generated by expressing a chimeric construct comprising the target sequence for a miRNA or siRNA to a sequence corresponding to all or part of the gene or genes to be silenced. In this embodiment, the dsRNA is "formed" when the target for the miRNA or siRNA interacts with the miRNA present in the cell. The resulting dsRNA can then reduce the level of expression of the gene or genes to be silenced. See, for example, US Application Publication 2007-0130653, entitled "Methods and Compositions for Gene Silencing", herein incorporated by reference. The construct can be designed to have a target for an endogenous miRNA or alternatively, a target for a heterologous and/or synthetic miRNA can be employed in the construct. If a heterologous and/or synthetic miRNA is employed, it can be introduced into the cell on the same nucleotide construct as the chimeric polynucleotide or on a separate construct. As discussed elsewhere herein, any method can be used to introduce the construct comprising the heterologous miRNA.

e. Silencing Elements

[00051] A silencing element may comprise a chimeric construction molecule comprising two or more sequences of the present invention. For example, the chimeric construction may be a hairpin or dsRNA as disclosed herein. A chimera may comprise two or more sequences of the present invention. Providing at least two different sequences in a single silencing element may allow for targeting multiple genes using one silencing element and/or for example, one expression cassette. Targeting multiple genes may allow for slowing or reducing the possibility of resistance by the pest, and providing the multiple targeting ability in one expressed molecule may reduce the expression burden of the transformed plant or plant product, or provide topical treatments that are capable of targeting multiple hosts with one application.

IV. Variants and Fragments

[00052] By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a polynucleotide that are useful as a silencing element do not need to encode

fragment proteins that retain biological activity. Thus, fragments of a nucleotide sequence may range from at least about 10, about 15, about 16, about 17, about 18, about 19, about 20 nucleotides, about 22 nucleotides, about 50 nucleotides, about 75 nucleotides, about 100 nucleotides, 200 nucleotides, 300 nucleotides, 400 nucleotides, 500 nucleotides, 600 nucleotides, 700 nucleotides and up to the full-length polynucleotide employed in the invention. Alternatively, fragments of a nucleotide sequence may range from 1-50, 25-75, 75-125, 50-100, 125-175, 175-225, 100-150, 150-200, 200-250, 225-275, 275-325, 250-300, 325-375, 375-425, 300-350, 350-400, 425-475, 400-450, 475-525, 450-500, 525-575, 575-625, 550-600, 625-675, 675-725, 600-650, 625-675, 675-725, 650-700, 725-825, 825-875, 750-800, 875-925, 925-975, 850-900, 925-975, 975-1025, 950-1000, 1000-1050, 1025-1075, 1075-1125, 1050-1100, 1125-1175, 1100-1200, 1175-1225, 1225-1275, 1200-1300, 1325-1375, 1375-1425, 1300-1400, 1425-1475, 1475-1525, 1400-1500, 1525-1575, 1575-1625, 1625-1675, 1675-1725, 1725-1775, 1775-1825, 1825-1875, 1875-1925, 1925-1975, 1975-2025, 2025-2075, 2075-2125, 2125-2175, 2175-2225, 1500-1600, 1600-1700, 1700-1800, 1800-1900, 1900-2000 of any one of SEQ ID NOS: 6-12, 18-40. Methods to assay for the activity of a desired silencing element are described elsewhere herein.

[00053] "Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native polynucleotide. A variant of a polynucleotide that is useful as a silencing element will retain the ability to reduce expression of the target polynucleotide and, in some embodiments, thereby control a pest of interest. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides employed in the invention. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis, but continue to retain the desired activity. Generally, variants of a particular polynucleotide of the invention (i.e., a silencing element) will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

[00054] Variants of a particular polynucleotide of the invention (i.e., the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides employed in the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

[00055] The following terms are used to describe the sequence relationships between two or more polynucleotides or polypeptides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", and, (d) "percentage of sequence identity."

[00056] (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

[00057] (b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two polynucleotides. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

[00058] Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison

program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

[00059] (c) As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Inc., Mountain View, California).

[00060] (d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

[00061] A method is provided for identifying a silencing element from the target polynucleotides set forth in SEQ ID NOS: 6-12, 18-40. Such methods comprise obtaining a candidate fragment of any one or more of SEQ ID NOS: 6-12, 18-40 which is of sufficient

length to act as a silencing element and thereby reduce the expression of the target polynucleotide and/or control a desired pest; expressing said candidate polynucleotide fragment in an appropriate expression cassette to produce a candidate silencing element and determining if said candidate polynucleotide fragment has the activity of a silencing element, thereby reducing the expression of the target polynucleotide and/or controlling a desired pest. Methods of identifying such candidate fragments based on the desired pathway for suppression are known. For example, various bioinformatics programs can be employed to identify the region of the target polynucleotides that could be exploited to generate a silencing element. See, for example, Elbahir *et al.* (2001) *Genes and Development* 15:188-200, Schwartz *et al.* (2003) *Cell* 115:199-208, Khvorova *et al.* (2003) *Cell* 115:209-216. See also, siRNA at Whitehead (jura.wi.mit.edu/bioc/siRNAext/) which calculates the binding energies for both sense and antisense siRNAs. See, also genscript.com/ssl-bin/app/rnai?op=known; Block-iT™ RNAi designer from Invitrogen and GenScript siRNA Construct Builder.

V. DNA Constructs

[00062] The use of the term "polynucleotide" is not intended to limit the present invention to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides of the invention also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

[00063] The polynucleotide encoding the silencing element(s) or in specific embodiments employed in the methods and compositions of the invention can be provided in expression cassettes for expression in a plant or organism of interest. It is recognized that multiple silencing elements including multiple identical silencing elements, multiple silencing elements targeting different regions of the target sequence, or multiple silencing elements from different target sequences can be used. In this embodiment, it is recognized that each silencing element can be contained in a single or separate cassette, DNA construct, or vector. As discussed, any means of providing the silencing element is contemplated. A plant or plant cell can be transformed with a single cassette comprising DNA encoding one or more silencing elements or separate cassettes comprising each silencing element can be used to transform a plant or plant cell or host cell. Likewise, a plant transformed with one component can be subsequently

transformed with the second component. One or more silencing elements can also be brought together by sexual crossing. That is, a first plant comprising one component is crossed with a second plant comprising the second component. Progeny plants from the cross will comprise both components.

[00064] The expression cassette can include 5' and 3' regulatory sequences operably linked to the polynucleotide of the invention. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of the invention and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of the invention. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked is intended that the coding regions are in the same reading frame. The cassette may additionally contain at least one additional polynucleotide to be co-transformed into the organism. Alternatively, the additional polypeptide(s) can be provided on multiple expression cassettes. Expression cassettes can be provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotide to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

[00065] The expression cassette can include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a polynucleotide comprising the silencing element employed in the methods and compositions of the invention, and a transcriptional and translational termination region (i.e., termination region) functional in plants. In another embodiment, the double stranded RNA is expressed from a suppression cassette. Such a cassette can comprise two convergent promoters that drive transcription of an operably linked silencing element. "Convergent promoters" refers to promoters that are oriented on either terminus of the operably linked silencing element such that each promoter drives transcription of the silencing element in opposite directions, yielding two transcripts. In such embodiments, the convergent promoters allow for the transcription of the sense and anti-sense strand and thus allow for the formation of a dsRNA.

[00066] The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotides employed in the invention may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the polynucleotide employed in the invention may be heterologous to the host cell or to each

other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

[00067] The termination region may be native with the transcriptional initiation region, may be native with the operably linked polynucleotide encoding the silencing element, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous) to the promoter, the polynucleotide comprising silencing element, the plant host, or any combination thereof. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

[00068] Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

[00069] In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this

purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

[00070] A number of promoters can be used in the practice of the invention. The polynucleotide encoding the silencing element can be combined with constitutive, tissue-preferred, or other promoters for expression in plants.

[00071] Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

[00072] An inducible promoter, for instance, a pathogen-inducible promoter could also be employed. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also WO 99/43819, herein incorporated by reference.

[00073] Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); wun1 and wun2, U.S. Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792; Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

[00074] Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the

objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. 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-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, 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(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

[00075] Tissue-preferred promoters can be utilized to target enhanced expression within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

[00076] Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

[00077] Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-

specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2): 343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

[00078] In one embodiment of this invention the plant-expressed promoter is a vascular-specific promoter such as a phloem-specific promoter. A "vascular-specific" promoter, as used herein, is a promoter which is at least expressed in vascular cells, or a promoter which is preferentially expressed in vascular cells. Expression of a vascular-specific promoter need not be exclusively in vascular cells, expression in other cell types or tissues is possible. A "phloem-specific promoter" as used herein, is a plant-expressible promoter which is at least expressed in phloem cells, or a promoter which is preferentially expressed in phloem cells.

[00079] Expression of a phloem-specific promoter need not be exclusively in phloem cells, expression in other cell types or tissues, e.g., xylem tissue, is possible. In one embodiment of this invention, a phloem-specific promoter is a plant-expressible promoter at least expressed in phloem cells, wherein the expression in non-phloem cells is more limited (or absent) compared to the expression in phloem cells. Examples of suitable vascular-specific or phloem-specific promoters in accordance with this invention include but are not limited to the promoters

selected from the group consisting of: the SCSV3, SCSV4, SCSV5, and SCSV7 promoters (Schunmann *et al.* (2003) *Plant Functional Biology* 30:453-60; the rolC gene promoter of *Agrobacterium rhizogenes*(Kiyokawa *et al.* (1994) *Plant Physiology* 104:801-02; Pandolfini *et al.* (2003) *BioMedCentral (BMC) Biotechnology* 3:7, (www.biomedcentral.com/1472-6750/3/7); Graham *et al.* (1997) *Plant Mol. Biol.* 33:729-35; Guivarc'h *et al.* (1996); Almon *et al.* (1997) *Plant Physiol.* 115:1599-607; the rolA gene promoter of *Agrobacterium rhizogenes* (Dehio *et al.* (1993) *Plant Mol. Biol.* 23:1199-210); the promoter of the *Agrobacterium tumefaciens* T-DNA gene 5 (Korber *et al.* (1991) *EMBO J.* 10:3983-91); the rice sucrose synthase RSs1 gene promoter (Shi *et al.* (1994) *J. Exp. Bot.* 45:623-31); the CoYMV or Commelina yellow mottle badnavirus promoter (Medberry *et al.* (1992) *Plant Cell* 4:185-92; Zhou *et al.* (1998) *Chin. J. Biotechnol.* 14:9-16); the CFDV or coconut foliar decay virus promoter (Rohde *et al.* (1994) *Plant Mol. Biol.* 27:623-28; Hehn and Rhode (1998) *J. Gen. Virol.* 79:1495-99); the RTBV or rice tungro bacilliform virus promoter (Yin and Beachy (1995) *Plant J.* 7:969-80; Yin *et al.* (1997) *Plant J.* 12:1179-80); the pea glutamine synthase GS3A gene (Edwards *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:3459-63; Brears *et al.* (1991) *Plant J.* 1:235-44); the inv CD111 and inv CD141 promoters of the potato invertase genes (Hedley *et al.* (2000) *J. Exp. Botany* 51:817-21); the promoter isolated from Arabidopsis shown to have phloem-specific expression in tobacco by Kertbundit *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5212-16); the VAHOX1 promoter region (Tornero *et al.* (1996) *Plant J.* 9:639-48); the pea cell wall invertase gene promoter (Zhang *et al.* (1996) *Plant Physiol.* 112:1111-17); the promoter of the endogenous cotton protein related to chitinase of US published patent application 20030106097, an acid invertase gene promoter from carrot (Ramloch-Lorenz *et al.* (1993) *The Plant J.* 4:545-54); the promoter of the sulfate transporter gene Sultr1;3 (Yoshimoto *et al.* (2003) *Plant Physiol.* 131:1511-17); a promoter of a sucrose synthase gene (Nolte and Koch (1993) *Plant Physiol.* 101:899-905); and the promoter of a tobacco sucrose transporter gene (Kuhn *et al.* (1997) *Science* 275-1298-1300).

[00080] Possible promoters also include the Black Cherry promoter for Prunasin Hydrolase (PH DL1.4 PRO) (US Patent No. 6,797, 859), thioredoxin H promoter from cucumber and rice (Fukuda A *et al.* (2005). *Plant Cell Physiol.* 46(11):1779-86), Rice (RSs1) (Shi, T. Wang *et al.* (1994). *J. Exp. Bot.* 45(274): 623-631) and maize sucrose synthase -1 promoters (Yang., N-S. *et al.* (1990) *PNAS* 87:4144-4148), PP2 promoter from pumpkin (Guo, H. *et al.* (2004) *Transgenic Research* 13:559-566), At SUC2 promoter (Truernit, E. *et al.* (1995) *Planta* 196(3):564-70., At SAM1 (S-adenosylmethionine synthetase) (Mijnsbrugge KV. *et al.*

(1996) *Planr. Cell. Physiol.* 37(8): 1108-1115), and the Rice tungro bacilliform virus (RTBV) promoter (Bhattacharyya-Pakrasi *et al. (*1993) *Plant J.* 4(1):71-79).

[00081] The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su et *al.* (2004) *Biotechnol Bioeng 85:*610-9 and Fetter *et al.* (2004) *Plant Cell 16:*215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science 117*:943-54 and Kato *et al.* (2002) *Plant Physiol 129*:913-42), and yellow florescent protein (PhiYFP™ from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science 117*:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

VI. Compositions Comprising Silencing Elements

[00082] One or more of the polynucleotides comprising a silencing element can be provided as an external composition such as a spray or powder to the plant, plant part, seed, a pest, or an area of cultivation. In another example, a plant is transformed with a DNA construct or expression cassette for expression of at least one silencing element. In either composition, the silencing element, when ingested by an insect, can reduce the level of a target pest sequence and thereby control the pest (i.e., a *Pentatomidae* plant pest including a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys*). It is recognized that the composition can comprise a cell (such as plant cell or a bacterial cell), in which a polynucleotide encoding one or more silencing elements is stably incorporated into the genome and operably linked to promoters active in the cell. Compositions comprising a mixture of cells, some cells expressing at least one silencing element are also encompassed. In other embodiments, compositions comprising the silencing elements are not contained in a cell. In such embodiments, the composition can be applied to an area inhabited by a pest. In one embodiment, the composition is applied externally to a plant (i.e., by spraying a field or area of cultivation) to protect the plant from the pest. Methods of applying nucleotides in such a manner are known to those skilled in the art.

[00083] The composition of the invention can further be formulated as bait. In this embodiment, the compositions comprise a food substance or an attractant which enhances the attractiveness of the composition to the pest.

[00084] The composition comprising the silencing element can be formulated in an agriculturally suitable and/or environmentally acceptable carrier. Such carriers can be any material that the animal, plant or environment to be treated can tolerate. Furthermore, the carrier must be such that the composition remains effective at controlling a pest. Examples of such carriers include water, saline, Ringer's solution, dextrose or other sugar solutions, Hank's solution, and other aqueous physiologically balanced salt solutions, phosphate buffer, bicarbonate buffer and Tris buffer. In addition, the composition may include compounds that increase the half-life of a composition.

[00085] It is recognized that the polynucleotides comprising sequences encoding the silencing element(s) can be used to transform organisms to provide for host organism production of these components, and subsequent application of the host organism to the environment of the target pest(s). Such host organisms include baculoviruses, bacteria, and the like. In this manner,

the combination of polynucleotides encoding the silencing element(s) may be introduced via a suitable vector into a microbial host, and said host applied to the environment, or to plants or animals.

[00086] The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be stably incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

[00087] Microbial hosts that are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplana) of one or more crops of interest may be selected. These microorganisms are selected so as to be capable of successfully competing in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the sequences encoding the silencing element, and desirably, provide for improved protection of the components from environmental degradation and inactivation.

[00088] Such microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms such as bacteria, e.g., *Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc,* and *Alcaligenes*, fungi, particularly yeast, e.g., *Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula,* and *Aureobasidium.* Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacteria, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, Clavibacter xyli* and *Azotobacter vinlandir,* and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces rosues, S. odorus, Kluyveromyces veronae,* and *Aureobasidium pollulans.* Of particular interest are the pigmented microorganisms.

[00089] A number of ways are available for introducing the polynucleotide comprising the silencing element(s) into the microbial host under conditions that allow for stable maintenance and expression of such nucleotide encoding sequences. For example, expression

cassettes can be constructed which include the nucleotide constructs of interest operably linked with the transcriptional and translational regulatory signals for expression of the nucleotide constructs, and a nucleotide sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system that is functional in the host, whereby integration or stable maintenance will occur.

[00090] Transcriptional and translational regulatory signals include, but are not limited to, promoters, transcriptional initiation start sites, operators, activators, enhancers, other regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, U.S. Patent Nos. 5,039,523 and 4,853,331; EPO 0480762A2; Sambrook *et al.* (2000); *Molecular Cloning: A Laboratory Manual* (3rd ed.; Cold Spring Harbor Laboratory Press, Plainview, NY); Davis *et al.* (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); and the references cited therein.

[00091] Suitable host cells include the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include *Enterobacteriaceae*, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, *and Proteus*; *Bacillaceae*; *Rhizobiceae*, such as *Rhizobium*; *Spirillaceae*, such as photobacterium, *Zymomonas* , *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum; Lactobacillaceae*; *Pseudomonadaceae*, such as *Pseudomonas* and *Acetobacter*; *Azotobacteraceae* and *Nitrobacteraceae.* Among eukaryotes are fungi, such as *Phycomycetes* and *Ascomycetes*, which includes yeast, such as *Saccharomyces* and *Schizosaccharomyces*; and *Basidiomycetes* yeast, such as *Rhodotorula*, *Aureobasidium*, *Sporobolomyces*, and the like.

[00092] Characteristics of particular interest in selecting a host cell for purposes of the invention include ease of introducing the coding sequence into the host, availability of expression systems, efficiency of expression, stability in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

[00093] Host organisms of particular interest include yeast, such as *Rhodotorula spp.*, *Aureobasidium spp.*, *Saccharomyces spp.*, and *Sporobolomyces spp.*, phylloplane organisms

such as *Pseudomonas spp.*, *Erwinia spp.*, and *Flavobacterium spp.*, and other such organisms, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Bacillus thuringiensis*, *Escherichia coli*, *Bacillus subtilis*, and the like.

[00094] The sequences encoding the silencing elements encompassed by the invention can be introduced into microorganisms that multiply on plants (epiphytes) to deliver these components to potential target pests. Epiphytes, for example, can be gram-positive or gram-negative bacteria.

[00095] The silencing element can be fermented in a bacterial host and the resulting bacteria processed and used as a microbial spray in the same manner that *Bacillus thuringiensis* strains have been used as insecticidal sprays. Any suitable microorganism can be used for this purpose. By way of example, *Pseudomonas* has been used to express *Bacillus thuringiensis* endotoxins as encapsulated proteins and the resulting cells processed and sprayed as an insecticide Gaertner *et al.* (1993), in *Advanced Engineered Pesticides,* ed. L. Kim (Marcel Decker, Inc.).

[00096] Alternatively, the components of the invention are produced by introducing heterologous genes into a cellular host. Expression of the heterologous sequences results, directly or indirectly, in the intracellular production of the silencing element. These compositions may then be formulated in accordance with conventional techniques for application to the environment hosting a target pest, e.g., soil, water, and foliage of plants. See, for example, EPA 0192319, and the references cited therein.

[00097] In the present invention, a transformed microorganism can be formulated with an acceptable carrier into separate or combined compositions that are, for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

[00098] Such compositions disclosed above may be obtained by the addition of a surface-active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protectant, a buffer, a flow agent or fertilizers, micronutrient donors, or other preparations that influence plant growth. One or more agrochemicals including, but not limited to, herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, acaracides, plant growth regulators, harvest aids, and fertilizers, can

be combined with carriers, surfactants or adjuvants customarily employed in the art of formulation or other components to facilitate product handling and application for particular target pests. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g., natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders, or fertilizers. The active ingredients of the present invention (i.e., at least one silencing element) are normally applied in the form of compositions and can be applied to the crop area, plant, or seed to be treated. For example, the compositions may be applied to grain in preparation for or during storage in a grain bin or silo, *etc.* The compositions may be applied simultaneously or in succession with other compounds. Methods of applying an active ingredient or a composition that contains at least one silencing element include, but are not limited to, foliar application, seed coating, and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest.

[00099] Suitable surface-active agents include, but are not limited to, anionic compounds such as a carboxylate of, for example, a metal; carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono- or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate, or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl aryl sulfonates such as alkyl-benzene sulfonates or lower alkylnaphtalene sulfonates, e.g., butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of sulfonated phenol-formaldehyde condensates; more complex sulfonates such as the amide sulfonates, e.g., the sulfonated condensation product of oleic acid and N-methyl taurine; or the dialkyl sulfosuccinates, e.g., the sodium sulfonate or dioctyl succinate. Non-ionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, e.g., sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, e.g., polyoxyethylene sorbitan fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetraethyl-5-decyn-4,7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di-, or polyamine such as an acetate, naphthenate or oleate; or oxygen-containing amine such as an amine oxide of polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

[000100] Examples of inert materials include, but are not limited to, inorganic minerals such as kaolin, phyllosilicates, carbonates, sulfates, phosphates, or botanical materials such as cork, powdered corncobs, peanut hulls, rice hulls, and walnut shells.

[000101] The compositions comprising the silencing element(s) can be in a suitable form for direct application or as a concentrate of primary composition that requires dilution with a suitable quantity of water or other dilutant before application.

[000102] The compositions (including the transformed microorganisms) can be applied to the environment of an insect pest (such as a *Pentatomidae* plant pest such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pest) by, for example, spraying, atomizing, dusting, scattering, coating or pouring, introducing into or on the soil, introducing into irrigation water, by seed treatment or general application or dusting at the time when the pest has begun to appear or before the appearance of pests as a protective measure. For example, the composition(s) and/or transformed microorganism(s) may be mixed with grain to protect the grain during storage. It is generally important to obtain good control of pests in the early stages of plant growth, as this is the time when the plant can be most severely damaged. The compositions can conveniently contain another insecticide if this is thought necessary. In an embodiment of the invention, the composition(s) is applied directly to the soil, at a time of planting, in granular form of a composition of a carrier and dead cells of a *Bacillus* strain or transformed microorganism of the invention. Another embodiment is a granular form of a composition comprising an agrochemical such as, for example, a herbicide, an insecticide, a fertilizer, in an inert carrier, and dead cells of a *Bacillus* strain or transformed microorganism of the invention.

VII. Plants, Plant Parts, and Methods of Introducing Sequences into Plants

[000103] In one embodiment, the methods of the invention involve introducing a polynucleotide into a plant. "Introducing" is intended to mean presenting to the plant the polynucleotide in such a manner that the sequence gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a sequence into a plant, only that the polynucleotides or polypeptides gain access to the interior of at least one cell of the plant. Methods for introducing polynucleotides into plants are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

[000104] "Stable transformation" is intended to mean that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by the progeny thereof. "Transient transformation" is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant or a polypeptide is introduced into a plant.

[000105] Transformation protocols as well as protocols for introducing polypeptides or polynucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing polypeptides and polynucleotides into plant cells include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (U.S. Patent No. 5,563,055 and U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent Nos. 4,945,050; U.S. Patent No. 5,879,918; U.S. Patent No. 5,886,244; and, 5,932,782; Tomes *et al.* (1995) in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Nos. 5,240,855; 5,322,783; and, 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

[000106] In specific embodiments, the silencing element sequences of the invention can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the protein or variants and fragments thereof directly into the plant or the introduction of the transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway *et al.* (1986) *Mol Gen. Genet. 202*:179-185; Nomura *et al.* (1986) *Plant Sci. 44:53-58*; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci. 91*: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science 107*:775-784, all of which are herein incorporated by reference. Alternatively, polynucleotides can be transiently transformed into the plant using techniques known in the art. Such techniques include viral vector systems and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, the transcription from the particle-bound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethyleneimine (PEI; Sigma-Aldrich Corp., St. Louis, MO, Catalog No. P3143).

[000107] In other embodiments, the polynucleotide of the invention may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing polynucleotides into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367, 5,316,931, and Porta *et al.* (1996) *Molecular Biotechnology 5*:209-221; herein incorporated by reference.

[000108] Methods are known in the art for the targeted insertion of a polynucleotide at a specific location in the plant genome. In one embodiment, the insertion of the polynucleotide at a desired genomic location is achieved using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference. Briefly, the polynucleotide of the invention can be contained in a transfer cassette flanked by two non-recombinogenic recombination sites. The transfer cassette is introduced into a plant having stably incorporated into its genome a target site which is flanked by two non-recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is

integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

[000109] The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting progeny having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a polynucleotide of the invention, for example, an expression cassette of the invention, stably incorporated into their genome.

[000110] As used herein, the term plant also includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides.

[000111] The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* 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*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

[000112] Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include 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 pulcherrima*), and chrysanthemum.

[000113] Conifers that 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*). In specific embodiments, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean plants and sugarcane plants are optimal, and in yet other embodiments corn plants are optimal.

[000114] Other plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

VIII. Methods of Use

[000115] Methods of the invention comprise methods for controlling a pest (i.e., a *Pentatomidae* plant pest, such as, *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pest). In one embodiment, the method comprises feeding to a pest a

composition comprising a silencing element of the invention, wherein said silencing element, when ingested by a pest (i.e., a *Pentatomidae* plant pest including *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys*), reduces the level of a target polynucleotide of the pest and thereby controls the pest. The pest can be fed the silencing element(s) in a variety of ways. For example, in one embodiment, a polynucleotide comprising the silencing element(s) is introduced into a plant. As the *Pentatomidae* plant pest such as, for example, a *N. viridula*, *Acrosternum hilare*, *Piezodorus guildini*, and/or *Halymorpha halys* plant pest feeds on the plant or part thereof expressing these sequences, the silencing element is delivered to the pest. When the silencing element is delivered to the plant in this manner, it is recognized that the silencing element can be expressed constitutively or alternatively, it may be produced in a stage-specific manner by employing the various inducible or tissue-preferred or developmentally regulated promoters that are discussed elsewhere herein. In specific embodiments, the silencing element(s) is expressed in the roots, stalk or stem, leaf including pedicel, xylem and phloem, fruit or reproductive tissue, silk, flowers and all parts therein or any combination thereof.

[000116] In another method, a composition comprising at least one silencing element of the invention is applied to a plant. In such embodiments, the silencing element can be formulated in an agronomically suitable and/or environmentally acceptable carrier, which is preferably, suitable for dispersal in fields. In addition, the carrier can also include compounds that increase the half-life of the composition. In specific embodiments, the composition comprising the silencing element is formulated in such a manner such that it persists in the environment for a length of time sufficient to allow it to be delivered to a pest. In such embodiments, the composition can be applied to an area inhabited by a pest. In one embodiment, the composition is applied externally to a plant (i.e., by spraying a field) to protect the plant from pests.

[000117] In certain embodiments, the constructs of the present invention can be stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired trait. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. For example, the polynucleotides of the present invention may be stacked with any other polynucleotides encoding polypeptides having pesticidal and/or insecticidal activity, such as other *Bacillus thuringiensis* toxic proteins (described in U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene* 48:109), lectins (Van Damme *et al.* (1994) *Plant Mol. Biol.* 24:825, pentin (described in U.S. Patent No. 5,981,722), and the like. The combinations generated can also include multiple

copies of any one of the polynucleotides of interest. The polynucleotides of the present invention can also be stacked with any other gene or combination of genes to produce plants with a variety of desired trait combinations including, but not limited to, traits desirable for animal feed such as high oil genes (e.g., U.S. Patent No. 6,232,529); balanced amino acids (e.g., hordothionins (U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,409); barley high lysine (Williamson *et al.* (1987) *Eur. J. Biochem.* 165:99-106; and WO 98/20122) and high methionine proteins (Pedersen *et al.* (1986) *J. Biol. Chem.* 261:6279; Kirihara *et al.* (1988) *Gene* 71:359; and Musumura *et al.* (1989) *Plant Mol. Biol.* 12:123)); increased digestibility (e.g., modified storage proteins (U.S. Application Serial No. 10/053,410, filed November 7, 2001); and thioredoxins (U.S. Application Serial No. 10/005,429, filed December 3, 2001)); the disclosures of which are herein incorporated by reference.

[000118] The polynucleotides of the present invention can also be stacked with traits desirable for disease or herbicide resistance (e.g., fumonisin detoxification genes (U.S. Patent No. 5,792,931); avirulence and disease resistance genes (Jones *et al.* (1994) *Science* 266:789; Martin *et al.* (1993) *Science* 262:1432; Mindrinos *et al.* (1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)); and traits desirable for processing or process products such as high oil (e.g., U.S. Patent No. 6,232,529); modified oils (e.g., fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., U.S. Patent No. 5.602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides providing agronomic traits such as male sterility (e.g., see U.S. Patent No. 5.583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (e.g., WO 99/61619, WO 00/17364, and WO 99/25821); the disclosures of which are herein incorporated by reference.

[000119] These stacked combinations can be created by any method including, but not limited to, cross-breeding plants by any conventional or TopCross methodology, or genetic transformation. If the sequences are stacked by genetically transforming the plants (i.e.,

molecular stacks), the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO99/25821, WO99/25854, WO99/25840, WO99/25855, and WO99/25853, all of which are herein incorporated by reference.

[000120] The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Selection of DNAs

[000121] DNAs were selected by two different methods. cDNA libraries were constructed using the SMART cDNA Synthesis Kit (Clontech) from mRNA isolated from second instar southern green stinkbug (Nezara viridula (Linnaeus)) or mRNA isolated from the head of second and third instar southern green stinkbugs. Select clones were sequenced and subject to BLAST analysis to create an expressed sequence tag (EST) library. The library was BLAST queried with sequences of interest and southern green stinkbug homologs were identified.

[000122] Additionally, a transcriptome of second instar southern green stinkbug was created using Illumina sequencing. Sequences were assembled using Oases (Schulz et al. 2012) and annotated using a proprietary functional annotation pipeline. The transcriptome was BLAST queried with sequences of interest and southern green stinkbug homologs were identified. DNAs were synthesized using RT-PCR. In brief, mRNA from second instar southern green stinkbug was reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen; catalog # 18080-051) using random primers. Sequences of interest were PCR amplified using

gene specific primers and ReadyMix Taq PCR Reaction Mix (Sigma-Aldrich Corp., St. Louis, MO, Catalog No. P4600). The resulting DNA was analyzed on TAE agarose gels and cloned into pCR2.1 (Invitrogen). The resulting clones were sequenced and sequence verified clones were used to produce double stranded RNA.

Example 2: Production of Double Stranded RNA

[000123] Either EST clones or clones derived from RT-PCR and cloned into pCR2.1 were used as template for PCR. Sequences flanking the insert were fused with the T7 promoter sequence (TAATACGACTCACTATAGGG, SEQ ID 1) and used to generate primers (Table 1) to PCR amplify DNA. This PCR amplified DNA was used to synthesize double stranded RNA (dsRNA) using the MEGAscript® kit (Ambion, Catalog No. AM1334) following the manufacturer's protocol. Products of PCR as well as dsRNA synthesis were run on 1% agarose gel to verify amplification. With the EST clones, after the initial screening, fragments of the EST clones were amplified using gene specific primers fused with the T7 promoter sequence.

TABLE 1

EST PRIMER 1	TAATACGACTCACTATAGGGATGCCCGGGAATTCGGCCATTACG	SEQ ID 2
EST PRIMER 2	TAATACGACTCACTATAGGGCGCGCCAAACGAATGGTCTAGAAA GC	SEQ ID 3
pCR2.1 Primer 1	TAATACGACTCACTATAGGGCTAGTAACGGCCGCCAGTGTGCTG	SEQ ID 4
pCR2.1 Primer 1	TAATACGACTCACTATAGGGGGCCGCCAGTGTGATGGATATCTG	SEQ ID 5

Example 3: Selected Clones

The following clones (Table 2) were selected for use in the bioassay.

TABLE 2

SEQ ID NO	Clone name	length bp	DESCRIPTION	Corresponding full-length DNA
SEQ ID NO 21	ta01222.002 Fragment 1	362	WD domain, G-beta repeat protein	ta01222.002_nezvi SEQ ID 6
SEQ ID NO 22	ta01222.002 Fragment 2	369	WD domain, G-beta repeat protein	ta01222.002_nezvi SEQ ID 6
SEQ ID NO 23	ta01222.002 Fragment 3	374	WD domain, G-beta repeat protein	ta01222.002_nezvi SEQ ID 6
SEQ ID NO 24	ta02948.001 Fragment 1	355	Coatomer protein complex, subunit beta 1,	ta02948.001_nezvi SEQ ID 7
SEQ ID NO 25	ta02948.001 Fragment 2	382	Coatomer protein complex, subunit beta 1,	ta02948.001_nezvi SEQ ID 7
SEQ ID NO 26	ta02948.001 Fragment 3	376	Coatomer protein complex, subunit beta 1,	ta02948.001_nezvi SEQ ID 7
SEQ ID NO 27	ta00781.001 Fragment 1	340	Coatomer, gamma subunit,	ta00781.001_nezvi SEQ ID 8
SEQ ID NO 28	ta00781.001 Fragment 2	383	Coatomer, gamma subunit,	ta00781.001_nezvi SEQ ID 8

SEQ ID NO 29	ta00781.001 Fragment 3	388	Coatomer, gamma subunit,	ta00781.001_nezvi SEQ ID 8
SEQ ID NO 30	nezvi_22408.WL.1 Fragment 3	412	Ryanodine receptor	nezvi_22408.WL.1 SEQ ID 9
SEQ ID NO 31	nezvi_22408.WL.1 Fragment 6	342	Ryanodine receptor	nezvi_22408.WL.1 SEQ ID 9
SEQ ID NO 32	nezvi_22408.WL.1 Fragment 7	432	Ryanodine receptor	nezvi_22408.WL.1 SEQ ID 9
SEQ ID NO 33	nezvi_22408.WL.1 Fragment 9	367	Ryanodine receptor	nezvi_22408.WL.1 SEQ ID 9
SEQ ID NO 34	nezvi_22408.WL.1 Fragment 14	396	Ryanodine receptor	nezvi_22408.WL.1 SEQ ID 9
SEQ ID NO 10	inv2c.pk011.b22.f	965	26S proteasome non-ATPase regulatory subunit 7	inv2c.pk011.b22.f SEQ ID 10
SEQ ID NO 35	inv2c.pk011.b22.f Fragment 1	557	26S proteasome non-ATPase regulatory subunit 7	inv2c.pk011.b22.f SEQ ID 10
SEQ ID NO 36	inv2c.pk011.b22f Fragment 2	530	26S proteasome non-ATPase regulatory subunit 7	inv2c.pk011.b22.f SEQ ID 10
SEQ ID NO 11	inv2c.pk020.l19.f	924	Proteasome subunit alpha type-2	inv2c.pk020.l19.f SEQ ID 11
SEQ ID NO 37	inv2c.pk020.l19.f Fragment 1	544	Proteasome subunit alpha type-2	inv2c.pk020.l19.f SEQ ID 11
SEQ ID NO 38	inv2c.pk020.l19.f Fragment 2	587	Proteasome subunit alpha type-2	inv2c.pk020.l19.f SEQ ID 11
SEQ ID NO 12	inv3c.pk002.i8.f	946	26S protease regulatory subunit 8-like	inv3c.pk002.i8.f SEQ ID 12
SEQ ID NO 39	inv3c.pk002.i8.f Fragment 1	550	26S protease regulatory subunit 8-like	inv3c.pk002.i8.f SEQ ID 12
SEQ ID NO 40	inv3c.pk002.i8.f Fragment 2	580	26S protease regulatory subunit 8-like	inv3c.pk002.i8.f SEQ ID 12

Example 4: Stinkbug Collection and Bioassay

[000124] Southern green stinkbug eggs were collected from a laboratory maintained colony and kept in an incubator at 27 °C with 65% relative humidity. After hatching, the insects were allowed to feed on green beans with or without the addition of green peas. Thereafter freshly molted second instar stinkbugs were transferred onto a modified artificial Lygus diet (Bioserve; Lygus Hesperus diet, catalog # F9644B) supplemented either with dsRNA or water (as control). Five second instar stinkbugs per bioassay were fed with 200 ppm dsRNA supplemented in the artificial diet. The diet with dsRNA or water was changed every two days and the bioassay observations on stunting and/or mortality were taken on day 7. All insects were weighed at the conclusion of the assay.

Example 5: Results of dsRNA Feeding

[000125] Five second instars per experiment were either fed upon a diet mixed with select dsRNA or water (control) and each experiment was replicated two to six times. The number of replicates is reported in column four (labeled N) of Table Three. Feeding of select dsRNAs to second instar southern green stinkbug significantly inhibited the growth when compared with controls. At the conclusion of the bioassay (day 7), the control stinkbugs developed into late third instars and weighed on an average 11.3 ± 0.9 mg (group A) or 8.4 ± 0.8 mg (group B). It is understood by those in the field that bioassay data can vary depending upon the time the assays are run which explains the differences in control weight. Insects fed selected dsRNA developed poorly and were still in second instar stage and only developed to 46-66% of the control weight. See Table 3.

TABLE 3

DNA	SEQ ID NO	Group	% control weight	N
ta01222.002 Fragment 1	21	A	66	4
ta01222.002 Fragment 2	22	A	59	4
ta01222.002 Fragment 3	23	A	51	4
ta02948.001 Fragment 1	24	A	49	4
ta02948.001 Fragment 2	25	A	57	4
ta02948.001 Fragment 3	26	A	63	4
ta00781.001 Fragment 1	27	A	52	4
ta00781.001 Fragment 2	28	A	59	4
ta00781.001 Fragment 3	29	A	58	4
nezvi_22408.WL.1 Fragment 7	32	A	58	3
inv2c.pk011.b22.f	10	B	46	2
inv2c.pk011.b22.f Fragment 1	35	B	48	2
inv2c.pk011.b22f Fragment 2	36	B	63	6
inv2c.pk020.l19.f	11	B	48	2
inv2c.pk020.l19.f Fragment 1	37	B	61	2
inv2c.pk020.l19.f Fragment 2	38	B	50	6
inv3c.pk002.i8.f	12	B	49	2
inv3c.pk002.i8.f Fragment 1	39	B	52	2
inv3c.pk002.i8.f Fragment 2	40	B	55	2
nezvi_22408.WL.1 Fragment 3	30	B	54	6
nezvi_22408.WL.1 Fragment 6	31	B	55	6
nezvi_22408.WL.1 Fragment 7	32	B	47	6
nezvi_22408.WL.1 Fragment 9	33	B	57	6
nezvi_22408.WL.1 Fragment 14	34	B	60	6

Example 6: Construction of hairpin constructs for plant transformation

[000126] A selection of the fragments that showed activity in the in vitro insect assay were used to make constructs for plant transformation. Fragments were amplified using gene specific primers flanked by sequence encoding an ATT B4 recombinase sequence

(CAACTTTGTATAGAAAAGTTG; SEQ ID 13) on one side and an ATT B3 recombinase sequence (CAACTTTGTATAATAAAGTTG; SEQ ID 14) on the other side. The resulting amplified DNA was cloned into pCR2.1 and clones were sequenced. Sequence verified clones were recombined into plasmid PHP36164 (Figure 1, SEQ ID 15) using a BP Gateway Reaction (Invitrogen). The resulting clones were then recombined into PHP59032 (Figure 2, SEQ ID 16) using a LR Gateway Reaction (Invitrogen). The resulting plasmid contains a hairpin-structured transcript controlled by the seed specific promoter kit. The cassette comprising a promoter and terminator separated by a unique Not I restriction endonuclease site comprises the KTi3 promoter, a unique Not I restriction endonuclease site, and the KTi3 terminator region. This cassette comprises about 2088 nucleotides of the KTi3 promoter, a unique Not I restriction endonuclease site, and about 202 nucleotides of the KTi3 transcription terminator. The gene encoding KTi3 has been described (Jofuku, K.D. and Goldberg, R.B., *Plant Cell* 1:1079-1093 (1989)).

[000127] It is understood that such a hairpin-structured transcript will form a dsRNA in vivo. The plasmid also contains a promoterless Glycine max acetolactate synthase (P178S) which is useful as a selectable marker. These two cassettes are flanked by FRT1 and FRT87 sites that are required for site specific integration during soybean transformation. An example of such a plasmid is PHP62151 (Figure 3, SEQ ID 17).

Example 7: Transformation and Regeneration of Soybean (Glycine max)

[000128] Transgenic soybean lines are generated by the method of particle gun bombardment (Klein et al., *Nature* (London) 327:70-73 (1987); U.S. Patent No. 4,945,050) using a BIORAD Biolistic PDS1000/He instrument and either plasmid or fragment DNA.

[000129] Integration of DNA into the soybean genome after particle gun-mediated transformation may be random, or it may be through site-specific integration (SSI), achieved by recombinase-mediated cassette exchange (RMCE) at a previously created transgenic target site (US patent application 7,102,055 issued September 5, 2006). Recombinase-mediated DNA cassette exchange RMCE using different recombinase systems have been achieved successfully in several plants (Nanto K, Yamada-Watanabet K, Ebinuma H(2005) Agrobacterium-mediated RMCE approach for gene replacement. Plant Biotechnol J, 3: 203–214; Louwerse JD et al. 2007. Stable recombinase-mediated cassette exchange in Arabidopsis using Agrobacterium tumefaciens. Plant Physiol 145: 1282–1293; Li Z. et al. 2009, Site-specific integration of

transgenes in soybean via recombinase-mediated DNA cassette exchange. Plant Physiol 151: 1087–1095). Groups of transgenes can be stacked to the same site through multiple rounds of RMCE (Li et al 2010, Published online before print August 2010, doi:10.1104/pp.110.160093; Plant Physiology October 2010 vol. 154 no. 2 622-631). Taking advantage of reversible DNA cassette exchange in RMCE, an RMCE product can be used as a new target for subsequent SSI transformation.

[000130] The transgenic target site for RMCE may contain a promoter followed by recombination sites surrounding a selectable marker gene such as the hygromycin phosphotransferase (HPT) gene, with or without additional components. After bombardment with donor DNA, the target DNA previously integrated into the soybean genome recombines with the donor DNA at recombination sites such as FRT1 and FRT87 with the help of a transiently expressed recombinase such as the FLP recombinase. The portion of the DNA cassette in the target which contains the original selectable marker gene flanked by dissimilar recombination sites such as FRT1 and FRT87 is replaced by the donor DNA cassette flanked by the same FRT1 and FRT87 sites, resulting in site-specific integration of the donor cassette to the exact same genomic site of the target. The promoter existing upstream of the recombination sites in the transgenic target remains after RMCE to regulate expression of the new selectable marker gene delivered to the site as part of the donor cassette. Successful RMCE events may be identified by chemical selection for cells expressing the selectable marker gene of the donor.

Culture media and stock solutions

[000131] The following stock solutions and media are used for transformation and regeneration of soybean plants:

Stock solutions:

[000132] Sulfate 100 X Stock:

37.0 g $MgSO_4.7H_2O$, 1.69 g $MnSO_4.H_2O$, 0.86 g $ZnSO_4.7H_2O$, 0.0025 g $CuSO_4.5H_2O$

Halides 100 X Stock:

30.0 g $CaCl_2.2H_2O$, 0.083 g KI, 0.0025 g $CoCl_2.6H_2O$

P, B, Mo 100X Stock:

18.5 g KH_2PO_4, 0.62 g H_3BO_3, 0.025 g $Na_2MoO_4.2H_2O$

Fe EDTA 100X Stock:

3.724 g Na_2EDTA, 2.784 g $FeSO_4.7H_2O$

2,4-D Stock:

10 mg/mL Vitamin

B5 vitamins, 1000X Stock:

100.0 g *myo*-inositol, 1.0 g nicotinic acid, 1.0 g pyridoxine HCl, 10 g thiamine.HCL.

Media (per Liter):

SB199 Solid Medium:

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066), 1 mL B5 vitamins 1000X stock, 30 g sucrose, 4 ml 2, 4-D (40 mg/L final concentration), pH 7.0, 2 g Gelrite™

SB1 Solid Medium:

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066), 1 mL B5 vitamins 1000X stock, 31.5 g Glucose, 2 mL 2, 4-D (20 mg/L final concentration), pH 5.7, 8 g TC agar

SB196:

10 mL of each of the above stock solutions 1-4, 1 mL B5 Vitamin stock, 0.463 g $(NH_4)_2SO_4$, 2.83 g KNO_3, 1 mL 2,4 D stock, 1 g asparagine, 10 g sucrose, pH 5.7

SB71-4:

Gamborg's B5 salts, 20 g sucrose, 5 g TC agar, pH 5.7.

SB103:

1 pk. Murashige & Skoog salts mixture, 1 mL B5 Vitamin stock, 750 mg $MgCl_2$ hexahydrate, 60 g maltose, 2 g Gelrite™, pH 5.7.

SB166:

SB103 supplemented with 5 g per liter activated charcoal.

Soybean Embryogenic Suspension Culture Initiation:

[000133] Pods with immature seeds from available soybean plants 45-55 days after planting are picked, removed from their shells and placed into a sterilized magenta box. The soybean seeds are sterilized by shaking them for 15 min in a 5% Clorox solution with soap or other surfactants at 1 drop per 100 mL solution. Seeds are rinsed with sterile distilled water, and those less than 4 mm are placed on a sterile surface under microscope. The small ends of seeds are cut, and the cotyledons are pressed out of the seed coats. Cotyledons are transferred to plates containing SB199 medium (25-30 cotyledons per plate) for 2 weeks, then transferred to SB1 for 2-4 weeks. Plates are wrapped with fiber tape and cultured for 8 weeks in growth chamber room

with temperature set at 24.4-26 ºC and light on a 16:8 h day/night photoperiod at an intensity of 45-65 µE/m2/s . After this time, secondary embryos are cut and placed into SB196 liquid medium for 7 days.

Culture Conditions:

[000134] Soybean embryogenic suspension cultures are maintained in 50 mL liquid medium SB196 on a rotary shaker at a speed of 100 - 150 rpm. The cultures are set in a growth chamber with temperature set at 24.4 -26 °C and light on a 16:8 h day/night photoperiod at intensity of 80-100 µE/m2/s for liquid culture and 80-120 µE/m2/s for maturation and germination. Cultures are subcultured every 7-14 days by inoculating up to ½ dime size quantity of tissue into 50 mL of fresh liquid SB196.

Preparation of DNA for Bombardment:

[000135] In particle gun bombardment procedures it is possible to use purified 1) entire plasmid DNA; or 2) DNA fragments containing only the recombinant DNA expression cassette(s) of interest. For every bombardment experiment, 85 µL of suspension is prepared containing 1 to 90 pg of plasmid DNA per base pair of DNA. To prepare for an SSI transformation, the donor plasmid is mixed with plasmid DNA containing the FLP recombinase gene cassette in a ratio such as 3:1. Both recombinant DNA plasmids are co-precipitated onto gold particles as follows. The DNAs in suspension are added to 50 µL of a 10 – 60 mg/mL 0.6 µm gold particle suspension and then combined with 50 µL $CaCl_2$ (2.5 M) and 20 µL spermidine (0.1 M). The mixture is vortexed for 5 sec, spun in a microcentrifuge for 5 sec, and the supernatant removed. The DNA-coated particles are then washed once with 150 µL of 100% ethanol, vortexed and spun in a microcentrifuge again, then resuspended in 85 µL of anhydrous ethanol. Five µL of the DNA-coated gold particles are then loaded onto each macrocarrier disk.

Tissue Preparation and Bombardment with DNA:

[000136] Approximately 100-200 mg of two-week-old suspension culture is placed in an empty 60 mm X 15 mm petri plate and the residual liquid removed from the tissue using a pipette. The tissue is placed about 3.5 inches away from the retaining screen. Membrane rupture pressure is set at 650 psi and the bombardment chamber of the particle gun is evacuated to –28 inches of Hg prior to bombardment. Typically, each plate of tissue is bombarded once.

Selection of Transformed Embryos and Plant Regeneration:

[000137] After bombardment, tissue from each bombarded plate is divided and placed into one to two flasks of SB196 liquid culture maintenance medium per plate of tissue, one flask per 100 mg tissue. Seven days post bombardment, the liquid medium in each flask is replaced with fresh SB196 culture maintenance medium supplemented with 100 ng/ml selective agent (selection medium). For selection of transformed soybean cells after random transformation or RMCE, the selective agent used can be a sulfonylurea (SU) compound with the chemical name, 2-chloro-N-((4-methoxy-6 methyl-1,3,5-triazine-2-yl)aminocarbonyl) benzenesulfonamide (common names: DPX-W4189 and chlorsulfuron). Chlorsulfuron is the active ingredient in the DuPont sulfonylurea herbicide, GLEAN®. The selection medium containing SU is replaced every two weeks for 8 weeks. After the 8 week selection period, islands of green, transformed tissue are observed growing from untransformed, necrotic embryogenic clusters. The putative transgenic randomly integrated or RMCE events are isolated and kept in SB196 liquid medium with SU at 100 ng/ml for another 5 weeks with media changes every 1-2 weeks to generate new, clonally propagated, transformed embryogenic suspension cultures. Embryos spend a total of around 13 weeks in contact with SU. Suspension cultures are subcultured and maintained as clusters of immature transgenic embryos and also regenerated into whole plants by maturation and germination of individual somatic embryos.

[000138] Transgenic somatic embryos become suitable for germination after four weeks on maturation medium (1 week on SB166 followed by 3 weeks on SB103). They are then removed from the maturation medium and dried in empty petri dishes, or with a small amount of medium, for approximately seven days. The dried embryos are then planted in SB71-4 medium where they are allowed to germinate under the same light and temperature conditions as described above. Germinated embryos are allowed to develop into small plantlets and are then transferred to potting medium and grown to maturity for seed production.

Example 8: Bioassay of Soybean Plants

[000139] After transformation, transgenic soybean plants will be grown in the greenhouse and seeds will be harvested from these transformed plants and designated as T1 seeds. T1 seeds will be chipped manually and DNA extracted from the chips will be used to determine zygosity using a quantitative PCR assay. Homozygous seeds will be sown in 2.5 inch pots, maintained in the growth chambers in 16:8 (light: dark) cycle in an insecticide free environment. After about 4

weeks, these plants will be transplanted to a larger pot and maintained at 14:10 (light: dark) cycle for 2 weeks. After two weeks, the plants will be maintained in 12:12 (light: dark) cycle to induce flowering and delivered for bioassay at R3 stage. Fertilizer will be provided as needed and chambers are maintained at 50% relative humidity. Ten second instar southern green stinkbugs will be used to infest soybean pods at various stages: R3 (beginning pod), R4 (full pod), R5 (beginning seed), R6 (full seed) and R7 (beginning maturity). Insects will be maintained on the pods using enclosures. Developmental stage, stunting (% control as outlined in example 5) and mortality will be recorded at 8-10 days after initial infest of the transgenic soybean pods.

Example 9: Alternative Sequences for nezvi_22408.WL.1 and inv2c.pk011.b22.f

[000140] A transcriptome is a collection of all the transcripts present in a given cell. As such, a transcriptome includes alternative spliced variants that are present within the cell. For nezvi_22408.WL.1 two alternatively spliced variants are predicted: nezvi_22408.WL.2 (SEQ ID 18) and nezvi_22408.WL.3 (SEQ ID 19). RT-PCR as described in Example 1 along with primers that were designed to amplify transcript specific sequences as well as cloning and sequence verification show that all three transcripts are real and exist in second instar southern green stinkbug mRNA.

[000141] Similarly, it is understood that cDNA library sequences may not encode the entire transcript. The sequence for the clone inv2c.pk011.b22.f (SEQ ID 10) was used to BLAST query the transcriptome and a longer sequence named nezvi_3755.WL.1 (SEQ ID 20) was found.

CLAIMS

What is claimed is:

1. An expression cassette, comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence comprising any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof;

(b) a nucleotide sequence comprising at least 90% sequence identity to any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest;

(c) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest; and,

(d) a nucleotide sequence that hybridizes under stringent conditions to the full length complement of the nucleotide sequence of a), wherein said stringent conditions comprise hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 °C to 65 °C, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest.

2. The expression cassette of Claim 1, wherein said *Pentatomidae* plant pest is a *N. viridula* plant pest.

3. The expression cassette of Claim 1, wherein said polynucleotide is operably linked to a heterologous promoter.

4. The expression cassette of Claim 1, wherein said polynucleotide is expressed as a double stranded RNA.

5. The expression cassette of claim 1, wherein said polynucleotide comprise a silencing element which is expressed as a hairpin RNA.

6. The expression cassette of claim 5, wherein the silencing element comprises, in the following order, a first segment, a second segment, and a third segment, wherein

a) said first segment comprises at least about 19 nucleotides having at least 90% sequence complementarity to a target sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof;

b) said second segment comprises a loop of sufficient length to allow the silencing element to be transcribed as a hairpin RNA; and,

c) said third segment comprises at least about 19 nucleotides having at least 85% complementarity to the first segment.

7. The expression cassette of Claim 1, wherein said polynucleotide is flanked by a first operably linked convergent promoter at one terminus of the polynucleotide and a second operably linked convergent promoter at the opposing terminus of the polynucleotide, wherein the first and the second convergent promoters are capable of driving expression of the polynucleotide.

8. A host cell comprising a heterologous expression cassette of any one of claims 1-7.

9. A plant cell having stably incorporated into its genome a heterologous polynucleotide comprising a silencing element, wherein said silencing element comprises

a) a fragment of at least 19 consecutive nucleotides of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof; or,

b) the nucleotide sequence comprising at least 90% sequence identity to any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof,

wherein said silencing element, when ingested by a *Pentatomidae* plant pest, reduces the level of a target sequence in said *Pentatomidae* plant pest and thereby controls the *Pentatomidae* plant pest.

10. The plant cell of Claim 9, wherein the *Pentatomidae* plant pest is a *N. viridula* plant pest.

11. The plant cell of Claim 9, wherein said silencing element comprises

a) a polynucleotide comprising the sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof;

b) a polynucleotide comprising at least 75 consecutive nucleotides of the sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof.

12. The plant cell of Claim 9, wherein said plant cell comprises the expression cassette of Claim 7.

13. The plant cell of any one of Claims 9-12, wherein said silencing element expresses a double stranded RNA.

14. The plant cell of any one of Claims 9-12, wherein said silencing element expresses a hairpin RNA.

15. The plant cell of Claim 14, wherein said polynucleotide comprising the silencing element comprises, in the following order, a first segment, a second segment, and a third segment, wherein

a) said first segment comprises at least about 19 nucleotides having at least 90% sequence complementarity to a target sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof;

b) said second segment comprises a loop of sufficient length to allow the silencing element to be transcribed as a hairpin RNA; and,

c) said third segment comprises at least about 19 nucleotides having at least 85% complementarity to the first segment.

16. The plant cell of any one of Claims 9-12, wherein said silencing element is operably linked to a heterologous promoter.

17. The plant cell of any one of Claims 9-12, wherein said plant cell is from a monocot.

18. The plant cell of Claim 17, wherein said monocot is maize, barley, millet, wheat or rice.

19. The plant cell of Claim 13, wherein said plant cell is from a monocot.

20. The plant cell of Claim 19, wherein said monocot is maize, barley, millet, wheat or rice.

21. The plant cell of Claim 14, wherein said plant cell is from a monocot.

22. The plant cell of Claim 21, wherein said monocot is maize, barley, millet, wheat or rice.

23. The plant cell of Claim 15, wherein said plant cell is from a monocot.

24. The plant cell of Claim 23, wherein said monocot is maize, barley, millet, wheat or rice.

25. The plant cell of any one of Claims 9-12, wherein said plant cell is from a dicot.

26. The plant cell of Claim 25, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

27. The plant cell of Claim 13, wherein said plant cell is from a dicot.

28. The plant cell of Claim 27, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

29. The plant cell of Claim 14, wherein said plant cell is from a dicot.

30. The plant cell of Claim 21, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

31. The plant cell of Claim 15, wherein said plant cell is from a dicot.

32. The plant cell of Claim 31, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

33. A plant or plant part comprising a plant cell of any one of Claims 9-12, or 18-32.

34. A transgenic seed from the plant of Claim 33.

35. A method for controlling a *Pentatomidae* plant pest comprising feeding to a *Pentatomidae* plant pest a composition comprising a silencing element, wherein said silencing element, when ingested by said *Pentatomidae* plant pest, reduces the level of a target *Pentatomidae* plant pest sequence and thereby controls the *Pentatomidae* plant pest, wherein said target *Pentatomidae* plant pest sequence comprise a nucleotide sequence comprising at least 90% sequence identity to any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof.

36. The method of Claim 35, wherein said *Pentatomidae* plant pest comprises a *N. viridula* plant pest.

37. The method of Claim 35, wherein said silencing element comprises

a) a fragment of at least 19 consecutive nucleotides of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof; or,

b) a nucleotide sequence comprising at least 90% sequence identity to any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof.

38. The method of any one of Claims 35-37, wherein said composition comprises a plant or plant part having stably incorporated into its genome a polynucleotide comprising said silencing element.

39. The method of Claim 38, wherein said silencing element comprises

a) a polynucleotide comprising the sense or antisense sequence of the sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof;

b) a polynucleotide comprising the sense or antisense sequence of a sequence having at least 95% sequence identity to the sequence set forth in SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof;

c) a polynucleotide comprising the sense or antisense sequence of a sequence having at least 75 contiguous nucleotides of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof.

40. The method of Claim 39, wherein said silencing element expresses a double stranded RNA.

41. The method of Claim 39, wherein said silencing element comprises a hairpin RNA.

42. The method of Claim 41, wherein said polynucleotide comprising the silencing element comprises, in the following order, a first segment, a second segment, and a third segment, wherein

a) said first segment comprises at least about 20 nucleotides having at least 90% sequence complementarity to the target polynucleotide;

b) said second segment comprises a loop of sufficient length to allow the silencing element to be transcribed as a hairpin RNA; and,

c) said third segment comprises at least about 20 nucleotides having at least 85% complementarity to the first segment.

43. The method of any one of Claims 35-37 or 39-42, wherein said silencing element is operably linked to a heterologous promoter.

44. The method of any one of Claims 35-37 or 39-42, wherein said silencing element is flanked by a first operably linked convergent promoter at one terminus of the silencing element and a second operably linked convergent promoter at the opposing terminus of the polynucleotide, wherein the first and the second convergent promoters are capable of driving expression of the silencing element.

45. The method of any one of Claims 35-37, wherein said plant is a monocot.

46. The method of Claim 45, wherein said monocot is maize, barley, millet, wheat or rice.

47. The method of Claim 38, wherein said plant is a monocot.

48. The method of Claim 47, wherein said monocot is maize, barley, millet, wheat or rice.

49. The method of Claim 39, wherein said plant is a monocot.

50. The method of Claim 49, wherein said monocot is maize, barley, millet, wheat or rice.

51. The method of Claim 40, wherein said plant is a monocot.

52. The method of Claim 51, wherein said monocot is maize, barley, millet, wheat or rice.

53. The method of Claim 41, wherein said plant is a monocot.

54. The method of Claim 53, wherein said monocot is maize, barley, millet, wheat or rice.

55. The method of any one of Claims 35-37, wherein said plant is a dicot.

56. The method of Claim 45, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

57. The method of Claim 38, wherein said plant is a dicot.

58. The method of Claim 47, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

59. The method of Claim 39, wherein said plant is a dicot.

60. The method of Claim 59, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

61. The method of Claim 40, wherein said plant is a dicot.

62. The method of Claim 61, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

63. The method of Claim 41, wherein said plant is a dicot.

64. The method of Claim 63, wherein said dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

65. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence comprising any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof;

(b) the nucleotide sequence comprising at least 90% sequence identity to any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest;

(c) the nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOS: 6-12, 18-40, a fragment or variant thereof, or a complement thereof, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest; and,

(d) the nucleotide sequence that hybridizes under stringent conditions to the full length complement of the nucleotide sequence of a), wherein said stringent conditions comprise hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 °C to 65 °C, wherein said polynucleotide encodes a silencing element having insecticidal activity against a *Pentatomidae* plant pest.

66. The polynucleotide of Claim 65, wherein said *Pentatomidae* plant pest is a *N. viridula* plant pest.

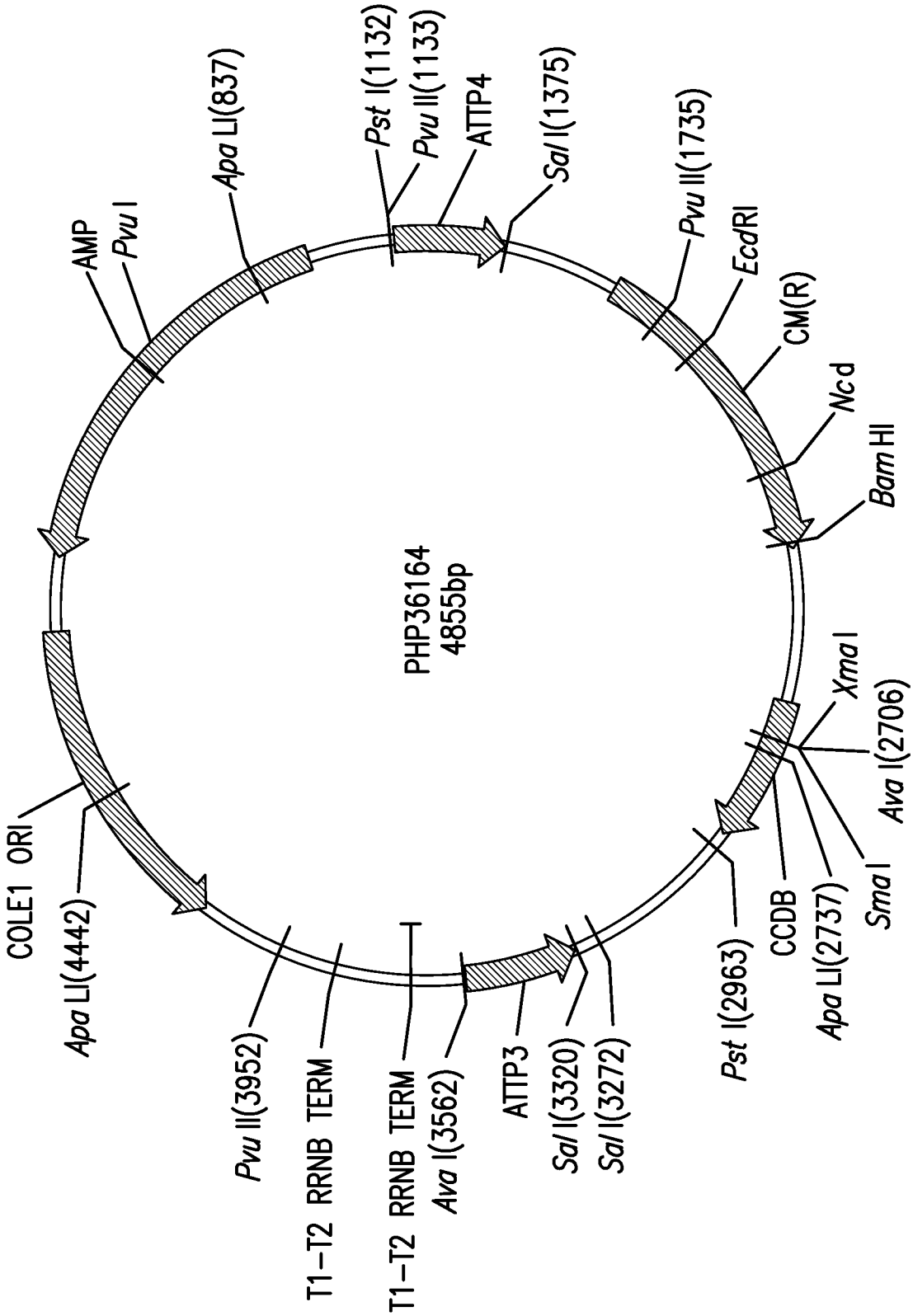

PHP36164
4855bp
AMP
Pvu I
Apa LI(837)
Pst I(1132)
Pvu II(1133)
ATTP4
Sal I(1375)
Pvu II(1735)
EcdRI
CM(R)
Ncd
BamHI
Ava I(2706)
Xma I
Sma I
Apa LI(2737)
CCDB
Pst I(2963)
Sal I(3272)
Sal I(3320)
ATTP3
Ava I(3562)
T1-T2 RRNB TERM
T1-T2 RRNB TERM
Pvu II(3952)
Apa LI(4442)
COLE1 ORI

FIG. 1

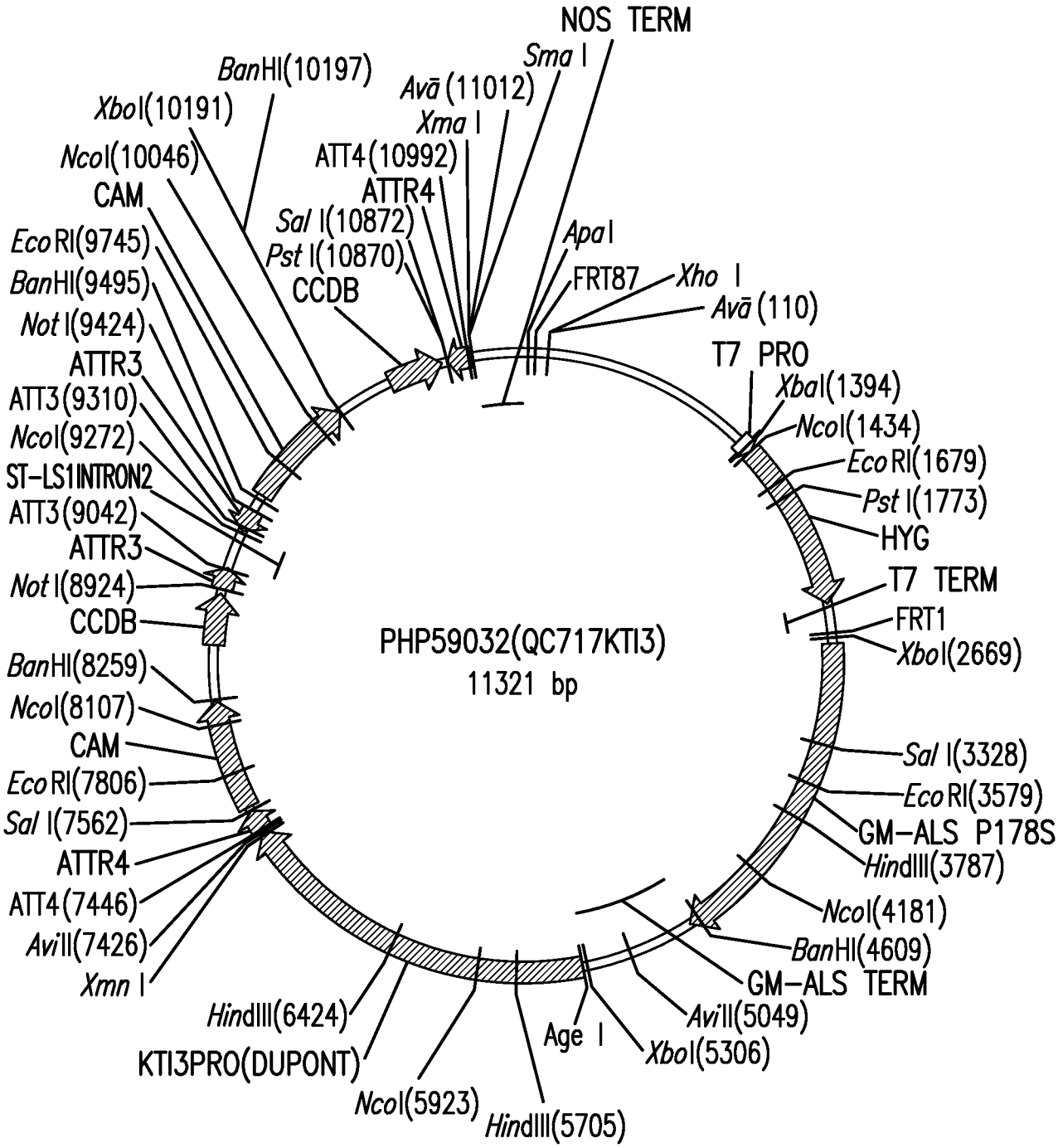

NOS TERM
Sma I
BanHI(10197)
Avā (11012)
Xbo I(10191)
Xma I
NcoI(10046)
ATT4(10992)
CAM
ATTR4
Sal I(10872)
Apa I
EcoRI(9745)
Pst I(10870)
FRT87
Xho I
BanHI(9495)
CCDB
Avā (110)
Not I(9424)
T7 PRO
ATTR3
XbaI(1394)
ATT3(9310)
NcoI(1434)
NcoI(9272)
EcoRI(1679)
ST-LS1INTRON2
Pst I(1773)
ATT3(9042)
HYG
ATTR3
T7 TERM
Not I(8924)
FRT1
CCDB
XboI(2669)
PHP59032(QC717KTI3)
11321 bp
BanHI(8259)
NcoI(8107)
Sal I(3328)
CAM
EcoRI(3579)
EcoRI(7806)
GM-ALS P178S
Sal I(7562)
HindIII(3787)
ATTR4
NcoI(4181)
ATT4(7446)
BanHI(4609)
AviII(7426)
GM-ALS TERM
Xmn I
AviII(5049)
HindIII(6424)
Age I
XboI(5306)
KTI3PRO(DUPONT)
NcoI(5923)
HindIII(5705)

FIG. 2

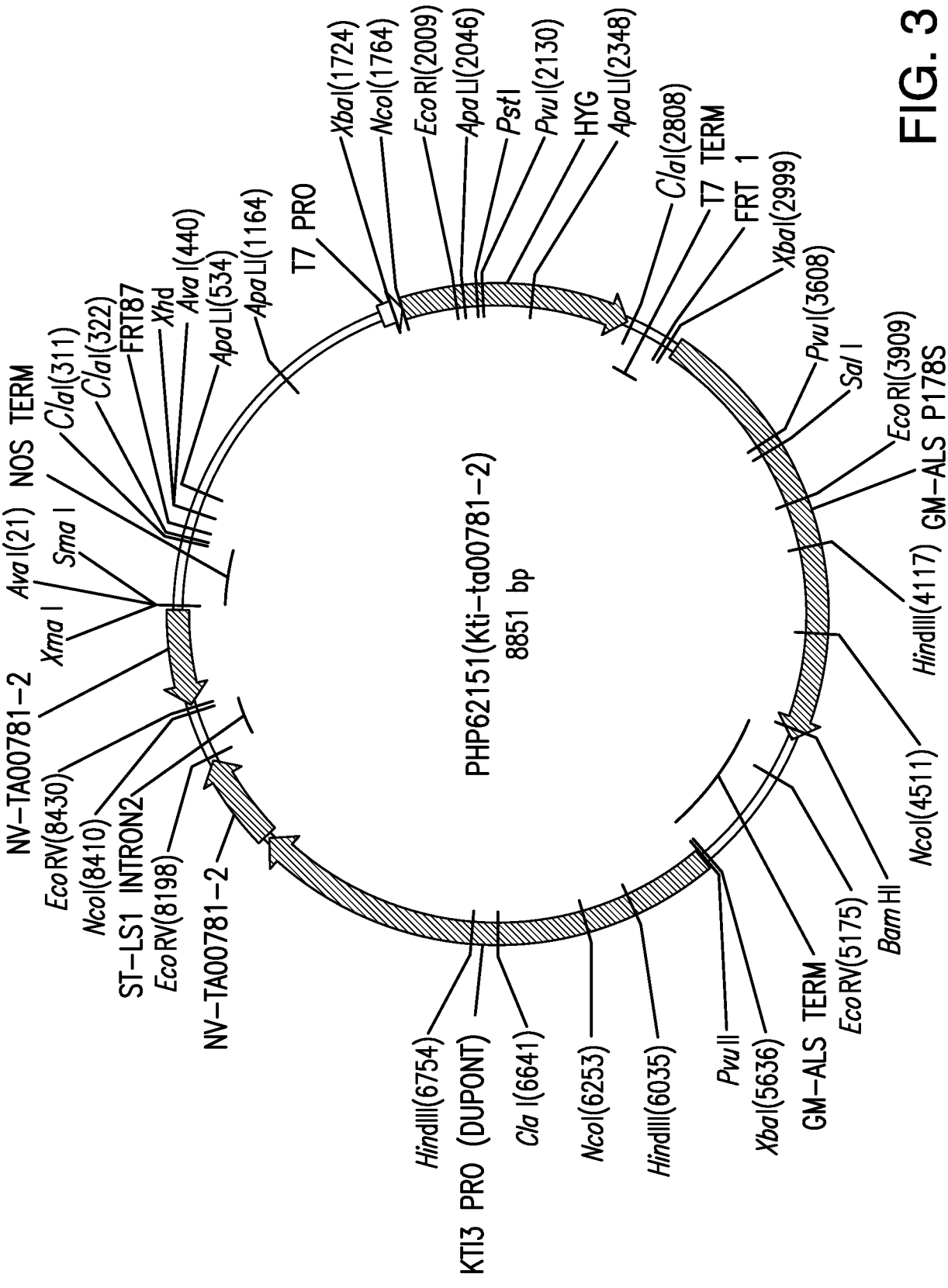

PHP62151(Kti-ta00781-2)
8851 bp
NV-TA00781-2
EcoRV(8430)
NcoI(8410)
ST-LS1 INTRON2
EcoRV(8198)
NV-TA00781-2
HindIII(6754)
KTI3 PRO (DUPONT)
Cla I(6641)
NcoI(6253)
HindIII(6035)
PvuII
XbaI(5636)
GM-ALS TERM
EcoRV(5175)
BamHI
NcoI(4511)
HindIII(4117)
GM-ALS P178S
EcoRI(3909)
SalI
PvuI(3608)
XbaI(2999)
FRT 1
T7 TERM
ClaI(2808)
ApaLI(2348)
HYG
PvuI(2130)
PstI
ApaLI(2046)
EcoRI(2009)
NcoI(1764)
XbaI(1724)
T7 PRO
ApaLI(1164)
ApaLI(534)
AvaI(440)
XhoI
FRT87
ClaI(322)
ClaI(311)
NOS TERM
AvaI(21)
SmaI
XmaI

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/25274

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/11, C12N 15/82, A01N 63/00, C12N 15/00 (2014.01)
USPC - 514/44A, 800/279, 800/288, 800/302, 424/93.2, 435/320.1, 435/419
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)
IPC - C12N 15/11, C12N 15/82, A01N 63/00, C12N 15/00 (2014.01)
USPC - 514/44A, 800/279, 800/288, 800/302, 424/93.2, 435/320.1, 435/419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 536/23.1, 536/24.5
CPC - A61K 38/00, C12N 15/86, C12N 15/8257, C12N 15/8282

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST; Google Scholar; PatBase
search terms - Pentatomidae, southern green, stinkbu*, viridula, nezvi, plant, pest, insect*, rna, silenc$, mirna, rnai, sirna, dsrna, shrna, hairpin, hair-pin, interfer$

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2007/0124836 A1 (BAUM et al.) 31 May 2007 (31.05.2007) para [0015]; [0017]; [0051]; [0074]; [0075]; [0162]; [0179]; [0236]; SEQ ID NO: 878.	1, 3-8, 65 ---------- 2, 66
Y	US 2012/0297501 A1 (BEGHYN et al.) 22 November 2012 (22.11.2012) para [0162]; [0164].	2, 66
A	US 2011/0301223 A1 (BROGLIE et al.) 08 December 2011 (08.12.2011) para [0064]; [0084].	1

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
06 August 2014 (06.08.2014)	26 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/25274

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.:
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:
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.

Group I+, claims 1-8 and 65-66, directed to an expression cassette/an isolated polynucleotide. The first invention of Group I+ is restricted to the first named nucleotide sequence SEQ ID NO: 6, and will be searched without additional fee. It is believed that claims 1-8 and 65-66 read on this first named invention. Applicants must indicate, if applicable, the claims which read on 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 inventions of Group I+ will result in only the first claimed invention to be searched/examined. Should additional fees be paid, Applicant is invited to elect additional nucleic acid sequences to be searched. An exemplary election comprises SEQ ID NO: 7 (claims 1-8, 65-66).

--continued on first extra sheet attached hereto--

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.:
1-8 and 65-66, limited to SEQ ID NO: 6

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.

continuation of Box No III:

Group II+, claims 9-32, directed to a plant cell having stably incorporated into its genome a heterologous polynucleotide comprising a silence element. Group II+ will be searched upon payment of additional fee(s). An exemplary election would be: the first named nucleotide sequence SEQ ID NO: 6. It is believed that claims 9-34 read on this named invention. Applicants must indicate, if applicable, the claims which read on this 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. Another exemplary election would be: SEQ ID NO: 7 (claims 9-34).

Group III+, claims 35-42, 45-64, directed to a method for controlling a Pentatomidae plant pest comprising feeding to a Pentatomidae plant pest a composition comprising a silencing element. Group III+ will be searched upon payment of additional fee(s). An exemplary election would be: the first named nucleotide sequence SEQ ID NO: 6. It is believed that claims 35-64 read on this named invention. Applicants must indicate, if applicable, the claims which read on this 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. Another exemplary election would be: SEQ ID NO: 7 (claims 35-64).

The inventions listed as Groups I+, II+ and III+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features
Group I+ includes the special technical feature of an isolated polynucleotide comprising an expression cassette, which is not required by Groups II+ or III+.
Group II+ includes the special technical feature of a plant cell having stably incorporated into its genome a heterologous polynucleotide comprsing a silence element, which is not required by Groups I+ or III+.
Group III+ includes the special technical feature of a method for controlling a Pentatomidae plant pest comprising feeding to a Pentatomidae plant pest a composition comprising a silencing element, which is not required by Groups I+ or II+.
Another special technical feature of each invention within each of Groups I+, II+ and III+ is the specific sequences recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among SEQ ID NOS: 6-12 and 18-40. Without a shared special technical feature, the inventions lack unity with one another.

Common technical features
Groups I+, II+ and III+ include the common technical feature of a nucleotide sequence, wherein said polynucleotide encodes a silencing element having insecticidal activity against a Pentatomidae plant pest. Groups II+ and III+ further share the common technical feature of wherein said silencing element, when ingested by a Pentatomidae plant pest, reduces the level of a target sequence in said Pentatomidae plant pest and thereby controls the Pentatomidae plant pest. However, these shared technical features do not represent a contribution over prior art, because the shared technical features are anticipated by reference US 2011/0301223 A1 to Broglie et al., (hereinafter Broglie).

Broglie teaches an expression cassette comprising a nucleotide sequence (para [0064]), wherein said polynucleotide encodes a silencing element having insecticidal activity against a Pentatomidae plant pest (abstract and para [0084]).

Broglie further teaches a plant cell having stably incorporated into its genome a heterologous polynucleotide comprising a silencing element (para [0084] 'the composition can comprise a cell (such as plant cell or a bacterial cell), in which a polynucleotide encoding the silencing element is stably incorporated into the genome'), wherein said silencing element, when ingested by a Pentatomidae plant pest, reduces the level of a target sequence in said Pentatomidae plant pest and thereby controls the Pentatomidae plant pest (abstract and para [0084] 'the silencing element, when ingested by an insect, can reduce the level of a target pest sequence and thereby control the pest (i.e., a Pentatomidae plant pest').

Broglie further teaches a method for controlling a Pentatomidae plant pest comprising feeding to a Pentatomidae plant pest a composition comprising a silencing element (abstract and para [0084]), wherein said silencing element, when ingested by said Pentatomidae plant pest, reduces the level of a target Pentatomidae plant pest sequence and thereby controls the Pentatomidae plant pest (abstract and para [0084]).

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+, II+ and III+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.