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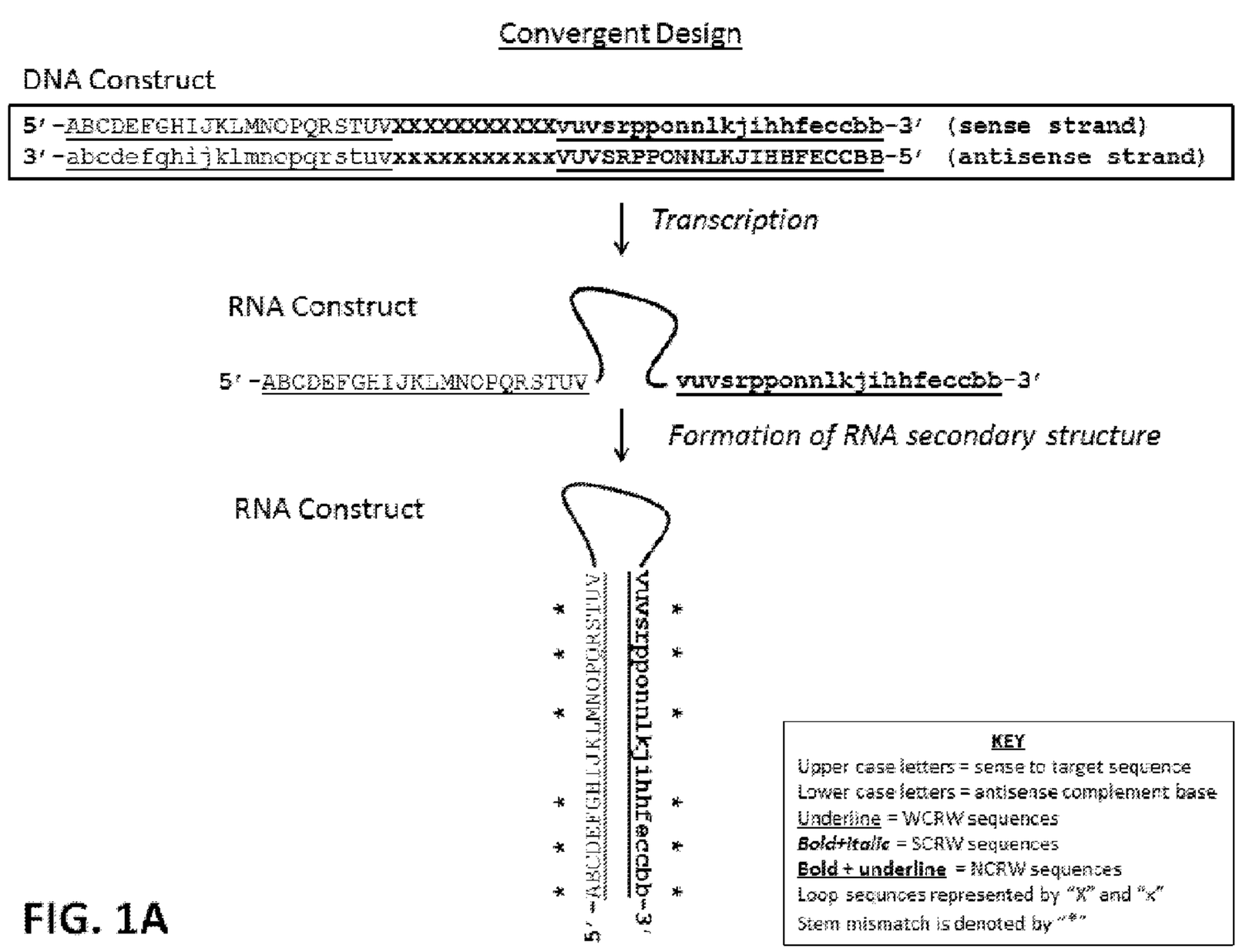

FIG. 1A

(57) Abrégé/Abstract:
Methods and compositions are provided which employ a silencing element that, when ingested by a plant insect pest, such as a Coleopteran plant pest or a Diabrotica plant pest, decrease the expression of a target sequence in the pest. Disclosed are various target polynucleotides set forth in any one of SEQ ID NOS: 1-33 disclosed herein, (but not including the forward and reverse primers.) or variants and fragments thereof, or complements thereof, wherein a decrease in expression of one or more of the sequences in the target pest controls the pest (i.e., has insecticidal activity). Plants, plant parts, 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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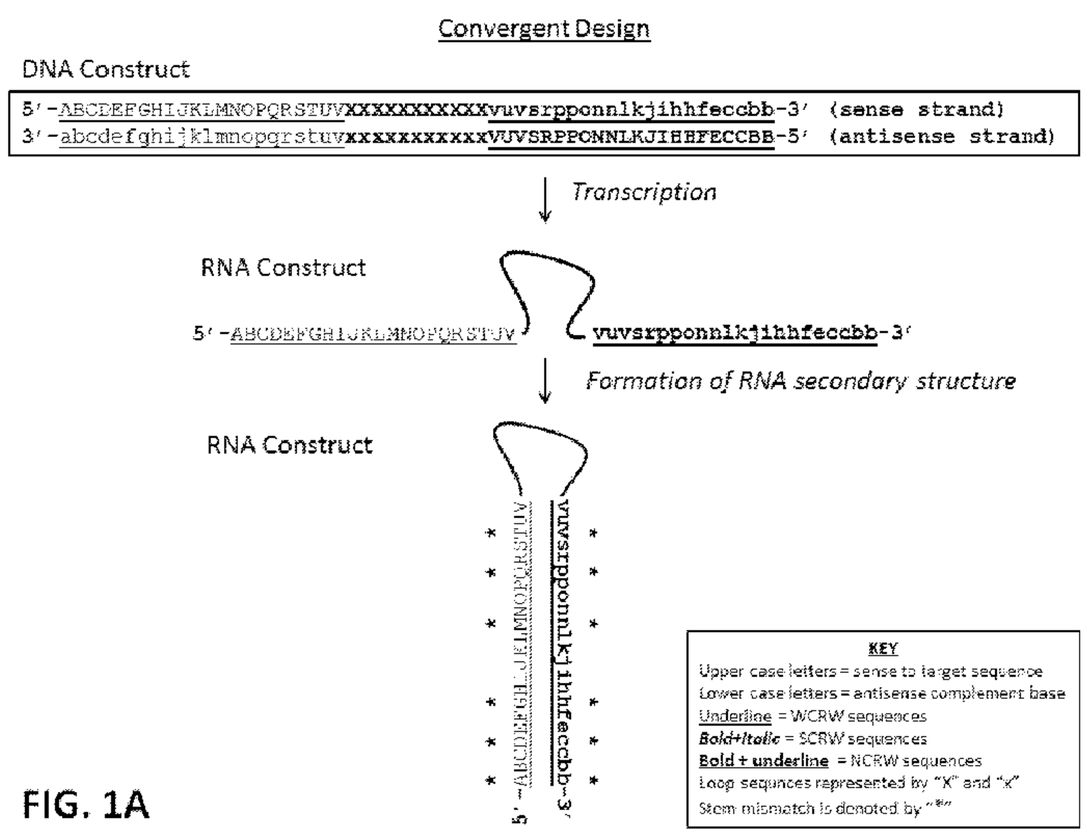

FIG. 1A

(57) **Abstract**: Methods and compositions are provided which employ a silencing element that, when ingested by a plant insect pest, such as a Coleopteran plant pest or a *Diabrotica* plant pest, decrease the expression of a target sequence in the pest. Disclosed are various target polynucleotides set forth in any one of SEQ ID NOS: 1-33 disclosed herein, (but not including the forward and reverse primers.) or variants and fragments thereof, or complements thereof, wherein a decrease in expression of one or more of the sequences in the target pest controls the pest (i.e., has insecticidal activity). Plants, plant parts, 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 TO CONTROL INSECT PESTS

FIELD

The present disclosure relates generally to methods of molecular biology and gene silencing to control pests.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS-WEB

The Sequence Listing created on August 12, 2015 as a text file named "6862_SeqList," and having a size of 34,733 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND

Plant insect pests are a serious problem in agriculture. They destroy millions of acres of staple crops such as corn, soybeans, peas, and cotton. Yearly, plant insect 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, in some instances these *Bt* insecticidal proteins may only protect plants from a relatively narrow range of pests. Thus, novel insect control compositions remain desirable..

BRIEF SUMMARY

Methods and compositions are provided which employ a silencing element that, when ingested by a plant insect pest, such as Coleopteran plant pests or *Diabrotica* plant pests or other plant insect pests, is capable of decreasing the expression of a target sequence in the pest. In one embodiment, design aspects for engineering silencing elements having improved activity are provided. In certain embodiments, the methods and compositions employ a silencing element comprising a first segment, a second segment, and a third segment, wherein the first segment and third segments are complementary comprising less than or equal to 20% base pair mismatches. The first and third segments comprise nucleotide sequences targeted to distinct insect species. In specific embodiments, the first segment comprises domains targeted to two or more insect species, and the third segment comprises complementary sequences targeted to two or more insect species.

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. Described herein are various target polynucleotides as set forth in SEQ ID NOs.: 1-33, or variants or fragments thereof, or complements thereof, wherein a decrease in expression of one or more of 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. Sprayable compositions comprising the silencing agents for topical applications to pest insects or substrates are also contemplated.

In another embodiment, a method for controlling a plant insect pest, such as a Coleopteran plant pest or a *Diabrotica* plant pest, is provided. The method comprises feeding to a plant insect 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 plant insect pest. Such methods comprise introducing into the plant or plant part a disclosed silencing element. When the plant expressing the silencing element is ingested by the pest, the level of

the target sequence is decreased and the pest is controlled.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (**FIG. 1A** and **FIG. 1B**) shows schematic representations of the convergent type of mismatch construct as disclosed herein. **FIG. 1A** shows a DNA construct that when transcribed to RNA comprises a segment 1 in the sense orientation to the target RNA, wherein segment 1 is consists of a hypothetical WCRW sequence; a segment 2 that consists of a sequence that is not self-complementary; and a segment 3 in the antisense orientation to the target RNA, wherein segment 3 is complementary to segment 1 and consists of a hypothetical NCRW sequence. **FIG. 1B** shows a DNA construct that when transcribed to RNA comprises a segment 1 in the sense orientation to the target RNA, wherein segment 1 is a chimera consists of hypothetical WCRW, SCRW, and NCRW sequences; a segment 2 that consists of a sequence that is not self-complementary; and a segment 3 in the antisense orientation to the target RNA, wherein segment 3 is complementary to segment 1 and consists of hypothetical WCRW and NCRW sequences.

Figure 2 (**FIG. 2A** and **FIG. 2B**) shows schematic representations of the divergent type of mismatch construct as disclosed herein. **FIG. 2A** shows a DNA construct that when transcribed to RNA comprises a segment 1 in the antisense orientation to the target RNA, wherein segment 1 is consists of a hypothetical WCRW sequence; a segment 2 that consists of a sequence that is not self-complementary; and a segment 3 in the sense orientation to the target RNA, wherein segment 3 is complementary to segment 1 and consists of a hypothetical NCRW sequence. **FIG. 2B** shows a DNA construct that when transcribed to RNA comprises a segment 1 in the antisense orientation to the target RNA, wherein segment 1 is a chimera consists of hypothetical WCRW and NCRW sequences; a segment 2 that consists of a sequence that is not self-complementary; and a segment 3 in the sense orientation to the target RNA, wherein segment 3 is complementary to segment 1 and consists of hypothetical WCRW, SCRW, and NCRW sequences.

Figure 3 (**FIG. 3A** and **FIG. 3B**) shows greenhouse bioassay obtained in maize plants transformed with DNA constructs comprising SEQ ID NOs.: 10-14. **FIG. 3A** shows representative data obtained in maize plants transformed with DNA constructs comprising SEQ ID NOs.: 11, 13, and 14 and compared to non-transformed control plants. **FIG. 3B**

shows… shows representative data obtained in maize plants transformed with DNA constructs comprising SEQ ID NOs.: 10 and 12 and compared to non-transformed control plants.

DETAILED DESCRIPTION

The disclosures herein will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all possible embodiments are shown. Indeed, disclosures 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.

Many modifications and other embodiments disclosed herein will come to mind to one skilled in the art to which the disclosed compositions and methods pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the disclosures 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.

It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting. As used in the specification and in the claims, the term "comprising" can include the aspect of "consisting of." Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosed compositions and methods belong. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined herein.

I. Overview

Frequently, RNAi discovery methods rely on evaluation of known classes of sensitive genes (transcription factors, housekeeping genes etc.). In contrast, target polynucleotides set forth herein were identified based solely on high throughput screens of all singletons and representatives of all gene clusters from a cDNA library of neonate and/or 3rd instar midgut western corn rootworms. This screen allowed for the discovery of many novel sequences,

many of which have extremely low or no homology to known sequences. This method provided the advantage of having no built in bias to genes that are frequently highly conserved across taxa. As a result, many novel targets for RNAi as well as known genes not previously shown to be sensitive to RNAi have been identified.

As such, methods and compositions are provided which employ one or more silencing elements that, when ingested by a plant insect pest, such as a Coleopteran plant pest or a *Diabrotica* 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. Dislcosed herein are target polynucleotides as set forth in SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof. Silencing elements comprising sequences, complementary sequences, active fragments or variants of these target polynucleotides are provided which, when ingested by or when contacting the pest, decrease the expression of one or more of the target sequences and thereby controls the pest (i.e., has insecticidal activity).

As used herein, by "controlling a plant insect pest" or "controls a plant insect pest" is intended any affect on a plant insect pest that results in limiting the damage that the pest causes. Controlling a plant insect 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, or in a manner for 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.

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 the invading pest. Reducing the level of expression of the target sequence of the pest will reduce the pest damage 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, methods disclosed herein can be utilized to control pests, including but not limited to, Coleopteran plant insect pests or a *Diabrotica* plant pest.

Assays measuring the control of a plant insect pest are commonly known in the art, as

are methods to record nodal injury score. See, for example, Oleson et al. (2005) J. Econ. Entomol. 98:1-8. See, for example, the examples below.

Disclosed herein are compositions and methods for protecting plants from a plant insect pest, or inducing resistance in a plant to a plant insect pest, such as Coleopteran plant pests or *Diabrotica* plant pests or other plant insect pests. Plant insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera Orthroptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Lepidoptera and Coleoptera.

Those skilled in the art will recognize that not all compositions are equally effective against all pests. Disclosed compositions, including the silencing elements disclosed herein, display activity against plant insect pests, which may include economically important agronomic, forest, greenhouse, nursery ornamentals, food and fiber, public and animal health, domestic and commercial structure, household and stored product pests.

As used herein "Coleopteran plant pest" is used to refer to any member of the Coleoptera order. Other plant insect pests that may be targeted by the methods and compositions disclosed herein, but are not limited to Mexican Bean Beetle (*Epilachna varivestis*), and Colorado potato beetle (*Leptinotarsa decemlineata*).

As used herein, the term "*Diabrotica* plant pest" is used to refer to any member of the *Diabrotica* genus. Accordingly, the compositions and methods are also useful in protecting plants against any *Diabrotica* plant pest including, for example, *Diabrotica adelpha; Diabrotica amecameca; Diabrotica balteata; Diabrotica barberi; Diabrotica biannularis; Diabrotica cristata; Diabrotica decempunctata; Diabrotica dissimilis; Diabrotica lemniscata; Diabrotica limitata* (including, for example, *Diabrotica limitata quindecimpuncata*); *Diabrotica longicornis; Diabrotica nummularis; Diabrotica porracea; Diabrotica scutellata; Diabrotica sexmaculata; Diabrotica speciosa* (including, for example, *Diabrotica speciosa speciosa); Diabrotica tibialis; Diabrotica undecimpunctata* (including, for example, Southern corn rootworm (*Diabrotica undecimpunctata*), *Diabrotica undecimpunctata duodecimnotata; Diabrotica undecimpunctata howardi* (spotted cucumber beetle); *Diabrotica undecimpunctata undecimpunctata* (western spotted cucumber beetle)); *Diabrotica virgifera* (including, for example, *Diabrotica virgifera virgifera* (western corn rootworm) and *Diabrotica virgifera zeae* (Mexican corn rootworm)); *Diabrotica viridula; Diabrotica wartensis; Diabrotica sp.*

JJG335; Diabrotica sp. JJG336; Diabrotica sp. JJG341; Diabrotica sp. JJG356; Diabrotica sp. JJG362; and, *Diabrotica sp. JJG365.*

In specific embodiments, the *Diabrotica* plant pest comprises *D. virgifera virgifera*, *D. barberi*, *D. virgifera zeae, D. speciosa*, or *D. undecimpunctata howardi*.

Larvae of the order Lepidoptera include, but are not limited to, armyworms, cutworms, loopers and heliothines in the family Noctuidae Spodoptera frugiperda JE Smith (fall armyworm); S. exigua Hübner (beet armyworm); S. litura Fabricius (tobacco cutworm, cluster caterpillar); Mamestra configurata Walker (bertha armyworm); M. brassicae Linnaeus (cabbage moth); Agrotis ipsilon Hufnagel (black cutworm); A. orthogonia Morrison (western cutworm); A. subterranea Fabricius (granulate cutworm); Alabama argillacea Hübner (cotton leaf worm); Trichoplusia ni Hübner (cabbage looper); Pseudoplusia includens Walker (soybean looper); Anticarsia gemmatalis Hübner (velvetbean caterpillar); Hypena scabra Fabricius (green cloverworm); Heliothis virescens Fabricius (tobacco budworm); Pseudaletia unipuncta Haworth (armyworm); Athetis mindara Barnes and Mcdunnough (rough skinned cutworm); Euxoa messoria Harris (darksided cutworm); Earias insulana Boisduval (spiny bollworm); E. vittella Fabricius (spotted bollworm); Helicoverpa armigera Hübner (American bollworm); H. zea Boddie (corn earworm or cotton bollworm); Melanchra picta Harris (zebra caterpillar); Egira (Xylomyges) curialis Grote (citrus cutworm); borers, casebearers, webworms, coneworms, and skeletonizers from the family Pyralidae Ostrinia nubilalis Hübner (European corn borer); Amyelois transitella Walker (naval orangeworm); Anagasta kuehniella Zeller (Mediterranean flour moth); Cadra cautella Walker (almond moth); Chilo suppressalis Walker (rice stem borer); C. partellus, (sorghum borer); Corcyra cephalonica Stainton (rice moth); Crambus caliginosellus Clemens (corn root webworm); C. teterrellus Zincken (bluegrass webworm); Cnaphalocrocis medinalis Guenée (rice leaf roller); Desmia funeralis Hübner (grape leaffolder); Diaphania hyalinata Linnaeus (melon worm); D. nitidalis Stoll (pickleworm); Diatraea grandiosella Dyar (southwestern corn borer), D. saccharalis Fabricius (surgarcane borer); Eoreuma loftini Dyar (Mexican rice borer); Ephestia elutella Hübner (tobacco (cacao) moth); Galleria mellonella Linnaeus (greater wax moth); Herpetogramma licarsisalis Walker (sod webworm); Homoeosoma electellum Hulst (sunflower moth); Elasmopalpus lignosellus Zeller (lesser cornstalk borer); Achroia grisella Fabricius (lesser wax moth); Loxostege sticticalis Linnaeus (beet webworm); Orthaga thyrisalis Walker (tea

tree web moth); Maruca testulalis Geyer (bean pod borer); Plodia interpunctella Hübner (Indian meal moth); Scirpophaga incertulas Walker (yellow stem borer); Udea rubigalis Guenée (celery leaftier); and leafrollers, budworms, seed worms and fruit worms in the family Tortricidae Acleris gloverana Walsingham (Western blackheaded budworm); A. variana Fernald (Eastern blackheaded budworm); Archips argyrospila Walker (fruit tree leaf roller); A. rosana Linnaeus (European leaf roller); and other Archips species, Adoxophyes orana Fischer von Rösslerstamm (summer fruit tortrix moth); Cochylis hospes Walsingham (banded sunflower moth); Cydia latiferreana Walsingham (filbertworm); C. pomonella Linnaeus (coding moth); Platynota flavedana Clemens (variegated leafroller); P. stultana Walsingham (omnivorous leafroller); Lobesia botrana Denis & Schiffermüller (European grape vine moth); Spilonota ocellana Denis & Schiffermüller (eyespotted bud moth); Endopiza viteana Clemens (grape berry moth); Eupoecilia ambiguella Hübner (vine moth); Bonagota salubricola Meyrick (Brazilian apple leafroller); Grapholita molesta Busck (oriental fruit moth); Suleima helianthana Riley (sunflower bud moth); Argyrotaenia spp.; Choristoneura spp..

Selected other agronomic pests in the order Lepidoptera include, but are not limited to, Alsophila pometaria Harris (fall cankerworm); Anarsia lineatella Zeller (peach twig borer); Anisota senatoria J.E. Smith (orange striped oakworm); Antheraea pernyi Guérin-Méneville (Chinese Oak Tussah Moth); Bombyx mori Linnaeus (Silkworm); Bucculatrix thurberiella Busck (cotton leaf perforator); Colias eurytheme Boisduval (alfalfa caterpillar); Datana integerrima Grote & Robinson (walnut caterpillar); Dendrolimus sibiricus Tschetwerikov (Siberian silk moth), Ennomos subsignaria Hübner (elm spanworm); Erannis tiliaria Harris (linden looper); Euproctis chrysorrhoea Linnaeus (browntail moth); Harrisina americana Guérin-Méneville (grapeleaf skeletonizer); Hemileuca oliviae Cockrell (range caterpillar); Hyphantria cunea Drury (fall webworm); Keiferia lycopersicella Walsingham (tomato pinworm); Lambdina fiscellaria fiscellaria Hulst (Eastern hemlock looper); L. fiscellaria lugubrosa Hulst (Western hemlock looper); Leucoma salicis Linnaeus (satin moth); Lymantria dispar Linnaeus (gypsy moth); Manduca quinquemaculata Haworth (five spotted hawk moth, tomato hornworm); M. sexta Haworth (tomato hornworm, tobacco hornworm); Operophtera brumata Linnaeus (winter moth); Paleacrita vernata Peck (spring cankerworm); Papilio cresphontes Cramer (giant swallowtail orange dog); Phryganidia californica Packard (California oakworm); Phyllocnistis citrella Stainton (citrus leafminer); Phyllonorycter

blancardella Fabricius (spotted tentiform leafminer); Pieris brassicae Linnaeus (large white butterfly); P. rapae Linnaeus (small white butterfly); P. napi Linnaeus (green veined white butterfly); Platyptilia carduidactyla Riley (artichoke plume moth); Plutella xylostella Linnaeus (diamondback moth); Pectinophora gossypiella Saunders (pink bollworm); Pontia protodice Boisduval and Leconte (Southern cabbageworm); Sabulodes aegrotata Guenée (omnivorous looper); Schizura concinna J.E. Smith (red humped caterpillar); Sitotroga cerealella Olivier (Angoumois grain moth); Thaumetopoea pityocampa Schiffermuller (pine processionary caterpillar); Tineola bisselliella Hummel (webbing clothesmoth); Tuta absoluta Meyrick (tomato leafminer); Yponomeuta padella Linnaeus (ermine moth); Heliothis subflexa Guenée; Malacosoma spp. and Orgyia spp.

Of interest are larvae and adults of the order Coleoptera including weevils from the families Anthribidae, Bruchidae and Curculionidae (including, but not limited to: Anthonomus grandis Boheman (boll weevil); Lissorhoptrus oryzophilus Kuschel (rice water weevil); Sitophilus granarius Linnaeus (granary weevil); S. oryzae Linnaeus (rice weevil); Hypera punctata Fabricius (clover leaf weevil); Cylindrocopturus adspersus LeConte (sunflower stem weevil); Smicronyx fulvus LeConte (red sunflower seed weevil); S. sordidus LeConte (gray sunflower seed weevil); Sphenophorus maidis Chittenden (maize billbug)); flea beetles, cucumber beetles, rootworms, leaf beetles, potato beetles and leafminers in the family Chrysomelidae (including, but not limited to: Leptinotarsa decemlineata Say (Colorado potato beetle); Diabrotica virgifera virgifera LeConte (western corn rootworm); D. barberi Smith and Lawrence (northern corn rootworm); D. undecimpunctata howardi Barber (southern corn rootworm); Chaetocnema pulicaria Melsheimer (corn flea beetle); Phyllotreta cruciferae Goeze (Crucifer flea beetle); Phyllotreta striolata (stripped flea beetle); Colaspis brunnea Fabricius (grape colaspis); Oulema melanopus Linnaeus (cereal leaf beetle); Zygogramma exclamationis Fabricius (sunflower beetle)); beetles from the family Coccinellidae (including, but not limited to: Epilachna varivestis Mulsant (Mexican bean beetle)); chafers and other beetles from the family Scarabaeidae (including, but not limited to: Popillia japonica Newman (Japanese beetle); Cyclocephala borealis Arrow (northern masked chafer, white grub); C. immaculata Olivier (southern masked chafer, white grub); Rhizotrogus majalis Razoumowsky (European chafer); Phyllophaga crinita Burmeister (white grub); Ligyrus gibbosus De Geer (carrot beetle)); carpet beetles from the family Dermestidae; wireworms from the family

Elateridae, Eleodes spp., Melanotus spp.; Conoderus spp.; Limonius spp.; Agriotes spp.; Ctenicera spp.; Aeolus spp.; bark beetles from the family Scolytidae and beetles from the family Tenebrionidae.

Adults and immatures of the order Diptera are of interest, including leafminers Agromyza parvicornis Loew (corn blotch leafminer); midges (including, but not limited to: Contarinia sorghicola Coquillett (sorghum midge); Mayetiola destructor Say (Hessian fly); Sitodiplosis mosellana Géhin (wheat midge); Neolasioptera murtfeldtiana Felt, (sunflower seed midge)); fruit flies (Tephritidae), Oscinella frit Linnaeus (fruit flies); maggots (including, but not limited to: Delia platura Meigen (seedcorn maggot); D. coarctata Fallen (wheat bulb fly) and other Delia spp., Meromyza americana Fitch (wheat stem maggot); Musca domestica Linnaeus (house flies); Fannia canicularis Linnaeus, F. femoralis Stein (lesser house flies); Stomoxys calcitrans Linnaeus (stable flies)); face flies, horn flies, blow flies, Chrysomya spp.; Phormia spp. and other muscoid fly pests, horse flies Tabanus spp.; bot flies Gastrophilus spp.; Oestrus spp.; cattle grubs Hypoderma spp.; deer flies Chrysops spp.; Melophagus ovinus Linnaeus (keds) and other Brachycera, mosquitoes Aedes spp.; Anopheles spp.; Culex spp.; black flies Prosimulium spp.; Simulium spp.; biting midges, sand flies, sciarids, and other Nematocera.

Included as insects of interest are adults and nymphs of the orders Hemiptera and Homoptera such as, but not limited to, adelgids from the family Adelgidae, plant bugs from the family Miridae, cicadas from the family Cicadidae, leafhoppers, Empoasca spp.; from the family Cicadellidae, planthoppers from the families Cixiidae, Flatidae, Fulgoroidea, Issidae and Delphacidae, treehoppers from the family Membracidae, psyllids from the family Psyllidae, whiteflies from the family Aleyrodidae, aphids from the family Aphididae, phylloxera from the family Phylloxeridae, mealybugs from the family Pseudococcidae, scales from the families Asterolecanidae, Coccidae, Dactylopiidae, Diaspididae, Eriococcidae Ortheziidae, Phoenicococcidae and Margarodidae, lace bugs from the family Tingidae, stink bugs from the family Pentatomidae, cinch bugs, Blissus spp.; and other seed bugs from the family Lygaeidae, spittlebugs from the family Cercopidae squash bugs from the family Coreidae and red bugs and cotton stainers from the family Pyrrhocoridae.

Agronomically important members from the order Homoptera further include, but are not limited to: Acyrthisiphon pisum Harris (pea aphid); Aphis craccivora Koch (cowpea

aphid); A. fabae Scopoli (black bean aphid); A. gossypii Glover (cotton aphid, melon aphid); A. maidiradicis Forbes (corn root aphid); A. pomi De Geer (apple aphid); A. spiraecola Patch (spirea aphid); Aulacorthum solani Kaltenbach (foxglove aphid); Chaetosiphon fragaefolii Cockerell (strawberry aphid); Diuraphis noxia Kurdjumov/Mordvilko (Russian wheat aphid); Dysaphis plantaginea Paaserini (rosy apple aphid); Eriosoma lanigerum Hausmann (woolly apple aphid); Brevicoryne brassicae Linnaeus (cabbage aphid); Hyalopterus pruni Geoffroy (mealy plum aphid); Lipaphis erysimi Kaltenbach (turnip aphid); Metopolophium dirrhodum Walker (cereal aphid); Macrosiphum euphorbiae Thomas (potato aphid); Myzus persicae Sulzer (peach-potato aphid, green peach aphid); Nasonovia ribisnigri Mosley (lettuce aphid); Pemphigus spp. (root aphids and gall aphids); Rhopalosiphum maidis Fitch (corn leaf aphid); R. padi Linnaeus (bird cherry-oat aphid); Schizaphis graminum Rondani (greenbug); Sipha flava Forbes (yellow sugarcane aphid); Sitobion avenae Fabricius (English grain aphid); Therioaphis maculata Buckton (spotted alfalfa aphid); Toxoptera aurantii Boyer de Fonscolombe (black citrus aphid) and T. citricida Kirkaldy (brown citrus aphid); Adelges spp. (adelgids); Phylloxera devastatrix Pergande (pecan phylloxera); Bemisia tabaci Gennadius (tobacco whitefly, sweetpotato whitefly); B. argentifolii Bellows & Perring (silverleaf whitefly); Dialeurodes citri Ashmead (citrus whitefly); Trialeurodes abutiloneus (bandedwinged whitefly) and T. vaporariorum Westwood (greenhouse whitefly); Empoasca fabae Harris (potato leafhopper); Laodelphax striatellus Fallen (smaller brown planthopper); Macrolestes quadrilineatus Forbes (aster leafhopper); Nephotettix cinticeps Uhler (green leafhopper); N. nigropictus Stål (rice leafhopper); Nilaparvata lugens Stål (brown planthopper); Peregrinus maidis Ashmead (corn planthopper); Sogatella furcifera Horvath (white-backed planthopper); Sogatodes orizicola Muir (rice delphacid); Typhlocyba pomaria McAtee (white apple leafhopper); Erythroneoura spp. (grape leafhoppers); Magicicada septendecim Linnaeus (periodical cicada); Icerya purchasi Maskell (cottony cushion scale); Quadraspidiotus perniciosus Comstock (San Jose scale); Planococcus citri Risso (citrus mealybug); Pseudococcus spp. (other mealybug complex); Cacopsylla pyricola Foerster (pear psylla); Trioza diospyri Ashmead (persimmon psylla).

Agronomically important species of interest from the order Hemiptera include, but are not limited to: Acrosternum hilare Say (green stink bug); Anasa tristis De Geer (squash bug); Blissus leucopterus leucopterus Say (chinch bug); Corythuca gossypii Fabricius (cotton lace

bug); Cyrtopeltis modesta Distant (tomato bug); Dysdercus suturellus Herrich-Schäffer (cotton stainer); Euschistus servus Say (brown stink bug); E. variolarius Palisot de Beauvois (one-spotted stink bug); Graptostethus spp. (complex of seed bugs); Leptoglossus corculus Say (leaf-footed pine seed bug); Lygus lineolaris Palisot de Beauvois (tarnished plant bug); L. Hesperus Knight (Western tarnished plant bug); L. pratensis Linnaeus (common meadow bug); L. rugulipennis Poppius (European tarnished plant bug); Lygocoris pabulinus Linnaeus (common green capsid); Nezara viridula Linnaeus (southern green stink bug); Oebalus pugnax Fabricius (rice stink bug); Oncopeltus fasciatus Dallas (large milkweed bug); Pseudatomoscelis seriatus Reuter (cotton fleahopper).

Furthermore, embodiments may be effective against Hemiptera such, Calocoris norvegicus Gmelin (strawberry bug); Orthops campestris Linnaeus; Plesiocoris rugicollis Fallen (apple capsid); Cyrtopeltis modestus Distant (tomato bug); Cyrtopeltis notatus Distant (suckfly); Spanagonicus albofasciatus Reuter (whitemarked fleahopper); Diaphnocoris chlorionis Say (honeylocust plant bug); Labopidicola allii Knight (onion plant bug); Pseudatomoscelis seriatus Reuter (cotton fleahopper); Adelphocoris rapidus Say (rapid plant bug); Poecilocapsus lineatus Fabricius (four-lined plant bug); Nysius ericae Schilling (false chinch bug); Nysius raphanus Howard (false chinch bug); Nezara viridula Linnaeus (Southern green stink bug); Eurygaster spp.; Coreidae spp.; Pyrrhocoridae spp.; Tinidae spp.; Blostomatidae spp.; Reduviidae spp. and Cimicidae spp.

Also included are adults and larvae of the order Acari (mites) such as Aceria tosichella Keifer (wheat curl mite); Petrobia latens Müller (brown wheat mite); spider mites and red mites in the family Tetranychidae, Panonychus ulmi Koch (European red mite); Tetranychus urticae Koch (two spotted spider mite); (T. mcdanieli McGregor (McDaniel mite); T. cinnabarinus Boisduval (carmine spider mite); T. turkestani Ugarov & Nikolski (strawberry spider mite); flat mites in the family Tenuipalpidae, Brevipalpus lewisi McGregor (citrus flat mite); rust and bud mites in the family Eriophyidae and other foliar feeding mites and mites important in human and animal health, i.e., dust mites in the family Epidermoptidae, follicle mites in the family Demodicidae, grain mites in the family Glycyphagidae, ticks in the order Ixodidae. Ixodes scapularis Say (deer tick); I. holocyclus Neumann (Australian paralysis tick); Dermacentor variabilis Say (American dog tick); Amblyomma americanum Linnaeus (lone star tick) and scab and itch mites in the families Psoroptidae, Pyemotidae and Sarcoptidae.

Insect pests of the order Thysanura are of interest, such as Lepisma saccharina Linnaeus (silverfish); Thermobia domestica Packard (firebrat).

Insect pest of interest include the superfamily of stink bugs and other related insects including but not limited to species belonging to the family Pentatomidae (Nezara viridula, Halyomorpha halys, Piezodorus guildini, Euschistus servus, Acrosternum hilare, Euschistus heros, Euschistus tristigmus, Acrosternum hilare, Dichelops furcatus, Dichelops melacanthus, and Bagrada hilaris (Bagrada Bug)), the family Plataspidae (Megacopta cribraria - Bean plataspid) and the family Cydnidae (Scaptocoris castanea - Root stink bug) and Lepidoptera species including but not limited to: diamond-back moth, e.g., Helicoverpa zea Boddie; soybean looper, e.g., Pseudoplusia includens Walker and velvet bean caterpillar e.g., Anticarsia gemmatalis Hübner.

II. Target Sequences

As used herein, a "target sequence" or "target polynucleotide" comprises any sequence in the pest that one desires to reduce the level of expression thereof. 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 include a polynucleotide set forth in SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof. As exemplified elsewhere herein, decreasing the level of expression of one or more of these target sequences in a Coleopteran plant pest or a *Diabrotica* plant pest controls the pest.

III. Silencing Elements

By "silencing element" is intended a polynucleotide which when contacted by or ingested by a plant insect pest, is capable of reducing or eliminating the level or expression of a target polynucleotide or the polypeptide encoded thereby, and a silencing element may include a polynucleotide that encodes the polynucleotide which when contacted by or ingested

by a pest, is capable of reducing or eliminating the level or expression of a target polynucleotide or the polypeptide encoded thereby. Accordingly, it is to be understood that "silencing element," as used herein, comprises polynucleotides such as RNA constructs, DNA constructs that encode the RNA constructs, expression constructs comprising the DNA constructs. In one embodiment, 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 disclosed methods 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*. It is to be understood that "silencing element," as used herein, is intended to comprise polynucleotides such as RNA constructs, DNA constructs that encode the RNA constructs, and/or expression constructs comprising the DNA constructs.

In specific embodiments, a silencing element may comprise a chimeric construction molecule comprising two or more disclosed sequences. For example, the chimeric construction may be a hairpin or dsRNA as disclosed herein. A chimera may comprise two or more disclosed sequences. In one embodiment, a chimera contemplates two complementary sequences set forth herein having some degree of mismatch between the complementary sequences such that the two sequences are not perfect complements of one another. 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.

In specific embodiments, the target sequence is not endogenous to the plant. In other embodiments, while the silencing element controls pests, preferably the silencing element has no effect on the normal plant or plant part.

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. In an embodiment, silencing elements may comprise a chimera where two or more disclosed sequences or active fragments or variants, or complements thereof, are found in the same RNA molecule. In various embodiments, a disclosed sequence 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. In a hairpin or dsRNA molecule, the location of a sense or antisense sequence in the molecule, for example, in which sequence is transcribed first or is located on a particular terminus of the RNA molecule, is not limiting to the disclosed sequences, and the dsRNA is not to be limited by disclosures herein of a particular location for such a sequence. Non-limiting examples of silencing elements that can be employed to decrease expression of these target sequences comprise fragments or variants of the sense or antisense sequence or consists of the sense or antisense sequence of a sequence set forth in SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof. The silencing element can further comprise additional sequences that advantageously effect transcription and/or the stability of a resulting transcript. For example, the silencing elements can comprise at least one thymine residue at the 3' end. This can aid in stabilization. Thus, the silencing elements can have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more thymine residues at the 3' end. As discussed in further detail below, enhancer suppressor elements can also be employed in conjunction with the silencing elements disclosed herein.

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 or come into contact with) the silencing element. In particular embodiments, reducing the polynucleotide level and/or the polypeptide level of the target sequence in a plant insect pest according to the disclosed methods 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.

i. Sense Suppression Elements

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.

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.: 1-33, or variants and fragments thereof, and complements thereof. In other embodiments, the sense suppression element can be, for example, about 15-25, 19-35, 19-50, 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.: 1-33, or variants and fragments thereof, and complements thereof.

ii. Antisense Suppression Elements

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, 16, 17, 18, 19, 20, 22, 25, 50, 100, 200, 300, 400, 450 nucleotides or greater of the sequence set forth in any of SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof 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 No. 5,942,657, which is herein incorporated by reference.

iii. Double Stranded RNA Suppression Element

A "double stranded RNA silencing element" or "dsRNA", which may also be referred to as "dsRNA construct", comprises at least one transcript that is capable of forming a dsRNA either before or after ingestion by a plant insect 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 at least two distinct RNA strands. The dsRNA molecule(s) employed in the disclosed methods and compositions 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 various embodiments, the dsRNA is capable of reducing or eliminating the level or expression of a target polynucleotide or the polypeptide encoded thereby in a plant insect

pest.

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.

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 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."

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.

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, 19, 18, 17, 16, 15, 10 nucleotides or less.

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.

The first and the third segment are at least about 1000, 500, 475, 450, 425, 400, 375, 350, 325, 300, 250, 225, 200, 175, 150, 125, 100, 75, 60, 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 10 to about 20 nucleotides, about 19 to about 50 nucleotides, about 50 nucleotides to about 100 nucleotides, about 100 nucleotides to about 150 nucleotides, about 100 nucleotides to about 300 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, about 600 nt, about 700 nt, about 800 nt, about 900 nt, about 1000 nt, about 1100 nt, about 1200 nt, 1300 nt, 1400 nt, 1500 nt, 1600 nt, 1700 nt, 1800 nt, 1900 nt, 2000 nt or longer. In other embodiments, the length of the first and/or the third segment comprises at least 10-19 nucleotides, 10-20 nucleotides; 19-35 nucleotides, 20-35 nucleotides; 30-45 nucleotides; 40-50 nucleotides; 50-100 nucleotides; 100-300 nucleotides; about 500 -700 nucleotides; about 700-900nucleotides; 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.

The disclosed hairpin molecules or double-stranded RNA molecules may have more than one disclosed sequence 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 disclosed sequence. 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.

In specific embodiments, the first and the third segment comprise at least 20 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.

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, 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 20-35 nucleotides, about 25-50 nucleotides, about 19 to 75 nucleotides, about 20 to 75 nucleotides, about 40-90 nucleotides about 15-100 nucleotides10-100 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 20 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 10-20 nucleotides, at least 10-19 nucleotides, 20-35 nucleotides, 30-45 nucleotides, 40-50 nucleotides, 50-100 nucleotides, or about 100-300 nucleotides.

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.

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 disclosed suppression cassettes 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 disclosed herein without altering the expression of the remaining wild-type allele. In other organisms, holistic sequence variability may be tolerated as long as some 22nt region of the sequence is represented in 100% homology between target polynucleitide and the suppression cassette.

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.

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.

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.

In other embodiments, the silencing element can comprise a small RNA (sRNA). sRNAs can comprise both micro RNA (miRNA) and short-interfering RNA (siRNA) (Meister and Tuschl (2004) *Nature* 431:343-349 and Bonetta *et al.* (2004) *Nature Methods* 1:79-86). 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 hairpin structure or partially base-paired structure containing 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. Specifically, the miRNA can comprise 19 nucleotides of the sequence having homology to a target polynucleotide in sense orientation and 19 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence. The miRNA can be an "artificial miRNA" or "amiRNA" which comprises a

miRNA sequence that is synthetically designed to silence a target sequence.

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 basepair 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.)

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.

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.

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.

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.

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.

The methods and compositions disclosed herein 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.

IV. Variants and Fragments

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, nucleotides, 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. Alternatively, fragments of a nucleotide sequence may range from 1-50, 25-75, 75-125, 50-100, 125-175, 175-225, 100-150, 100-300, 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.: 1-33, or variants and fragments thereof, and complements thereof. Methods to assay for the activity of a desired silencing element are described elsewhere herein.

"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 plant insect 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 disclosed polypeptides. Variant polynucleotides also include synthetically derived polynucleotide, such as those generated, for example, by using site-directed mutagenesis, but continue to retain the desired activity. Generally, variants of a particular disclosed polynucleotide (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.

Variants of a particular disclosed polynucleotide (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 disclosed polynucleotides employed 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.

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."

(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.

(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.

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.

(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, Mountain View, California).

(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.

A method is further provided for identifying a silencing element from the target polynucleotides set forth in SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof. Such methods comprise obtaining a candidate fragment of any one of SEQ ID NOs.: 1-33, or variants and fragments thereof, and complements thereof, 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 is said candidate polynucleotide fragment has the activity of a silencing element and thereby reduce the expression of the target polynucleotide and/or controls 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. In various aspects, it is to be understand that the term "…SEQ ID NOs: 1-33, or variants or fragments thereof, or complements thereof…" is intended to mean that the disclosed sequences comprise SEQ ID NOs: 1-33, and/or fragments of SEQ ID NOs: 1-33, and/or variants of SEQ ID NOs: 1-33, and/or the complements of SEQ ID NOs: 1-33, the variants of SEQ ID NOs: 1-33, and/or the fragments of SEQ ID NOs: 1-33, individually (or) or inclusive of some or all listed sequences.

V. DNA constructs

The use of the term “polynucleotide” is not intended to be limiting 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 disclosed polynucleotides 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.

The polynucleotide encoding the silencing element or in specific embodiments employed in the disclosed methods and compositions 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.

The expression cassette can include 5' and 3' regulatory sequences operably linked to the polynucleotide of the disclosure. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a polynucleotide of the disclosure and a regulatory sequence (i.e., a promoter) is a functional link that allows for expression of the polynucleotide of the disclosure. 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 cotransformed 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.

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 disclosure, and a transcriptional and translational termination region (i.e., termination region) functional in plants. In other 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. Such a cassette may also comprise two divergent promoters that drive transcription of one or more operably linked silencing elements. “Divergent promoters” refers to promoters that are oriented in opposite directions of each other, driving transcription of the one or more silencing

elements in opposite directions. In such embodiments, the divergent promoters allow for the transcription of the sense and antisense strands and allow for the formation of a dsRNA. In such embodiments, the divergent promoters also allow for the transcription of at least two separate hairpin RNAs. In another embodiment, one cassette comprising two or more silencing elements under the control of two separate promoters in the same orientation is present in a construct. In another embodiment, two or more individual cassettes, each comprising at least one silencing element under the control of a promoter, are present in a construct in the same orientation.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

In an embodiment, 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.

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 glutamin 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 geneSultr1; 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).

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 synthese -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 SAM-1 (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).

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

One or more of the polynucleotides comprising the silencing element can be provided as an external composition such as a spray or powder to the plant, plant part, seed, a plant insect 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 Coleopteran plant pest including a *Diabrotica* plant pest, such as, *D. virgifera virgifera*, *D. barberi*, *D. virgifera zeae, D. speciosa*, or *D. undecimpunctata howardi*). It is recognized that 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 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 plant insect 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 of skill in the art.

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.

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 plant insect 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. Various insecticidal formulations can also be found in, for example, US Publications 2008/0275115, 2008/0242174, 2008/0027143, 2005/0042245, and 2004/0127520, each of which is herein incorporated by reference.

It is recognized that the polynucleotides comprising sequences encoding the silencing element 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 may be introduced via a suitable vector into a microbial host, and said host applied to the environment, or to plants or animals.

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).

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.

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.

A number of ways are available for introducing the polynucleotide comprising the silencing element 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.

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.

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.

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.

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.

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.

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.).

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.

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.

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.

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.

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.

The compositions comprising the silencing element 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.

The compositions (including the transformed microorganisms) can be applied to the environment of an insect pest (such as a Coleoptera plant pest or a *Diabrotica* 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, an 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*

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 polynucleotide or polypeptides gains 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.

"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.

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.

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 or 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 polyethylimine (PEI; Sigma #P3143).

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.

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 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.

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.

As used herein, the term plant 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.

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.

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.

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.

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. Stacking of Traits in Transgenic Plant

Transgenic plants may comprise a stack of one or more target polynucleotides as set forth in SEQ ID NOs.: 1-33, or variants or fragments thereof, or complements thereof, as

disclosed herein with one or more additional polynucleotides resulting in the production or suppression of multiple polypeptide sequences. Transgenic plants comprising stacks of polynucleotide sequences can be obtained by either or both of traditional breeding methods or through genetic engineering methods. These methods include, but are not limited to, breeding individual lines each comprising a polynucleotide of interest, transforming a transgenic plant comprising an expression construct comprising various target polynucleotides as set forth in SEQ ID NOs.: 1-33, or variants or fragments thereof, or complements thereof, as disclosed herein with a subsequent gene and co-transformation of genes into a single plant cell. As used herein, the term "stacked" includes having the multiple traits present in the same plant (i.e., both traits are incorporated into the nuclear genome, one trait is incorporated into the nuclear genome and one trait is incorporated into the genome of a plastid or both traits are incorporated into the genome of a plastid). In one non-limiting example, "stacked traits" comprise a molecular stack where the sequences are physically adjacent to each other. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. Co-transformation of polynucleotides can be carried out using single transformation vectors comprising multiple polynucleotides or polynucleotides carried separately on multiple vectors. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. 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. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. *See*, for example, WO 1999/25821, WO 1999/25854, WO 1999/25840, WO 1999/25855 and WO 1999/25853, all of which are herein incorporated by reference.

In some embodiments the various target polynucleotides as set forth in SEQ ID NOs.: 1-33, variantsor variants or fragments thereof, or complements thereof, as disclosed herein, alone or stacked with one or more additional insect resistance traits can be stacked with one or more additional input traits (e.g., herbicide resistance, fungal resistance, virus resistance,

stress tolerance, disease resistance, male sterility, stalk strength, and the like) or output traits (e.g., increased yield, modified starches, improved oil profile, balanced amino acids, high lysine or methionine, increased digestibility, improved fiber quality, drought resistance, and the like). Thus, the polynucleotide embodiments can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

Transgenes useful for stacking include, but are not limited to, to those as described herein below.

i. Transgenes that Confer Resistance to Insects or Disease

(A) Plant disease resistance genes. Plant defenses are often activated by specific interaction between the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence (Avr) gene in the pathogen. A plant variety can be transformed with cloned resistance gene to engineer plants that are resistant to specific pathogen strains. *See*, for example, Jones, et al., (1994) Science 266:789 (cloning of the tomato Cf-9 gene for resistance to Cladosporium fulvum); Martin, et al., (1993) Science 262:1432 (tomato Pto gene for resistance to Pseudomonas syringae pv. tomato encodes a protein kinase); Mindrinos, et al., (1994) Cell 78:1089 (Arabidopsis RSP2 gene for resistance to Pseudomonas syringae), McDowell and Woffenden, (2003) Trends Biotechnol. 21(4):178-83 and Toyoda, et al., (2002) Transgenic Res. 11(6):567-82. A plant resistant to a disease is one that is more resistant to a pathogen as compared to the wild type plant.

(B) Genes encoding a Bacillus thuringiensis protein, a derivative thereof or a synthetic polypeptide modeled thereon. *See*, for example, Geiser, et al., (1986) Gene 48:109, who disclose the cloning and nucleotide sequence of a Bt delta-endotoxin gene. Moreover, DNA molecules encoding delta-endotoxin genes can be purchased from American Type Culture Collection (Rockville, Md.), for example, under ATCC Accession Numbers 40098, 67136, 31995 and 31998. Other non-limiting examples of Bacillus thuringiensis transgenes being genetically engineered are given in the following patents and patent applications and hereby are incorporated by reference for this purpose: U.S. Pat. Nos. 5,188,960; 5,689,052; 5,880,275; 5,986,177; 6,023,013, 6,060,594, 6,063,597, 6,077,824, 6,620,988, 6,642,030, 6,713,259, 6,893,826, 7,105,332; 7,179,965, 7,208,474; 7,227,056, 7,288,643, 7,323,556, 7,329,736, 7,449,552, 7,468,278, 7,510,878, 7,521,235, 7,544,862, 7,605,304, 7,696,412,

7,629,504, 7,705,216, 7,772,465, 7,790,846, 7,858,849 and WO 1991/14778; WO 1999/31248; WO 2001/12731; WO 1999/24581 and WO 1997/40162.

Genes encoding pesticidal proteins may also be stacked including but are not limited to: insecticidal proteins from Pseudomonas sp. such as PSEEN3174 (Monalysin, (2011) PLoS Pathogens, 7:1-13), from Pseudomonas protegens strain CHA0 and Pf-5 (previously fluorescens) (Pechy-Tarr, (2008) Environmental Microbiology 10:2368-2386: Gen Bank Accession No. EU400157); from Pseudomonas Taiwanensis (Liu, et al., (2010) J. Agric. Food Chem. 58:12343-12349) and from Pseudomonas pseudoalcligenes (Zhang, et al., (2009) Annals of Microbiology 59:45-50 and Li, et al., (2007) Plant Cell Tiss. Organ Cult. 89:159-168); insecticidal proteins from Photorhabdus sp. and Xenorhabdus sp. (Hinchliffe, et al., (2010) The Open Toxinology Journal 3:101-118 and Morgan, et al., (2001) Applied and Envir. Micro. 67:2062-2069), U.S. Pat. No. 6,048,838, and U.S. Pat. No. 6,379,946; and .delta.-endotoxins including, but not limited to, the Cry1, Cry2, Cry3, Cry4, Cry5, Cry6, Cry7, Cry8, Cry9, Cry10, Cry11, Cry12, Cry13, Cry14, Cry15, Cry16, Cry17, Cry18, Cry19, Cry20, Cry21, Cry22, Cry23, Cry24, Cry25, Cry26, Cry27, Cry 28, Cry 29, Cry 30, Cry31, Cry32, Cry33, Cry34, Cry35, Cry36, Cry37, Cry38, Cry39, Cry40, Cry41, Cry42, Cry43, Cry44, Cry45, Cry 46, Cry47, Cry49, Cry 51 and Cry55 classes of delta-endotoxin genes and the B. thuringiensis cytolytic Cyt1 and Cyt2 genes. Members of these classes of B. thuringiensis insecticidal proteins include, but are not limited to Cry1Aa1 (Accession #Accession #M11250), Cry1Aa2 (Accession #M10917), Cry1Aa3 (Accession #D00348), Cry1Aa4 (Accession #X13535), Cry1Aa5 (Accession #D17518), Cry1Aa6 (Accession #U43605), Cry1Aa7 (Accession #AF081790), Cry1Aa8 (Accession #I26149), Cry1Aa9 (Accession #AB026261), Cry1Aa10 (Accession #AF154676), Cry1Aa11 (Accession #Y09663), Cry1Aa12 (Accession #AF384211), Cry1Aa13 (Accession #AF510713), Cry1Aa14 (Accession #AY197341), Cry1Aa15 (Accession #DQ062690), Cry1Ab1 (Accession #M13898), Cry1Ab2 (Accession #M12661), Cry1Ab3 (Accession #M15271), Cry1Ab4 (Accession #D00117), Cry1Ab5 (Accession #X04698), Cry1Ab6 (Accession #M37263), Cry1Ab7 (Accession #X13233), Cry1Ab8 (Accession #M16463), Cry1Ab9 (Accession #X54939), Cry1Ab10 (Accession #A29125), Cry1Ab11 (Accession #I12419), Cry1Ab12 (Accession #AF059670), Cry1Ab13 (Accession #AF254640), Cry1Ab14 (Accession #U94191), Cry1Ab15 (Accession #AF358861), Cry1Ab16 (Accession #AF375608),

Cry1Ab17 (Accession #AAT46415), Cry1Ab18 (Accession #AAQ88259), Cry1Ab19 (Accession #AY847289), Cry1Ab20 (Accession #DQ241675), Cry1Ab21 (Accession #EF683163), Cry1Ab22 (Accession #ABW87320), Cry1Ab-like (Accession #AF327924), Cry1Ab-like (Accession #AF327925), Cry1Ab-like (Accession #AF327926), Cry1Ab-like (Accession #DQ781309), Cry1Ac1 (Accession #M11068), Cry1Ac2 (Accession #M35524), Cry1Ac3 (Accession #X54159), Cry1Ac4 (Accession #M73249), Cry1Ac5 (Accession #M73248), Cry1Ac6 (Accession #U43606), Cry1Ac7 (Accession #U87793), Cry1Ac8 (Accession #U87397), Cry1Ac9 (Accession #U89872), Cry1Ac10 (Accession #AJ002514), Cry1Ac11 (Accession #AJ130970), Cry1Ac12 (Accession #I12418), Cry1Ac13 (Accession #AF148644), Cry1Ac14 (Accession #AF492767), Cry1Ac15 (Accession #AY122057), Cry1Ac16 (Accession #AY730621), Cry1Ac17 (Accession #AY925090), Cry1Ac18 (Accession #DQ023296), Cry1Ac19 (Accession #DQ195217), Cry1Ac20 (Accession #DQ285666), Cry1Ac21 (Accession #DQ062689), Cry1Ac22 (Accession #EU282379), Cry1Ac23 (Accession #AM949588), Cry1Ac24 (Accession #ABL01535), Cry1Ad1 (Accession #M73250), Cry1Ad2 (Accession #A27531), Cry1Ae1 (Accession #M65252), Cry1Af1 (Accession #U82003), Cry1Ag1 (Accession #AF081248), Cry1Ah1 (Accession #AF281866), Cry1Ah2 (Accession #DQ269474), Cry1Ai1 (Accession #AY174873), Cry1A-like (Accession #AF327927), Cry1Ba1 (Accession #X06711), Cry1Ba2 (Accession #X95704), Cry1Ba3 (Accession #AF368257), Cry1Ba4 (Accession #AF363025), Cry1Ba5 (Accession #AB020894), Cry1Ba6 (Accession #ABL60921), Cry1Bb1 (Accession #L32020), Cry1Bc1 (Accession #Z46442), Cry1Bd1 (Accession #U70726), Cry1Bd2 (Accession #AY138457), Cry1Be1 (Accession #AF077326), Cry1Be2 (Accession #AAQ52387), Cry1Bf1 (Accession #AX189649), Cry1Bf2 (Accession #AAQ52380), Cry1Bg1 (Accession #AY176063), Cry1Ca1 (Accession #X07518), Cry1Ca2 (Accession #X13620), Cry1Ca3 (Accession #M73251), Cry1Ca4 (Accession #A27642), Cry1Ca5 (Accession #X96682), Cry1Ca6 [1] (Accession #AF215647), Cry1Ca7 (Accession #AY015492), Cry1Ca8 (Accession #AF362020), Cry1Ca9 (Accession #AY078160), Cry1Ca10 (Accession #AF540014), Cry1Ca11 (Accession #AY955268), Cry1Cb1 (Accession #M97880), Cry1Cb2 (Accession #AY007686), Cry1Cb3 (Accession #EU679502), Cry1Cb-like (Accession #AAX63901), Cry1Da1 (Accession #X54160), Cry1Da2 (Accession #I76415), Cry1Db1 (Accession #Z22511), Cry1 Db2 (Accession #AF358862), Cry1 Dc1 (Accession #EF059913),

Cry1Ea1 (Accession #X53985), Cry1Ea2 (Accession #X56144), Cry1Ea3 (Accession #M73252), Cry1Ea4 (Accession #U94323), Cry1Ea5 (Accession #A15535), Cry1Ea6 (Accession #AF202531), Cry1 Ea7 (Accession #AAW72936), Cry1 Ea8 (Accession #ABX11258), Cry1Eb1 (Accession #M73253), Cry1Fa1 (Accession #M63897), Cry1Fa2 (Accession #M73254), Cry1Fb1 (Accession #Z22512), Cry1Fb2 (Accession #AB012288), Cry1Fb3 (Accession #AF062350), Cry1Fb4 (Accession #I73895), Cry1Fb5 (Accession #AF336114), Cry1Fb6 (Accession #EU679500), Cry1Fb7 (Accession #EU679501), Cry1Ga1 (Accession #Z22510), Cry1Ga2 (Accession #Y09326), Cry1Gb1 (Accession #U70725), Cry1Gb2 (Accession #AF288683), Cry1Gc (Accession #AAQ52381), Cry1Ha1 (Accession #Z22513), Cry1Hb1 (Accession #U35780), Cry1H-like (Accession #AF182196), Cry1Ia1 (Accession #X62821), Cry1Ia2 (Accession #M98544), Cry1Ia3 (Accession #L36338), Cry1Ia4 (Accession #L49391), Cry1Ia5 (Accession #Y08920), Cry1Ia6 (Accession #AF076953), Cry1Ia7 (Accession #AF278797), Cry1Ia8 (Accession #AF373207), Cry1Ia9 (Accession #AF521013), Cry1Ia10 (Accession #AY262167), Cry1Ia11 (Accession #AJ315121), Cry1Ia12 (Accession #AAV53390), Cry1Ia13 (Accession #ABF83202), Cry1Ia14 (Accession #EU887515), Cry1Ib1 (Accession #U07642), Cry1Ib2 (Accession #ABW88019), Cry1Ib3 (Accession #EU677422), Cry1Ic1 (Accession #AF056933), Cry1Ic2 (Accession #AAE71691), Cry1Id1 (Accession #AF047579), Cry1Ie1 (Accession #AF211190), Cry1If1 (Accession #AAQ52382), Cry1I-like (Accession #I90732), Cry1I-like (Accession #DQ781310), Cry1Ja1 (Accession #L32019), Cry1Jb1 (Accession #U31527), Cry1Jc1 (Accession #I90730), Cry1Jc2 (Accession #AAQ52372), Cry1Jd1 (Accession #AX189651), Cry1Ka1 (Accession #U28801), Cry1La1 (Accession #AAS60191), Cry1-like (Accession #I90729), Cry2Aa1 (Accession #M31738), Cry2Aa2 (Accession #M23723), Cry2Aa3 (Accession #D86064), Cry2Aa4 (Accession #AF047038), Cry2Aa5 (Accession #AJ 132464), Cry2Aa6 (Accession #AJ 132465), Cry2Aa7 (Accession #AJ132463), Cry2Aa8 (Accession #AF252262), Cry2Aa9 (Accession #AF273218), Cry2Aa10 (Accession #AF433645), Cry2Aa11 (Accession #AAQ52384), Cry2Aa12 (Accession #DQ977646), Cry2Aa13 (Accession #ABL01536), Cry2Aa14 (Accession #ACF04939), Cry2Ab1 (Accession #M23724), Cry2Ab2 (Accession #X55416), Cry2Ab3 (Accession #AF164666), Cry2Ab4 (Accession #AF336115), Cry2Ab5 (Accession #AF441855), Cry2Ab6 (Accession #AY297091), Cry2Ab7 (Accession #DQ119823), Cry2Ab8 (Accession #DQ361266),

Cry2Ab9 (Accession #DQ341378), Cry2Ab10 (Accession #EF157306), Cry2Ab11 (Accession #AM691748), Cry2Ab12 (Accession #ABM21764), Cry2Ab13 (Accession #EU909454), Cry2Ab14 (Accession #EU909455), Cry2Ac1 (Accession #X57252), Cry2Ac2 (Accession #AY007687), Cry2Ac3 (Accession #AAQ52385), Cry2Ac4 (Accession #DQ361267), Cry2Ac5 (Accession #DQ341379), Cry2Ac6 (Accession #DQ359137), Cry2Ac7 (Accession #AM292031), Cry2Ac8 (Accession #AM421903), Cry2Ac9 (Accession #AM421904), Cry2Ac10 (Accession #BI 877475), Cry2Ac11 (Accession #AM689531), Cry2Ac12 (Accession #AM689532), Cry2Ad1 (Accession #AF200816), Cry2Ad2 (Accession #DQ358053), Cry2Ad3 (Accession #AM268418), Cry2Ad4 (Accession #AM490199), Cry2Ad5 (Accession #AM765844), Cry2Ae1 (Accession #AAQ52362), Cry2Af1 (Accession #EF439818), Cry2Ag (Accession #ACH91610), Cry2Ah (Accession #EU939453), Cry3Aa1 (Accession #M22472), Cry3Aa2 (Accession #J02978), Cry3Aa3 (Accession #Y00420), Cry3Aa4 (Accession #M30503), Cry3Aa5 (Accession #M37207), Cry3Aa6 (Accession #U10985), Cry3Aa7 (Accession #AJ237900), Cry3Aa8 (Accession #AAS79487), Cry3Aa9 (Accession #AAWO5659), Cry3Aa10 (Accession #AAU29411), Cry3Aa11 (Accession #AY882576), Cry3Aa12 (Accession #ABY49136), Cry3Ba1 (Accession #X17123), Cry3Ba2 (Accession #A07234), Cry3Bb1 (Accession #M89794), Cry3Bb2 (Accession #U31633), Cry3Bb3 (Accession #I15475), Cry3Ca1 (Accession #X59797), Cry4Aa1 (Accession #Y00423), Cry4Aa2 (Accession #D00248), Cry4Aa3 (Accession #AL731825), Cry4A-like (Accession #DQ078744), Cry4Ba1 (Accession #X07423), Cry4Ba2 (Accession #X07082), Cry4Ba3 (Accession #M20242), Cry4Ba4 (Accession #D00247), Cry4Ba5 (Accession #AL731825), Cry4Ba-like (Accession #ABC47686), Cry4Ca1 (Accession #EU646202), Cry5Aa1 (Accession #L07025), Cry5Ab1 (Accession #L07026), Cry5Ac1 (Accession #I34543), Cry5Ad1 (Accession #EF219060), Cry5Ba1 (Accession #U19725), Cry5Ba2 (Accession #EU121522), Cry6Aa1 (Accession #L07022), Cry6Aa2 (Accession #AF499736), Cry6Aa3 (Accession #DQ835612), Cry6Ba1 (Accession #L07024), Cry7Aa1 (Accession #M64478), Cry7Ab1 (Accession #U04367), Cry7Ab2 (Accession #U04368), Cry7Ab3 (Accession #BI 1015188), Cry7Ab4 (Accession #EU380678), Cry7Ab5 (Accession #ABX9555), Cry7Ab6 (Accession #FJ194973), Cry7Ba1 (Accession #ABB70817), Cry7Ca1 (Accession #EF486523), Cry8Aa1 (Accession #U04364), Cry8Ab1 (Accession #EU044830), Cry8Ba1 (Accession #U04365), Cry8Bb1 (Accession #AX543924), Cry8Bc1 (Accession

#AX543926), Cry8Ca1 (Accession #U04366), Cry8Ca2 (Accession #AAR98783), Cry8Ca3 (Accession #EU625349), Cry8Da1 (Accession #AB089299), Cry8Da2 (Accession #BD133574), Cry8Da3 (Accession #BD133575), Cry8 Db1 (Accession #AB303980), Cry8Ea1 (Accession #AY329081), Cry8Ea2 (Accession #EU047597), Cry8Fa1 (Accession #AY551093), Cry8Ga1 (Accession #AY590188), Cry8Ga2 (Accession #DQ318860), Cry8Ga3 (Accession #FJ198072), Cry8Ha1 (Accession #EF465532), Cry81a1 (Accession #EU381044), Cry8Ja1 (Accession #EU625348), Cry8 like (Accession #ABS53003), Cry9Aa1 (Accession #X58120), Cry9Aa2 (Accession #X58534), Cry9Aa like (Accession #AAQ52376), Cry9Ba1 (Accession #X75019), Cry9Bb1 (Accession #AY758316), Cry9Ca1 (Accession #Z37527), Cry9Ca2 (Accession #AAQ52375), Cry9Da1 (Accession #D85560), Cry9Da2 (Accession #AF042733), Cry9 Db1 (Accession #AY971349), Cry9Ea1 (Accession #AB011496), Cry9Ea2 (Accession #AF358863), Cry9Ea3 (Accession #EF157307), Cry9Ea4 (Accession #EU760456), Cry9Ea5 (Accession #EU789519), Cry9Ea6 (Accession #EU887516), Cry9Eb1 (Accession #AX189653), Cry9Ec1 (Accession #AF093107), Cry9Ed1 (Accession #AY973867), Cry9 like (Accession #AF093107), Cry10Aa1 (Accession #M12662), Cry10Aa2 (Accession #E00614), Cry10Aa3 (Accession #AL731825), Cry10A like (Accession #DQ167578), Cry1IAa1 (Accession #M31737), Cry1IAa2 (Accession #M22860), Cry1IAa3 (Accession #AL731825), Cry1IAa-like (Accession #DQ166531), Cry11Ba1 (Accession #X86902), Cry11Bb1 (Accession #AF017416), Cry12Aa1 (Accession #L07027), Cry13Aa1 (Accession #L07023), Cry14Aa1 (Accession #U13955), Cry15Aa1 (Accession #M76442), Cry16Aa1 (Accession #X94146), Cry17Aa1 (Accession #X99478), Cry18Aa1 (Accession #X99049), Cry18Ba1 (Accession #AF169250), Cry18Ca1 (Accession #AF169251), Cry19Aa1 (Accession #Y07603), Cry19Ba1 (Accession #D88381), Cry20Aa1 (Accession #U82518), Cry21Aa1 (Accession #I32932), Cry21Aa2 (Accession #I66477), Cry21Ba1 (Accession #AB088406), Cry22Aa1 (Accession #134547), Cry22Aa2 (Accession #AX472772), Cry22Aa3 (Accession #EU715020), Cry22Ab1 (Accession #AAK50456), Cry22Ab2 (Accession #AX472764), Cry22Ba1 (Accession #AX472770), Cry23Aa1 (Accession #AAF76375), Cry24Aa1 (Accession #U88188), Cry24Ba1 (Accession #BAD32657), Cry24Ca1 (Accession #AM158318), Cry25Aa1 (Accession #U88189), Cry26Aa1 (Accession #AF122897), Cry27Aa1 (Accession #AB023293), Cry28Aa1 (Accession #AF132928), Cry28Aa2 (Accession #AF285775), Cry29Aa1 (Accession

#AJ251977), Cry30Aa1 (Accession #AJ251978), Cry30Ba1 (Accession #BAD00052), Cry30Ca1 (Accession #BAD67157), Cry30Da1 (Accession #EF095955), Cry30 Db1 (Accession #BAE80088), Cry30Ea1 (Accession #EU503140), Cry30Fa1 (Accession #EU751609), Cry30Ga1 (Accession #EU882064), Cry31Aa1 (Accession #AB031065), Cry31Aa2 (Accession #AY081052), Cry31Aa3 (Accession #AB250922), Cry31Aa4 (Accession #AB274826), Cry31Aa5 (Accession #AB274827), Cry31Ab1 (Accession #AB250923), Cry31Ab2 (Accession #AB274825), Cry31Ac1 (Accession #AB276125), Cry32Aa1 (Accession #AY008143), Cry32Ba1 (Accession #BAB78601), Cry32Ca1 (Accession #BAB78602), Cry32Da1 (Accession #BAB78603), Cry33Aa1 (Accession #AAL26871), Cry34Aa1 (Accession #AAG50341), Cry34Aa2 (Accession #AAK64560), Cry34Aa3 (Accession #AY536899), Cry34Aa4 (Accession #AY536897), Cry34Ab1 (Accession #AAG41671), Cry34Ac1 (Accession #AAG50118), Cry34Ac2 (Accession #AAK64562), Cry34Ac3 (Accession #AY536896), Cry34Ba1 (Accession #AAK64565), Cry34Ba2 (Accession #AY536900), Cry34Ba3 (Accession #AY536898), Cry35Aa1 (Accession #AAG50342), Cry35Aa2 (Accession #AAK64561), Cry35Aa3 (Accession #AY536895), Cry35Aa4 (Accession #AY536892), Cry35Ab1 (Accession #AAG41672), Cry35Ab2 (Accession #AAK64563), Cry35Ab3 (Accession #AY536891), Cry35Ac1 (Accession #AAG50117), Cry35Ba1 (Accession #AAK64566), Cry35Ba2 (Accession #AY536894), Cry35Ba3 (Accession #AY536893), Cry36Aa1 (Accession #AAK64558), Cry37Aa1 (Accession #AAF76376), Cry38Aa1 (Accession #AAK64559), Cry39Aa1 (Accession #BAB72016), Cry40Aa1 (Accession #BAB72018), Cry40Ba1 (Accession #BAC77648), Cry40Ca1 (Accession #EU381045), Cry40Da1 (Accession #EU596478), Cry41Aa1 (Accession #AB116649), Cry41Ab1 (Accession #AB116651), Cry42Aa1 (Accession #AB116652), Cry43Aa1 (Accession #AB115422), Cry43Aa2 (Accession #AB176668), Cry43Ba1 (Accession #AB115422), Cry43-like (Accession #AB115422), Cry44Aa (Accession #BAD08532), Cry45Aa (Accession #BAD22577), Cry46Aa (Accession #BAC79010), Cry46Aa2 (Accession #BAG68906), Cry46Ab (Accession #BAD35170), Cry47Aa (Accession #AY950229), Cry48Aa (Accession #AJ841948), Cry48Aa2 (Accession #AM237205), Cry48Aa3 (Accession #AM237206), Cry48Ab (Accession #AM237207), Cry48Ab2 (Accession #AM237208), Cry49Aa (Accession #AJ841948), Cry49Aa2 (Accession #AM237201), Cry49Aa3 (Accession #AM237203), Cry49Aa4 (Accession

#AM237204), Cry49Ab1 (Accession #AM237202), Cry50Aa1 (Accession #AB253419), Cry51Aa1 (Accession #DQ836184), Cry52Aa1 (Accession #EF613489), Cry53Aa1 (Accession #EF633476), Cry54Aa1 (Accession #EU339367), Cry55Aa1 (Accession #EU121521), Cry55Aa2 (Accession #AAE33526).

Examples of delta-endotoxins also include but are not limited to Cry1A proteins of U.S. Pat. Nos. 5,880,275 and 7,858,849; a DIG-3 or DIG-11 toxin (N-terminal deletion of alpha-helix 1 and/or alpha-helix 2 variants of Cry proteins such as Cry1A) of U.S. Pat. Nos. 8,304,604 and 8,304,605, Cry1B of U.S. patent application Ser. No. 10/525,318; Cry1C of U.S. Pat. No. 6,033,874; Cry1F of U.S. Pat. Nos. 5,188,960, 6,218,188; Cry1A/F chimeras of U.S. Pat. Nos. 7,070,982; 6,962,705 and 6,713,063); a Cry2 protein such as Cry2Ab protein of U.S. Pat. No. 7,064,249); a Cry3A protein including but not limited to an engineered hybrid insecticidal protein (eHIP) created by fusing unique combinations of variable regions and conserved blocks of at least two different Cry proteins (US Patent Application Publication Number 2010/0017914); a Cry4 protein; a Cry5 protein; a Cry6 protein; Cry8 proteins of U.S. Pat. Nos. 7,329,736, 7,449,552, 7,803,943, 7,476,781, 7,105,332, 7,378,499 and 7,462,760; a Cry9 protein such as such as members of the Cry9A, Cry9B, Cry9C, Cry9D, Cry9E, and Cry9F families; a Cry15 protein of Naimov, et al., (2008) Applied and Environmental Microbiology 74:7145-7151; a Cry22, a Cry34Ab1 protein of U.S. Pat. Nos. 6,127,180, 6,624,145 and 6,340,593; a CryET33 and CryET34 protein of U.S. Pat. Nos. 6,248,535, 6,326,351, 6,399,330, 6,949,626, 7,385,107 and 7,504,229; a CryET33 and CryET34 homologs of US Patent Publication Number 2006/0191034, 2012/0278954, and PCT Publication Number WO 2012/139004; a Cry35Ab1 protein of U.S. Pat. Nos. 6,083,499, 6,548,291 and 6,340,593; a Cry46 protein, a Cry 51 protein, a Cry binary toxin; a TIC901 or related toxin; TIC807 of US 2008/0295207; ET29, ET37, TIC809, TIC810, TIC812, TIC127, TIC128 of PCT US 2006/033867; AXMI-027, AXMI-036, and AXMI-038 of U.S. Pat. No. 8,236,757; AXMI-***031,*** AXMI-039, AXMI-040, AXMI-049 of U.S. Pat. No. 7,923,602; AXMI-018, AXMI-020, and AXMI-021 of WO 2006/083891; AXMI-010 of WO 2005/038032; AXMI-003 of WO 2005/021585; AXMI-008 of US 2004/0250311; AXMI-006 of US 2004/0216186; AXMI-007 of US 2004/0210965; AXMI-009 of US 2004/0210964; AXMI-014 of US 2004/0197917; AXMI-004 of US 2004/0197916; AXMI-028 and AXMI-029 of WO 2006/119457; AXMI-007, AXMI-008, AXMI-0080rf2, AXMI-009, AXMI-014

and AXMI-004 of WO 2004/074462; AXMI-150 of U.S. Pat. No. 8,084,416; AXMI-205 of US20110023184; AXMI-011, AXMI-012, AXMI-013, AXMI-015, AXMI-019, AXMI-044, AXMI-037, AXMI-043, AXMI-033, AXMI-034, AXMI-022, AXMI-023, AXMI-041, AXMI-063, and AXMI-064 of US 2011/0263488; AXMI-R1 and related proteins of US 2010/0197592; AXMI221Z, AXMI222z, AXMI223z, AXMI224z and AXMI225z of WO 2011/103248; AXMI218, AXMI219, AXMI220, AXMI226, AXMI227, AXMI228, AXMI229, AXMI230, and AXMI231 of WO11/103,247; AXMI-115, AXMI-113, AXMI-005, AXMI-163 and AXMI-184 of U.S. Pat. No. 8,334,431; AXMI-001, AXMI-002, AXMI-030, AXMI-035, and AXMI-045 of US 2010/0298211; AXMI-066 and AXMI-076 of US20090144852; AXMI128, AXMI130, AXMI131, AXMI133, AXMI140, AXMI141, AXMI142, AXMI143, AXMI144, AXMI146, AXMI148, AXMI149, AXMI152, AXMI153, AXMI154, AXMI155, AXMI156, AXMI157, AXMI158, AXMI162, AXMI165, AXMI166, AXMI167, AXMI168, AXMI169, AXMI170, AXMI171, AXMI172, AXMI173, AXMI174, AXMI175, AXMI176, AXMI177, AXMI178, AXMI179, AXMI180, AXMI181, AXMI182, AXMI185, AXMI186, AXMI187, AXMI188, AXMI189 of U.S. Pat. No. 8,318,900; AXMI079, AXMI080, AXMI081, AXMI082, AXMI091, AXMI092, AXMI096, AXMI097, AXMI098, AXMI099, AXMI100, AXMI101, AXMI102, AXMI103, AXMI104, AXMI107, AXMI108, AXMI109, AXMI110, AXMI111, AXMI112, AXMI114, AXMI116, AXMI117, AXMI118, AXMI119, AXMI120, AXMI121, AXMI122, AXMI123, AXMI124, AXMI1257, AXMI1268, AXMI127, AXMI129, AXMI164, AXMI151, AXMI161, AXMI183, AXMI132, AXMI138, AXMI137 of US 2010/0005543; Cry proteins such as Cry1A and Cry3A having modified proteolytic sites of U.S. Pat. No. 8,319,019; and a Cry1Ac, Cry2Aa and Cry1Ca toxin protein from Bacillus thuringiensis strain VBTS 2528 of US Patent Application Publication Number 2011/0064710. Other Cry proteins are well known to one skilled in the art (see, Crickmore, et al., "Bacillus thuringiensis toxin nomenclature" (2011), at lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/which can be accessed on the world-wide web using the "www" prefix). The insecticidal activity of Cry proteins is well known to one skilled in the art (for review, see, van Frannkenhuyzen, (2009) J. Invert. Path. 101:1-16). The use of Cry proteins as transgenic plant traits is well known to one skilled in the art and Cry-transgenic plants including but not limited to Cry1Ac, Cry1Ac+Cry2Ab, Cry1Ab, Cry1A.105, Cry1F, Cry1Fa2, Cry1F+Cry1Ac, Cry2Ab, Cry3A, mCry3A, Cry3Bb1, Cry34Ab1,

Cry35Ab1, Vip3A, mCry3A, Cry9c and CBI-Bt have received regulatory approval (see, Sanahuja, (2011) Plant Biotech Journal 9:283-300 and the CERA (2010) GM Crop Database Center for Environmental Risk Assessment (CERA), ILSI Research Foundation, Washington D.C. at cera-gmc.org/index.php?action=gm_crop_database which can be accessed on the world-wide web using the "www" prefix). Pesticidal proteins also include insecticidal lipases including lipid acyl hydrolases of U.S. Pat. No. 7,491,869, and cholesterol oxidases such as from Streptomyces (Purcell et al. (1993) Biochem Biophys Res Commun 15:1406-1413). Pesticidal proteins also include VIP (vegetative insecticidal proteins) toxins of U.S. Pat. Nos. 5,877,012, 6,107,279, 6,137,033, 7,244,820, 7,615,686, and 8,237,020, and the like. Other VIP proteins are well known to one skilled in the art (see, lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html which can be accessed on the world-wide web using the "www" prefix). Pesticidal proteins also include toxin complex (TC) proteins, obtainable from organisms such as Xenorhabdus, Photorhabdus and Paenibacillus (see, U.S. Pat. Nos. 7,491,698 and 8,084,418). Some TC proteins have "stand alone" insecticidal activity and other TC proteins enhance the activity of the stand-alone toxins produced by the same given organism. The toxicity of a "stand-alone" TC protein (from Photorhabdus, Xenorhabdus or Paenibacillus, for example) can be enhanced by one or more TC protein "potentiators" derived from a source organism of a different genus. There are three main types of TC proteins. As referred to herein, Class A proteins ("Protein A") are stand-alone toxins. Class B proteins ("Protein B") and Class C proteins ("Protein C") enhance the toxicity of Class A proteins. Examples of Class A proteins are TcbA, TcdA, XptA1 and XptA2. Examples of Class B proteins are TcaC, TcdB, XptB1Xb and XptC1Wi. Examples of Class C proteins are TccC, XptC1Xb and XptB1Wi. Pesticidal proteins also include spider, snake and scorpion venom proteins. Examples of spider venom peptides include but are not limited to lycotoxin-1 peptides and mutants thereof (U.S. Pat. No. 8,334,366).

(C) A polynucleotide encoding an insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone, a variant thereof, a mimetic based thereon or an antagonist or agonist thereof. See, for example, the disclosure by Hammock, et al., (1990) Nature 344:458, of baculovirus expression of cloned juvenile hormone esterase, an inactivator of juvenile hormone.

(D) A polynucleotide encoding an insect-specific peptide which, upon expression,

disrupts the physiology of the affected pest. For example, see the disclosures of, Regan, (1994) J. Biol. Chem. 269:9 (expression cloning yields DNA coding for insect diuretic hormone receptor); Pratt, et al., (1989) Biochem. Biophys. Res. Comm. 163:1243 (an allostatin is identified in Diploptera puntata); Chattopadhyay, et al., (2004) Critical Reviews in Microbiology 30(1):33-54; Zjawiony, (2004) J Nat Prod 67(2):300-310; Carlini and Grossi-de-Sa, (2002) Toxicon 40(11):1515-1539; Ussuf, et al., (2001) Curr Sci. 80(7):847-853 and Vasconcelos and Oliveira, (2004) Toxicon 44(4):385-403. See also, U.S. Pat. No. 5,266,317 to Tomalski, et al., who disclose genes encoding insect-specific toxins.

(E) A polynucleotide encoding an enzyme responsible for a hyperaccumulation of a monoterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.

(F) A polynucleotide encoding an enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural or synthetic. *See*, PCT Application WO 1993/02197 in the name of Scott, et al., which discloses the nucleotide sequence of a callase gene. DNA molecules which contain chitinase-encoding sequences can be obtained, for example, from the ATCC under Accession Numbers 39637 and 67152. *See* also, Kramer, et al., (1993) Insect Biochem. Molec. Biol. 23:691, who teach the nucleotide sequence of a cDNA encoding tobacco hookworm chitinase and Kawalleck, et al., (1993) Plant Molec. Biol. 21:673, who provide the nucleotide sequence of the parsley ubi4-2 polyubiquitin gene, and U.S. Pat. Nos. 6,563,020; 7,145,060 and 7,087,810.

(G) A polynucleotide encoding a molecule that stimulates signal transduction. For example, see the disclosure by Botella, et al., (1994) Plant Molec. Biol. 24:757, of nucleotide sequences for mung bean calmodulin cDNA clones, and Griess, et al., (1994) Plant Physiol. 104:1467, who provide the nucleotide sequence of a maize calmodulin cDNA clone.

(H) A polynucleotide encoding a hydrophobic moment peptide. *See*, PCT Application WO 1995/16776 and U.S. Pat. No. 5,580,852 disclosure of peptide derivatives of Tachyplesin which inhibit fungal plant pathogens) and PCT Application WO 1995/18855 and U.S. Pat. No. 5,607,914 (teaches synthetic antimicrobial peptides that confer disease resistance).

(I) A polynucleotide encoding a membrane permease, a channel former or a channel blocker. For example, see the disclosure by Jaynes, et al., (1993) Plant Sci. 89:43, of heterologous expression of a cecropin-beta lytic peptide analog to render transgenic tobacco plants resistant to Pseudomonas solanacearum.

(J) A gene encoding a viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. See, Beachy, et al., (1990) Ann. Rev. Phytopathol. 28:451. Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus. Id.

(K) A gene encoding an insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect. Cf. Taylor, et al., Abstract #497, SEVENTH INT'L SYMPOSIUM ON MOLECULAR PLANT-MICROBE INTERACTIONS (Edinburgh, Scotland, 1994) (enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).

(L) A gene encoding a virus-specific antibody. See, for example, Tavladoraki, et al., (1993) Nature 366:469, who show that transgenic plants expressing recombinant antibody genes are protected from virus attack.

(M) A polynucleotide encoding a developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, fungal endo alpha-1,4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo-alpha-1,4-D-galacturonase. See, Lamb, et al., (1992) Bio/Technology 10:1436. The cloning and characterization of a gene which encodes a bean endopolygalacturonase-inhibiting protein is described by Toubart, et al., (1992) Plant J. 2:367.

(N) A polynucleotide encoding a developmental-arrestive protein produced in nature by a plant. For example, Logemann, et al., (1992) Bio/Technology 10:305, have shown that transgenic plants expressing the barley ribosome-inactivating gene have an increased resistance to fungal disease.

(O) Genes involved in the Systemic Acquired Resistance (SAR) Response and/or the

pathogenesis related genes. Briggs, (1995) Current Biology 5(2), Pieterse and Van Loon, (2004) Curr. Opin. Plant Bio. 7(4):456-64 and Somssich, (2003) Cell 113(7):815-6.

(P) Antifungal genes (Cornelissen and Melchers, (1993) Pl. Physiol. 101:709-712 and Parijs, et al., (1991) Planta 183:258-264 and Bushnell, et al., (1998) Can. J. of Plant Path. 20(2):137-149. Also see, U.S. patent application Ser. Nos. 09/950,933; 11/619,645; 11/657,710; 11/748,994; 11/774,121 and U.S. Pat. Nos. 6,891,085 and 7,306,946. LysM Receptor-like kinases for the perception of chitin fragments as a first step in plant defense response against fungal pathogens (US 2012/0110696).

(Q) Detoxification genes, such as for fumonisin, beauvericin, moniliformin and zearalenone and their structurally related derivatives. For example, see, U.S. Pat. Nos. 5,716,820; 5,792,931; 5,798,255; 5,846,812; 6,083,736; 6,538,177; 6,388,171 and 6,812,380.

(R) A polynucleotide encoding a Cystatin and cysteine proteinase inhibitors. See, U.S. Pat. No. 7,205,453.

(S) Defensin genes. See, WO 2003/000863 and U.S. Pat. Nos. 6,911,577; 6,855,865; 6,777,592 and 7,238,781.

(T) Genes conferring resistance to nematodes. See, e.g., PCT Application WO 1996/30517; PCT Application WO 1993/19181, WO 2003/033651 and Urwin, et al., (1998) Planta 204:472-479, Williamson, (1999) Curr Opin Plant Bio. 2(4):327-31; U.S. Pat. Nos. 6,284,948 and 7,301,069 and miR164 genes (WO 2012/058266).

(U) Genes that confer resistance to Phytophthora Root Rot, such as the Rps 1, Rps 1-a, Rps 1-b, Rps 1-c, Rps 1-d, Rps 1-e, Rps 1-k, Rps 2, Rps 3-a, Rps 3-b, Rps 3-c, Rps 4, Rps 5, Rps 6, Rps 7 and other Rps genes. See, for example, Shoemaker, et al., Phytophthora Root Rot Resistance Gene Mapping in Soybean, Plant Genome IV Conference, San Diego, Calif. (1995).

(V) Genes that confer resistance to Brown Stem Rot, such as described in U.S. Pat. No. 5,689,035 and incorporated by reference for this purpose.

(W) Genes that confer resistance to Colletotrichum, such as described in US Patent Application Publication US 2009/0035765 and incorporated by reference for this purpose. This includes the Rcg locus that may be utilized as a single locus conversion.

ii. Transgenes that Confer Resistance to a Herbicide.

(A) A polynucleotide encoding resistance to a herbicide that inhibits the growing point or meristem, such as an imidazolinone or a sulfonylurea. Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee, et al., (1988) EMBO J. 7:1241 and Miki, et al., (1990) Theor. Appl. Genet. 80:449, respectively. See also, U.S. Pat. Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937 and 5,378,824; U.S. patent application Ser. No. 11/683,737 and International Publication WO 1996/33270.

(B) A polynucleotide encoding a protein for resistance to Glyphosate (resistance imparted by mutant 5-enolpyruvl-3-phosphikimate synthase (EPSP) and aroA genes, respectively) and other phosphono compounds such as glufosinate (phosphinothricin acetyl transferase (PAT) and Streptomyces hygroscopicus phosphinothricin acetyl transferase (bar) genes), and pyridinoxy or phenoxy proprionic acids and cyclohexones (ACCase inhibitor-encoding genes). See, for example, U.S. Pat. No. 4,940,835 to Shah, et al., which discloses the nucleotide sequence of a form of EPSPS which can confer glyphosate resistance. U.S. Pat. No. 5,627,061 to Barry, et al., also describes genes encoding EPSPS enzymes. See also, U.S. Pat. Nos. 6,566,587; 6,338,961; 6,248,876 B1; 6,040,497; 5,804,425; 5,633,435; 5,145,783; 4,971,908; 5,312,910; 5,188,642; 5,094,945, 4,940,835; 5,866,775; 6,225,114 B1; 6,130,366; 5,310,667; 4,535,060; 4,769,061; 5,633,448; 5,510,471; Re. 36,449; RE 37,287 E and 5,491,288 and International Publications EP 1173580; WO 2001/66704; EP 1173581 and EP 1173582, which are incorporated herein by reference for this purpose.

Glyphosate resistance is also imparted to plants that express a gene encoding a glyphosate oxido-reductase enzyme as described more fully in U.S. Pat. Nos. 5,776,760 and 5,463,175, which are incorporated herein by reference for this purpose. In addition glyphosate resistance can be imparted to plants by the over expression of genes encoding glyphosate N-acetyltransferase. See, for example, U.S. Pat. Nos. 7,462,481; 7,405,074 and US Patent Application Publication Number US 2008/0234130. A DNA molecule encoding a mutant aroA gene can be obtained under ATCC Accession Number 39256, and the nucleotide sequence of the mutant gene is disclosed in U.S. Pat. No. 4,769,061 to Comai. EP Application Number 0 333 033 to Kumada, et al., and U.S. Pat. No. 4,975,374 to Goodman, et al., disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such

as L-phosphinothricin. The nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in EP Application Numbers 0 242 246 and 0 242 236 to Leemans, et al.; De Greef, et al., (1989) Bio/Technology 7:61, describe the production of transgenic plants that express chimeric bar genes coding for phosphinothricin acetyl transferase activity. See also, U.S. Pat. Nos. 5,969,213; 5,489,520; 5,550,318; 5,874,265; 5,919,675; 5,561,236; 5,648,477; 5,646,024; 6,177,616 B1 and 5,879,903, which are incorporated herein by reference for this purpose. Exemplary genes conferring resistance to phenoxy proprionic acids and cyclohexones, such as sethoxydim and haloxyfop, are the Acc1-S1, Acc1-S2 and Acc1-S3 genes described by Marshall, et al., (1992) Theor. Appl. Genet. 83:435.

(C) A polynucleotide encoding a protein for resistance to herbicide that inhibits photosynthesis, such as a triazine (psbA and gs+genes) and a benzonitrile (nitrilase gene). Przibilla, et al., (1991) Plant Cell 3:169, describe the transformation of Chlamydomonas with plasmids encoding mutant psbA genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648 to Stalker and DNA molecules containing these genes are available under ATCC Accession Numbers 53435, 67441 and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes, et al., (1992) Biochem. J. 285:173.

(D) A polynucleotide encoding a protein for resistance to Acetohydroxy acid synthase, which has been found to make plants that express this enzyme resistant to multiple types of herbicides, has been introduced into a variety of plants (see, e.g., Hattori, et al., (1995) Mol Gen Genet. 246:419). Other genes that confer resistance to herbicides include: a gene encoding a chimeric protein of rat cytochrome P4507A1 and yeast NADPH-cytochrome P450 oxidoreductase (Shiota, et al., (1994) Plant Physiol 106:17), genes for glutathione reductase and superoxide dismutase (Aono, et al., (1995) Plant Cell Physiol 36:1687) and genes for various phosphotransferases (Datta, et al., (1992) Plant Mol Biol 20:619).

(E) A polynucleotide encoding resistance to a herbicide targeting Protoporphyrinogen oxidase (protox) which is necessary for the production of chlorophyll. The protox enzyme serves as the target for a variety of herbicidal compounds. These herbicides also inhibit growth of all the different species of plants present, causing their total destruction. The development of plants containing altered protox activity which are resistant to these herbicides are described in U.S. Pat. Nos. 6,288,306 B1; 6,282,837 B1 and 5,767,373 and International

Publication WO 2001/12825.

(F) The aad-1 gene (originally from Sphingobium herbicidovorans) encodes the aryloxyalkanoate dioxygenase (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides. The aad-1 gene, itself, for herbicide tolerance in plants was first disclosed in WO 2005/107437 (see also, US 2009/0093366). The aad-12 gene, derived from Delftia acidovorans, which encodes the aryloxyalkanoate dioxygenase (AAD-12) protein that confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides by deactivating several herbicides with an aryloxyalkanoate moiety, including phenoxy auxin (e.g., 2,4-D, MCPA), as well as pyridyloxy auxins (e.g., fluoroxypyr, triclopyr).

(G) A polynucleotide encoding a herbicide resistant dicamba monooxygenase disclosed in US Patent Application Publication 2003/0135879 for imparting dicamba tolerance.

(H) A polynucleotide molecule encoding bromoxynil nitrilase (Bxn) disclosed in U.S. Pat. No. 4,810,648 for imparting bromoxynil tolerance.

(I) A polynucleotide molecule encoding phytoene (crtl) described in Misawa, et al., (1993) Plant J. 4:833-840 and in Misawa, et al., (1994) Plant J. 6:481-489 for norflurazon tolerance.

iii. Transgenes that Confer or Contribute to an Altered Grain Characteristic

(A) Altered fatty acids, for example, by (1) Down-regulation of stearoyl-ACP to increase stearic acid content of the plant. See, Knultzon, et al., (1992) Proc. Natl. Acad. Sci. USA 89:2624 and WO 1999/64579 (Genes to Alter Lipid Profiles in Corn); (2) Elevating oleic acid via FAD-2 gene modification and/or decreasing linolenic acid via FAD-3 gene modification (see, U.S. Pat. Nos. 6,063,947; 6,323,392; 6,372,965 and WO 1993/11245); (3) Altering conjugated linolenic or linoleic acid content, such as in WO 2001/12800; (4) Altering LEC1, AGP, Dek1, Superal1, mil ps, various Ipa genes such as Ipa1, Ipa3, hpt or hggt. For example, see, WO 2002/42424, WO 1998/22604, WO 2003/011015, WO 2002/057439, WO 2003/011015, U.S. Pat. Nos. 6,423,886, 6,197,561, 6,825,397 and US Patent Application Publication Numbers US 2003/0079247, US 2003/0204870 and Rivera-Madrid, et al., (1995)

Proc. Natl. Acad. Sci. 92:5620-5624; (5) Genes encoding delta-8 desaturase for making long-chain polyunsaturated fatty acids (U.S. Pat. Nos. 8,058,571 and 8,338,152), delta-9 desaturase for lowering saturated fats (U.S. Pat. No. 8,063,269), Primula .DELTA.6-desaturase for improving omega-3 fatty acid profiles; (6) Isolated nucleic acids and proteins associated with lipid and sugar metabolism regulation, in particular, lipid metabolism protein (LMP) used in methods of producing transgenic plants and modulating levels of seed storage compounds including lipids, fatty acids, starches or seed storage proteins and use in methods of modulating the seed size, seed number, seed weights, root length and leaf size of plants (EP 2404499); (7) Altering expression of a High-Level Expression of Sugar-Inducible 2 (HSI2) protein in the plant to increase or decrease expression of HSI2 in the plant. Increasing expression of HSI2 increases oil content while decreasing expression of HSI2 decreases abscisic acid sensitivity and/or increases drought resistance (US Patent Application Publication Number 2012/0066794); (8) Expression of cytochrome b5 (Cb5) alone or with FAD2 to modulate oil content in plant seed, particularly to increase the levels of omega-3 fatty acids and improve the ratio of omega-6 to omega-3 fatty acids (US Patent Application Publication Number 2011/0191904); and (9) Nucleic acid molecules encoding wrinkled1-like polypeptides for modulating sugar metabolism (U.S. Pat. No. 8,217,223).

(B) Altered phosphorus content, for example, by the (1) introduction of a phytase-encoding gene would enhance breakdown of phytate, adding more free phosphate to the transformed plant. For example, see, Van Hartingsveldt, et al., (1993) Gene 127:87, for a disclosure of the nucleotide sequence of an Aspergillus niger phytase gene; and (2) modulating a gene that reduces phytate content. In maize, this, for example, could be accomplished, by cloning and then re-introducing DNA associated with one or more of the alleles, such as the LPA alleles, identified in maize mutants characterized by low levels of phytic acid, such as in WO 2005/113778 and/or by altering inositol kinase activity as in WO 2002/059324, US Patent Application Publication Number 2003/0009011, WO 2003/027243, US Patent Application Publication Number 2003/0079247, WO 1999/05298, U.S. Pat. No. 6,197,561, U.S. Pat. No. 6,291,224, U.S. Pat. No. 6,391,348, WO 2002/059324, US Patent Application Publication Number 2003/0079247, WO 1998/45448, WO 1999/55882, WO 2001/04147.

(C) Altered carbohydrates affected, for example, by altering a gene for an enzyme that

affects the branching pattern of starch or, a gene altering thioredoxin such as NTR and/or TRX (see, U.S. Pat. No. 6,531,648. which is incorporated by reference for this purpose) and/or a gamma zein knock out or mutant such as cs27 or TUSC27 or en27 (see, U.S. Pat. No. 6,858,778 and US Patent Application Publication Number 2005/0160488, US Patent Application Publication Number 2005/0204418, which are incorporated by reference for this purpose). See, Shiroza, et al., (1988) J. Bacteriol. 170:810 (nucleotide sequence of Streptococcus mutant fructosyltransferase gene), Steinmetz, et al., (1985) Mol. Gen. Genet. 200:220 (nucleotide sequence of Bacillus subtilis levansucrase gene), Pen, et al., (1992) Bio/Technology 10:292 (production of transgenic plants that express Bacillus licheniformis alpha-amylase), Elliot, et al., (1993) Plant Molec. Biol. 21:515 (nucleotide sequences of tomato invertase genes), Sogaard, et al., (1993) J. Biol. Chem. 268:22480 (site-directed mutagenesis of barley alpha-amylase gene) and Fisher, et al., (1993) Plant Physiol. 102:1045 (maize endosperm starch branching enzyme II), WO 1999/10498 (improved digestibility and/or starch extraction through modification of UDP-D-xylose 4-epimerase, Fragile 1 and 2, Refl, HCHL, C4H), U.S. Pat. No. 6,232,529 (method of producing high oil seed by modification of starch levels (AGP)). The fatty acid modification genes mentioned herein may also be used to affect starch content and/or composition through the interrelationship of the starch and oil pathways.

(D) Altered antioxidant content or composition, such as alteration of tocopherol or tocotrienols. For example, see, U.S. Pat. No. 6,787,683, US Patent Application Publication Number 2004/0034886 and WO 2000/68393 involving the manipulation of antioxidant levels and WO 2003/082899 through alteration of a homogentisate geranyl geranyl transferase (hggt).

(E) Altered essential seed amino acids. For example, see, U.S. Pat. No. 6,127,600 (method of increasing accumulation of essential amino acids in seeds), U.S. Pat. No. 6,080,913 (binary methods of increasing accumulation of essential amino acids in seeds), U.S. Pat. No. 5,990,389 (high lysine), WO 1999/40209 (alteration of amino acid compositions in seeds), WO 1999/29882 (methods for altering amino acid content of proteins), U.S. Pat. No. 5,850,016 (alteration of amino acid compositions in seeds), WO 1998/20133 (proteins with enhanced levels of essential amino acids), U.S. Pat. No. 5,885,802 (high methionine), U.S. Pat. No. 5,885,801 (high threonine), U.S. Pat. No. 6,664,445 (plant amino acid biosynthetic

enzymes), U.S. Pat. No. 6,459,019 (increased lysine and threonine), U.S. Pat. No. 6,441,274 (plant tryptophan synthase beta subunit), U.S. Pat. No. 6,346,403 (methionine metabolic enzymes), U.S. Pat. No. 5,939,599 (high sulfur), U.S. Pat. No. 5,912,414 (increased methionine), WO 1998/56935 (plant amino acid biosynthetic enzymes), WO 1998/45458 (engineered seed protein having higher percentage of essential amino acids), WO 1998/42831 (increased lysine), U.S. Pat. No. 5,633,436 (increasing sulfur amino acid content), U.S. Pat. No. 5,559,223 (synthetic storage proteins with defined structure containing programmable levels of essential amino acids for improvement of the nutritional value of plants), WO 1996/01905 (increased threonine), WO 1995/15392 (increased lysine), US Patent Application Publication Number 2003/0163838, US Patent Application Publication Number 2003/0150014, US Patent Application Publication Number 2004/0068767, U.S. Pat. No. 6,803,498, WO 2001/79516.

iv. Genes that Control Male-Sterility

There are several methods of conferring genetic male sterility available, such as multiple mutant genes at separate locations within the genome that confer male sterility, as disclosed in U.S. Pat. Nos. 4,654,465 and 4,727,219 to Brar, et al., and chromosomal translocations as described by Patterson in U.S. Pat. Nos. 3,861,709 and 3,710,511. In addition to these methods, Albertsen, et al., U.S. Pat. No. 5,432,068, describe a system of nuclear male sterility which includes: identifying a gene which is critical to male fertility; silencing this native gene which is critical to male fertility; removing the native promoter from the essential male fertility gene and replacing it with an inducible promoter; inserting this genetically engineered gene back into the plant; and thus creating a plant that is male sterile because the inducible promoter is not "on" resulting in the male fertility gene not being transcribed. Fertility is restored by inducing or turning "on", the promoter, which in turn allows the gene that confers male fertility to be transcribed. Non-limiting examples include: (A) Introduction of a deacetylase gene under the control of a tapetum-specific promoter and with the application of the chemical N-Ac-PPT (WO 2001/29237); (B) Introduction of various stamen-specific promoters (WO 1992/13956, WO 1992/13957); and (C) Introduction of the barnase and the barstar gene (Paul, et al., (1992) Plant Mol. Biol. 19:611-622). For additional examples of nuclear male and female sterility systems and genes, see also, U.S. Pat. Nos.

5,859,341; 6,297,426; 5,478,369; 5,824,524; 5,850,014 and 6,265,640, all of which are hereby incorporated by reference.

v. Genes that Create a Site for Site Specific DNA Integration.

This includes the introduction of FRT sites that may be used in the FLP/FRT system and/or Lox sites that may be used in the Cre/Loxp system. For example, see, Lyznik, et al., (2003) Plant Cell Rep 21:925-932 and WO 1999/25821, which are hereby incorporated by reference. Other systems that may be used include the Gln recombinase of phage Mu (Maeser, et al., (1991) Vicki Chandler, The Maize Handbook ch. 118 (Springer-Verlag 1994), the Pin recombinase of E. coli (Enomoto, et al., 1983) and the R/RS system of the pSRi plasmid (Araki, et al., 1992).

vi. Genes that affect abiotic stress resistance

Including but not limited to flowering, ear and seed development, enhancement of nitrogen utilization efficiency, altered nitrogen responsiveness, drought resistance or tolerance, cold resistance or tolerance and salt resistance or tolerance and increased yield under stress. Non-limiting examples include: (A) For example, see: WO 2000/73475 where water use efficiency is altered through alteration of malate; U.S. Pat. Nos. 5,892,009, 5,965,705, 5,929,305, 5,891,859, 6,417,428, 6,664,446, 6,706,866, 6,717,034, 6,801,104, WO 2000/060089, WO 2001/026459, WO 2001/035725, WO 2001/034726, WO 2001/035727, WO 2001/036444, WO 2001/036597, WO 2001/036598, WO 2002/015675, WO 2002/017430, WO 2002/077185, WO 2002/079403, WO 2003/013227, WO 2003/013228, WO 2003/014327, WO 2004/031349, WO 2004/076638, WO 199809521; (B) WO 199938977 describing genes, including CBF genes and transcription factors effective in mitigating the negative effects of freezing, high salinity and drought on plants, as well as conferring other positive effects on plant phenotype; (C) US Patent Application Publication Number 2004/0148654 and WO 2001/36596 where abscisic acid is altered in plants resulting in improved plant phenotype such as increased yield and/or increased tolerance to abiotic stress; (D) WO 2000/006341, WO 2004/090143, U.S. Pat. Nos. 7,531,723 and 6,992,237 where cytokinin expression is modified resulting in plants with increased stress tolerance, such as drought tolerance, and/or increased yield. Also see, WO 2002/02776, WO

2003/052063, JP 2002/281975, U.S. Pat. No. 6,084,153, WO 2001/64898, U.S. Pat. No. 6,177,275 and U.S. Pat. No. 6,107,547 (enhancement of nitrogen utilization and altered nitrogen responsiveness); (E) For ethylene alteration, see, US Patent Application Publication Number 2004/0128719, US Patent Application Publication Number 2003/0166197 and WO 2000/32761; (F) For plant transcription factors or transcriptional regulators of abiotic stress, see, e.g., US Patent Application Publication Number 2004/0098764 or US Patent Application Publication Number 2004/0078852; (G) Genes that increase expression of vacuolar pyrophosphatase such as AVP1 (U.S. Pat. No. 8,058,515) for increased yield; nucleic acid encoding a HSFA4 or a HSFA5 (Heat Shock Factor of the class A4 or A5) polypeptides, an oligopeptide transporter protein (OPT4-like) polypeptide; a plastochron2-like (PLA2-like) polypeptide or a Wuschel related homeobox 1-like (WOX1-like) polypeptide (U. Patent Application Publication Number US 2011/0283420); (H) Down regulation of polynucleotides encoding poly (ADP-ribose) polymerase (PARP) proteins to modulate programmed cell death (U.S. Pat. No. 8,058,510) for increased vigor; (I) Polynucleotide encoding DTP21 polypeptides for conferring drought resistance (US Patent Application Publication Number US 2011/0277181); (J) Nucleotide sequences encoding ACC Synthase 3 (ACS3) proteins for modulating development, modulating response to stress, and modulating stress tolerance (US Patent Application Publication Number US 2010/0287669); (K) Polynucleotides that encode proteins that confer a drought tolerance phenotype (DTP) for conferring drought resistance (WO 2012/058528); (L) Tocopherol cyclase (TC) genes for conferring drought and salt tolerance (US Patent Application Publication Number 2012/0272352); (M) CAAX amino terminal family proteins for stress tolerance (U.S. Pat. No. 8,338,661); (N) Mutations in the SAL1 encoding gene have increased stress tolerance, including increased drought resistant (US Patent Application Publication Number 2010/0257633); (O) Expression of a nucleic acid sequence encoding a polypeptide selected from the group consisting of: GRF polypeptide, RAA1-like polypeptide, SYR polypeptide, ARKL polypeptide, and YTP polypeptide increasing yield-related traits (US Patent Application Publication Number 2011/0061133); and (P) Modulating expression in a plant of a nucleic acid encoding a Class III Trehalose Phosphate Phosphatase (TPP) polypeptide for enhancing yield-related traits in plants, particularly increasing seed yield (US Patent Application Publication Number 2010/0024067).

Other genes and transcription factors that affect plant growth and agronomic traits

such as yield, flowering, plant growth and/or plant structure, can be introduced or introgressed into plants, see e.g., WO 1997/49811 (LHY), WO 1998/56918 (ESD4), WO 1997/10339 and U.S. Pat. No. 6,573,430 (TFL), U.S. Pat. No. 6,713,663 (FT), WO 1996/14414 (CON), WO 1996/38560, WO 2001/21822 (VRN1), WO 2000/44918 (VRN2), WO 1999/49064 (GI), WO 2000/46358 (FR1), WO 1997/29123, U.S. Pat. No. 6,794,560, U.S. Pat. No. 6,307,126 (GAI), WO 1999/09174 (D8 and Rht) and WO 2004/076638 and WO 2004/031349 (transcription factors).

vii. Genes that Confer Increased Yield

Non-limiting examples of genes that confer increased yield are: (A) A transgenic crop plant transformed by a 1-AminoCyclopropane-1-Carboxylate Deaminase-like Polypeptide (ACCDP) coding nucleic acid, wherein expression of the nucleic acid sequence in the crop plant results in the plant's increased root growth, and/or increased yield, and/or increased tolerance to environmental stress as compared to a wild type variety of the plant (U.S. Pat. No. 8,097,769); (B) Over-expression of maize zinc finger protein gene (Zm-ZFP1) using a seed preferred promoter has been shown to enhance plant growth, increase kernel number and total kernel weight per plant (US Patent Application Publication Number 2012/0079623); (C) Constitutive over-expression of maize lateral organ boundaries (LOB) domain protein (Zm-LOBDP1) has been shown to increase kernel number and total kernel weight per plant (US Patent Application Publication Number 2012/0079622); (D) Enhancing yield-related traits in plants by modulating expression in a plant of a nucleic acid encoding a VIM1 (Variant in Methylation 1)-like polypeptide or a VTC2-like (GDP-L-galactose phosphorylase) polypeptide or a DUF1685 polypeptide or an ARF6-like (Auxin Responsive Factor) polypeptide (WO 2012/038893); (E) Modulating expression in a plant of a nucleic acid encoding a Ste20-like polypeptide or a homologue thereof gives plants having increased yield relative to control plants (EP 2431472); and (F) Genes encoding nucleoside diphosphatase kinase (NDK) polypeptides and homologs thereof for modifying the plant's root architecture (US Patent Application Publication Number 2009/0064373).

IX. Methods of Use

Methods disclosed herein comprise methods for controlling a plant insect pest (i.e., a Coleopteran plant pest, including a *Diabrotica* plant pest, such as, *D. virgifera virgifera*, *D. barberi*, *D. virgifera zeae, D. speciosa*, or *D. undecimpunctata howardi*). In one embodiment, the method comprises feeding or applying to a plant insect pest a composition comprising a silencing element of the invention, wherein said silencing element, when ingested or contacted by a plant insect pest (i.e., but not limited to, a Coleopteran plant pest including a *Diabrotica* plant pest, such as, *D. virgifera virgifera*, *D. barberi*, *D. virgifera zeae, D. speciosa*, or *D. undecimpunctata howardi)*, reduces the level of a target polynucleotide of the pest and thereby controls the pest. The pest can be fed the silencing element in a variety of ways. For example, in an embodiment, the polynucleotide comprising the silencing element is introduced into a plant. As the 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 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.

In another method, a composition comprising at least one silencing element disclosed herein 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 plant insect pest. In such embodiments, the composition can be applied to an area inhabited by a plant insect pest. In one embodiment, the composition is applied externally to a plant (i.e., by spraying a field) to protect the plant from pests.

In certain embodiments, the disclosed polynucleotides or constructs 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.

Disclosed 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 disclosure with polynucleotides providing agronomic traits such as male sterility (e.g., see U.S. Patent No. 5.583,210), stalk strength, drought resistance (e.g., U.S. Patent No. 7,786,353), 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.

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.

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

EXPERIMENTAL

Example 1: Sequences Having Insecticidal Activity.

Nucleic acid sequences disclosed herein comprise the following nucleic acid sequences. Certain sequences are exemplary and were shown to have insecticidal activity against corn rootworms using the assay methods described in Example 1 as set forth below. Such sequences or their complements can be used in the methods of the present disclosure as described herein above and below. Methods for making inhibitory sequences are known in the art. DNA constructs, vectors, transgenic cells, plants, seeds or products described herein may comprise one or more of the following nucleic acid or amino acid sequences, or a portion of one or more of the disclosed sequences. Non-limiting examples of target polynucleotides are set forth below in Tables 1 and 2, or variants and fragments thereof, and complements thereof, including, for example, SEQ ID NOs.:1-9, and variants and fragments therof, and complements thereof, and SEQ ID NOs.: 15-23, and variants and fragments therof, and complements thereof. The official copy of the sequence listing is submitted with the specification as a text file via EFS-Web, in compliance with the American Standard Code for Information Interchange (ASCII). The sequence listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein, and comprises SEQ ID. NOs. 1-33.

Table 1: RYANR RNAi target fragments.

Target ID	Fragment length (bp)	Mismatches*	SEQ ID NO.
WCRW frag5	503	n/a	**1**
NCRW frag5	503	12	**2**
SCRW frag5	503	27	**3**
WCRW frag1	211	n/a	**4**
NCRW frag1	211	7	**5**
SCRW frag1	211	16	**6**
WCRW frag74	211	n/a	**7**
NCRW frag74	211	4	**8**
SCRW frag74	211	8	**9**

* The number of mismatched base pairs (bp) between the homologous WCRW sequence and the target ID sequence. "n/a" indicates "not applicable," as there are no mismatches between the sense and antisense strands of WCRW itself.

Table 2. RYANR Homologues.

Sources	Common name	ORF*	ORF Length**	Transcript*	Transcript Length**
Spodoptera frugiperda	Fall Army Worm	15	474	19	1147
Spodoptera exigua	Beet Armyworm	16	474	20	1052
Phyllotreta cruciferae	Crucifer flea beetle	17	480	21	1564
Phyllotreta striolata	Striped flea beetle	18	480	22	1212
Diabrotica virgifera (field)	Western corn rooworm	19	480	23	1302

* The number given in the column for "ORF" or "Transcript," as indicated, is the SEQ ID NO corresponding to the RYANR homologue. "ORF" is the coding region or open reading frame for protein encoded by the given RYANR homologue. "Transcript" corresponds to the full-length mRNA transcript for the given RYANR homologue.

† Length in base pairs (bp).

Example 2: *In vitro* Transcript dsRNA Screening Method.

Two previously identified RNAi active targets RYANR and HP2 showed 54% and 49% identity to Drosophila snakeskin (Ssk) and Mesh, respectively. The Mesh-Ssk protein complex is required for septate junction formation in the Drosophila midgut.

Regions (fragments) of WCRW, NCRW and SCRW RYANR genes were produced by PCR using target specific primers to generate DNA templates and, followed by in vitro transcription (IVT) to produce long double stranded RNAs of the sequences described in Table 1. The target specific primers also contain T7 RNA polymerase sites (T7 sequence at 5′ end of both or one side primer(s)). dsRNA duplex molecules were also made by mixing and annealing two single strand RNA molecules (as shown in Table 3 below). Following enzymatic digestion and removal of the DNA template, the IVT reaction products were incorporated into artificial insect diet as described below.

Example 3. WCRW Insecticidal Activity (IC_{50}).

dsRNAs were incorporated into standard WCRW artificial diet in a 96 well microtiter plate format. 5 µl of the IVT reaction were added to a given well of a 96 well microtiter plate. 25 µl of molten lowmelt Western corn rootworm diet were added to the sample and shaken on an orbital shaker to mix the sample and diet. Once the diet has solidified, eight wells were used for each RNA sample. Preconditioned 1st instar WCRW (neonate insects were placed on neutral diet for 24 hours prior to transfer to test material) were added to the 96 well microtiter plates at a rate of 3-5 insects/well. Plates were sealed with Mylar® which was then punctured twice above each well of the microtiter plate using a superfine insect collection pin. To prevent drying of the diet, plates were first placed inside a plastic bag with a slightly damp cloth and the bags were placed inside an incubator set at 28 ˚C and 70% RH. The assay was scored for mortality and stunting affects after 7 days and an average was determined based on assignment of numeric values to each category of impact (3 = mortality, 2 = severe stunting, 1 = stunting, 0 = no affect). The number reported in this and all diet assay tables reflect the average score across all observations. A score of 3 represents complete mortality across all observations. A score of 2.5 would indicate half the wells demonstrating mortality and half scored as severe stunting.

Subregions of RYANR dsRNAs were designed to improve insecticidal activities in diet and dsRNA expression *in planta*. Regions demonstrating a severe impact on larval phenotype (mortality or severe growth retardation) were used for informal inhibitory concentration (IC_{50}) assays. IC_{50} assays used doses starting at 50 ppm and progressing downward by ½ step dilutions through 25, 12.5, 6, 3, 1.5, and 0.75 ppm. 12 observations were included for each rate. . Calculations of inhibition relied on scoring for both mortality and severe stunting (IC_{50}) values were calculated and described in Table 3, which showed strong insecticidal activities among all combinations of dsRNA duplexes containing RYANR frag1 from WCRW, NCRW and/or SCRW. Without wishing to be bound by a particular theory, these data support the value of mismatch hairpin for improving dsRNA accumulation *in planta*.

Table 3. RNA Duplex Insecticidal Activity.

RNA Duplex*	Strand		IC_{50}	
	Sense	Antisense	1st test	2nd test
ww	WCRW	WCRW	0.01639	0.02482
nw	NCRW	WCRW	0.01304	0.03255
sw	SCRW	WCRW	0.03255	0.11788
wn	WCRW	NCRW	0.00566	0.05497
ws	WCRW	SCRW	0.02167	0.08954
nn	NCRW	NCRW	0.00861	0.02005
ss	SCRW	SCRW	0.01357	0.06862

* "ww" is an RNA duplex of the sense and antisense strands of WCRW frag1 (SEQ ID NO.: 4); "nw" is an RNA duplex of the sense NCRW frag1 (SEQ ID NO.: 5) and antisense strand of WCRW frag1 (SEQ ID NO.: 4); "sw" is an RNA duplex of the sense SCRW frag1 (SEQ ID NO.: 6) and antisense strand of WCRW frag1 (SEQ ID NO.: 4); "wn" is an RNA duplex of the sense WCRW frag1 (SEQ ID NO.: 4) and antisense strand of NCRW frag1 (SEQ ID NO.: 5); "ws" is an RNA duplex of the sense WCRW frag1 (SEQ ID NO.: 4) and antisense strand of SCRW frag1 (SEQ ID NO.: 6); "nn" is an RNA duplex of the sense and antisense strands of NCRW frag1 (SEQ ID NO.: 5); and, "ss" is an RNA duplex of the sense and antisense strands of SCRW frag1 (SEQ ID NO.: 6).

Example 4: Mismatch Construct Design and Features.

The mismatch RNA constructs as described herein comprise at least a double-strand RNA (dsRNA) molecule wherein the strands comprise one or more mismatches. The level of mismatch between the strands can be adjusted to modulate the accumulation of dsRNA in a target cell or pest and/or to target multiple species in a single dsRNA molecule. In the design of the disclosed mismatch RNA constructs, there are a number of criteria useful in the selection of the appropriate nucleotide sequences to use, including the level of mismatch between segment 1 and segment 3 (see below for further discussion of segments 1, 2, and 3) and the level of complementary between the RNA construct and the plant insect pest target sequence. For example, desirable levels of mismatch between the two RNA strands, i.e. the level of mismatch between segments 1 and 3, can vary between about 0.1% to about 20%. The strands of the dsRNA molecule can be connected by an RNA strand that is not self-complementary, i.e. segment 2 as discussed herein below, thus forming a loop connecting the strands of the dsRNA molecule. As the results described herein surprisingly demonstrate, desirable mismatches can be formed between two strands wherein one strand is a nucleic acid

sequence from one pest insect species and the complementary strand is a nucleic acid sequence from a second insect species, wherein the nucleic acid sequences are derived from homologous transcript or ORF sequences. The second criteria, as mentioned above is the level of complementarity between the RNA construct, e.g. segment 1 or segment 3 as discussed herein below, and the plant insect pest target sequence. For example, the level of such complementarity can vary between about 90% to about 100%.

The disclosed RNA mismatch constructs have multiple advantages, including, but not limited to, the ability to positively modulate the accumulation of RNA in the target plant insect pest. A further advantage of the disclosed RNA mismatch constructs is that these constructs provide the ability to target multiple plant insect pests with a single RNA construct molecule. Thus, a single RNA construct molecule can be designed to be used in a field infested with distinct plant insect pests. Alternatively, a single RNA construct molecule can be manufactured that can be utilized in field use over a broad geographic range.

As used herein, "convergent design" or "convergent orientation" for a mismatch construct refers to a construct, e.g. a DNA construct that when transcribed provides an RNA construct or alternatively, the RNA construct itself, such that the leading strand of a RNA duplex or dsRNA comprising a stem-loop structure is the sense strand in relation to the target pest RNA.

As used herein, "divergent design" or "divergent orientation" for a mismatch construct refers to a construct, e.g. a DNA construct that when transcribed provides an RNA construct or alternatively, the RNA construct itself, such that the leading strand of a RNA duplex, or dsRNA comprising a stem-loop structure, is the antisense strand in relation to the target pest RNA.

In general, a DNA construct encoding an RNA mismatch construct comprises on the sense strand of the DNA construct a sequence comprising three sequence elements as follows: a first segment, a second segment, and a third segment, which are sequentially ordered as given. The first and third segments are capable of forming a duplex structure in an RNA molecule transcribed from the sense strand of the DNA construct. The second segment, situatied between the first and third segments, is capable of forming a loop structure between the duplex formed between the first and third segments. Thus, an RNA transcribed from the sense strand of such a DNA constructs is an RNA sequence which has at the 5' end the first

RNA segment that can be in either the sense or antisense orientation relative to target sequence, followed by the second RNA segment which is a non-complementary sequence and is capable of forming a loop, and then the third RNA segment that is capable of base pairing with first RNA segment. The third RNA segment is in the opposite orientation of the first RNA segment to the target sequence. Thus, it should be understood that reference to complementarity between the first and third segments is intended to mean that they are complementary except for the desired level of mismatch such as described above.

Various aspects of the mismatch constructs described herein are shown in FIGs. 1A and 1B, which shows two possible convergent design constructs. For example, FIG. 1A shows a DNA construct comprising a first segment consisting of a WCRW sequence that is the sense orientation relative to target sequence, followed by the second segment consisting of a sequence that is not self-complementary and is capable of forming a loop structure in the transcribed RNA, and then followed by the third segment consisting of a NCRW sequence. It should be noted for the schematic examples shown in FIG. 1A, 1B, 2A, and 2B, sequences comprise, for the purposes of illustration, bases that are represented by letters of the alphabet wherein uppercase letters are complementary to their lowercase counterparts. Thus, "A" is complementary to "a" and non-complementary to all other lowercase letters. Accordingly, the RNA transcribed from the DNA construct of FIG. 1A is capable of forming a stem-loop RNA molecule with mismatches as indicated in the stem structure which arise from the sequence divergence of the related WCRW and NCRW sequences used to construct the mismatch construct illustrated. As shown in the figure, the transcript produced from this DNA construct is an RNA sequence which has at the 5' end an RNA sequence that is the sense orientation relative to target sequence

A second possible convergent design mismatch construct is shown in FIG. 1B wherein the first segment comprises sequences that are in the sense orientation to the target sense, and wherein the first segment is a chimera of sequences from the WCRW and SCRW, as shown in FIG. 1B. Accordingly, the third segment comprises a first portion that is derived from SCRW sequences and which are complementary to the WCRW sequences of the first segment. The SCRW sequence of the third segment are then followed in this example by a NCRW sequence which is complementary to the WCRW sequence of the first segment.

Further various aspects of the mismatch construct described herein are shown in FIGs.

2A and 2B, which illustrate possible divergent design mismatch constructs.

It should be understood that although the schematic examples referenced in FIGs. 1A, 1B, 2A, and 2B are non-limiting examples, and reference to Corn Rootworm sequences in segments 1 and 3 should be understood to be only a specific embodiment of the disclosed mismatch RNA constructs. Accordingly, segments 1 and 3 can be designed to comprise sequences complementary to target RYANR (or RYANR homologue) sequences from closely related species, geographically distinct species, subspecies, or species from different families or genera with sufficiently similar target gene sequences. In further non-limiting examples, segments 1 and 3 can comprise sequences complementary to target RYANR sequences in the following species:

Helicoverpa zea and *armigera*;
Ostrinia nubilalis and *furnicalis*;
Phylotreta striolata and *cruciferae*;
Lygus hesperus and *lineolaris*;
Spodoptera exigua and *frugiperda*;
Tribolium confusum and *castaneum*;
Tetranychus urticae and *cinnabarinus*;
Anopheles gambiae and *barberi*; or
Musca domestica and *autumnalis*.

As described above, in one embodiment, the first and third segments can comprise a mistmatch level about 0.1% to about 20% of the base pairs in segments 1 and 3. The mismatch level can be varied to modulate various parameters, including, but not limited to, the number of species targeted by the RNA construct and/or the RNA levels that accumulate in the target plant insect pest. The effect of the mismatch level in the design of the RNA construct can be assessed using the methods disclosed herein. For example, the mismatch level may be about 0.1% to about 19% of the base pairs in segments 1 and 3; about 0.1% to about 18% of the base pairs in segments 1 and 3; about 0.1% to about 17% of the base pairs in segments 1 and 3; about 0.1% to about 16% of the base pairs in segments 1 and 3; about 0.1% to about 15% of the base pairs in segments 1 and 3; about 0.1% to about 14% of the base pairs in segments 1 and 3; about 0.1% to about 13% of the base pairs in segments 1 and 3; about 0.1% to about 12% of the base pairs in segments 1 and 3; about 0.1% to about 11% of the base

pairs in segments 1 and 3; about 0.1% to about 10% of the base pairs in segments 1 and 3; about 0.1% to about 9% of the base pairs in segments 1 and 3; about 0.1% to about 8% of the base pairs in segments 1 and 3; about 0.1% to about 7% of the base pairs in segments 1 and 3; or about 0.1% to about 5% of the base pairs in segments 1 and 3. In various additional specific embodiments, the mismatch level may be about 0.5% to about 19% of the base pairs in segments 1 and 3; about 0.5% to about 18% of the base pairs in segments 1 and 3; about 0.5% to about 17% of the base pairs in segments 1 and 3; about 0.5% to about 16% of the base pairs in segments 1 and 3; about 0.5% to about 15% of the base pairs in segments 1 and 3; about 0.5% to about 14% of the base pairs in segments 1 and 3; about 0.5% to about 13% of the base pairs in segments 1 and 3; about 0.5% to about 12% of the base pairs in segments 1 and 3; about 0.5% to about 11% of the base pairs in segments 1 and 3; about 0.5% to about 10% of the base pairs in segments 1 and 3; about 0.5% to about 9% of the base pairs in segments 1 and 3; about 0.5% to about 8% of the base pairs in segments 1 and 3; about 0.5% to about 7% of the base pairs in segments 1 and 3; or about 0.5% to about 5% of the base pairs in segments 1 and 3. In additional particular embodiments, the mismatch level may be about 1% to about 19% of the base pairs in segments 1 and 3; about 1% to about 18% of the base pairs in segments 1 and 3; about 1% to about 17% of the base pairs in segments 1 and 3; about 1% to about 16% of the base pairs in segments 1 and 3; about 1% to about 15% of the base pairs in segments 1 and 3; about 1% to about 14% of the base pairs in segments 1 and 3; about 1% to about 13% of the base pairs in segments 1 and 3; about 1% to about 12% of the base pairs in segments 1 and 3; about 1% to about 11% of the base pairs in segments 1 and 3; about 1% to about 10% of the base pairs in segments 1 and 3; about 1% to about 9% of the base pairs in segments 1 and 3; about 1% to about 8% of the base pairs in segments 1 and 3; about 1% to about 7% of the base pairs in segments 1 and 3; or about 1% to about 5% of the base pairs in segments 1 and 3. In various specific embodiments, the mismatch level may be about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% of the base pairs in segments 1 and 3.

Example 5: Mismatch Constructs for Broad Spectrum Control of Corn Rootworm.

Exemplary RNAi mismatch constructs were designed and prepared to test *in planta* against WCRW larvae (see Table 4 below).

Table 4: Exemplary RYANR Constructs.*

SEQ ID NO.	Stem-1	Stem-2	Mismatch† (%)	Orientation
10	SCRW frag1	WCRW frag1	7.6	convergent
11	NCRW frag1	WCRW frag1	3.5	convergent
12	WCRW frag1	SCRW frag1	7.6	convergent
13	WCRW frag1	NCRW frag1	3.5	convergent
14	WCRW frag1	NCRW frag1	3.5	divergent

* The constructs comprised the indicated Stem-1 and Stem-2 sequences, as well as suitable transciptional promoter and terminator sequences, as well as a suitable loop segment.

† Percent mismatch between Stem-1 and Stem-2.

Example 6: Prospective Mismatch Constructs for Broad Spectrum Control of Corn Rootworm.

Prospective exemplary constructs are described herein (see Table 4 below). The constructs of Table 5 can be prepared and tested using the methods described herein.

Table 5: Prospective Exemplary RYANR Constructs.*

Construct ID	Stem-1	Stem-2	Mismatch† (%)	Orientation
A	NCRW frag1 + WCRW-frag74	NCRW-frag74 + WCRW frag1	2.6	convergent
B	NCRW frag1 + WCRW-frag74	NCRW-frag74 + WCRW frag1	2.6	divergent
C	SCRW-frag1 + WCRW-frag74	SCRW-frag74 + WCRW frag1	5.6	convergent

* The constructs comprised the indicated Stem-1 and Stem-2 sequences, as well as suitable transciptional promoter and terminator sequences, as well as suitable loop segment.

† Percent mismatch between Stem-1 and Stem-2.

Example 7. *Agrobacterium*-mediated Transformation of Maize

For *Agrobacterium*-mediated maize transformation with the disclosed polynucleotide

constructs comprising a silencing element as disclosed herein, the method of Zhao was employed (US Patent Number 5,981,840 and International Patent Publication Number WO 1998/32326, the contents of which are hereby incorporated by reference). Briefly, immature embryos were isolated from maize and the embryos contacted with an *Agrobacterium* suspension, where the bacteria were capable of transferring the desired disclosed polynucleotide constructs comprising a silencing element as disclosed herein (step 1: the infection step). In this step the immature embryos were immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos were co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos were cultured on solid medium following the infection step. Following this co-cultivation period an optional resting step was contemplated. In this resting step, the embryos were incubated in the presence of at least one antibiotic known to inhibit *Agrobacterium* growth without a plant transformant selective agent (step 3: resting step). The immature embryos were cultured on solid medium with antibiotic, but without a selecting agent, for *Agrobacterium* elimination and for a resting phase for the infected cells. Next, inoculated embryos were cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). The immature embryos were cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus was then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium were cultured on solid medium to regenerate the plants.

Example 8. Greenhouse Bioassay.

The silencing elements were expressed in a maize plant as hairpins using the transformation techniques described herein above in Example 7, and the plant was tested for insecticidal activity against corn root worms. The data from these studies are shown in Table 6, FIG. 3A, and FIG. 3B.

Maize plants were transformed with plasmids containing genes listed in Table 5, and plants expressing the silencing elements were transplanted from 272V plates into greenhouse flats containing Fafard Superfine potting mix. Approximately 10 to 14 days after transplant, plants (now at growth stage V2-V3) were transplanted into treepots containing Fafard Superfine potting mix. At 14 days post greenhouse send date, plants were infested with 200

eggs of Western Corn Rootworms (WCRW) per plant. For later sets, a second infestation of 200 eggs WCRW per plant was done 7 days after the first infestation and scoring was at 14 days after the second infestation. 21 days post infestation, plants were scored using CRWNIS. Those plants with a score of ≤ 0.5 were transplanted into large pots containing SB300 for T1 seed. The data in Table 5, FIG. 3A, and FIG. 3B show that SEQ ID NOs: 10-14 showed reduced CRWNIS compared to non-transgenic control plants (untransformed negative control identified in FIG. 3A and 3B as "HC69").

Table 6. T0 Greenhouse Insect Bioassay Results.

Experiment	SEQ ID NO.*	Mismatch	Orientation	T0 plants (N)	CRWNIS (median)
test-A	11	3.5	convergent	24	1
test-A	13	3.5	convergent	11	0.75
test-A	14	3.5	divergent	13	0.8
test-A	Control	n/a	n/a	25	1.5
test-B	10	7.6	convergent	28	1.8
test-B	12	7.6	convergent	14	1.35
test-B	Control	n/a	n/a	16	2

* "Control" is untransformed negative control.

The sequences referred to herein, SEQ. ID NOs: 1-33 are filed concurrently herewith in a textfile and are incorporated herein in their entirety.

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.

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

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims.

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THAT WHICH IS CLAIMED:

1. A ribonucleic acid construct, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment and is complementary to the nucleotide sequence of a target sequence from an insect species different from the species of the target sequence of segment 1, wherein the first and third segments form at least a partially double stranded region, and wherein a first or third segment may comprise

a) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

b) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof;

c) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof;

wherein the ribonucleic acid construct has insecticidal activity against an insect pest.

2. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Western Corn Rootworm.

3. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Southern Corn Rootworm.

4. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Northern Corn Rootworm.

5. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to one or more target sequences comprising at least a portion of a sequence of Western Corn Rootworm, Southern Corn Rootworm or Northern Corn Rootworm, or two or more of these.

6. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 85% complementary to the first segment.

7. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 90% complementary to the first segment.

8. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 95% complementary to the first segment.

9. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 98% complementary to the first segment.

10. A ribonucleic acid construct, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and

a)wherein a first segment comprises

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof; or

iii a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; and

b) a third segment comprises

i) a nucleotide sequence comprising any one of SEQ ID NOs 15-24 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 15-24, or variants or fragments thereof, or complements thereof; or

iii a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 15-24, or fragments or variants thereof, or complements thereof; and

wherein the ribonucleic acid construct has insecticidal activity against an insect pest.

11. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Western Corn Rootworm.

12. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Southern Corn Rootworm.

13. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to a target sequence comprising at least a portion of a sequence of Northern Corn Rootworm.

14. The ribonucleic acid construct of Claim 1, wherein the first segment is complementary to one or more target sequences comprising at least a portion of a sequence of Western Corn Rootworm, Southern Corn Rootworm or Northern Corn Rootworm, or two or more of these.

15. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 85% complementary to the first segment.

16. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 90% complementary to the first segment.

17. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 95% complementary to the first segment.

18. The ribonucleic acid construct of Claim 1, wherein the third segment is at least 98% complementary to the first segment.

19. A ribonucleic acid construct, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target

sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and wherein the ribonucleic acid construct nucleotide sequence comprises SEQ ID NOs 10-14, and wherein the ribonucleic acid construct has wherein the ribonucleic acid construct has insecticidal activity against an insect pest.

20. The ribonucleic acid construct of any one of claims 1, 10 or 19, wherein the insect pest comprises a Coleoptera plant pest.

21. The ribonucleic acid construct of claim 20, wherein the Coleoptera plant pest comprises a *Diabrotica* plant pest.

22. The ribonucleic acid construct of claim 21, wherein the *Diabrotica* plant pest comprises *D. virgifera virgifera*, *D. virgifera zeae*, *D. speciosa, D. barberi*, *D. virgifera zeae,* or *D. undecimpunctata howardi*.

23. The ribonucleic acid construct of any one of claims 1, 10 or 19, wherein the insect pest comprises *Acyrthosiphon* spp., *Aedes* spp., *Anopheles* spp., *Bombyx* spp., *Chrysopa* spp., *Culex* spp., *Dendroctonus* spp., *Euschistus* spp., *Euschistus* spp., *Halyomorpha* spp., *Helicoverpa* spp., *Lygus* spp., *Manduca* spp., *Megachile* spp., *Megacopta* spp., *Nasonia* spp., *Nezara* spp., *Ostrinia* spp., *Pediculus* spp., *Phlebotomus* spp., *Phyllotreta* spp., *Phyllotreta* spp., *Piezodorus* spp., *Spodoptera* spp., *Spodoptera* spp., or *Tribolium* spp.

24. The ribonucleic acid construct of any one of claims 1, 10 or 19, wherein the RNA construct is a hairpin loop.

25. A DNA construct comprising a nucleotide sequence encoding the RNA construct of any one of claims 1-33.

26. An expression construct comprising a DNA construct of claim 25.

27. The expression construct of claim 26, wherein the polynucleotide is operably linked to a heterologous promoter.

28. The expression construct of claim 26, wherein the 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.

29. A host cell comprising the ribonucleic acid construct of any of claims 1-33, the DNA construct of claim 25, or the expression construct of any one of claims 26-28.

30. The host cell of claim 29, wherein the host cell is a bacterial cell.

31. The host cell of claim 30, wherein the bacterial cell is an inactivated bacterial cell.

32. The host cell of any of claims 29-31, wherein the host cell comprises the expression construct of claim 26.

33. The host cell of claim 32, wherein the expression construct comprises a transcriptional promoter operably linked to the DNA construct of claim 25.

34. The host cell of claim 33, wherein the transcriptional promoter is inducible by exposure of the host cell to an exogenous molecule.

35. A composition comprising the ribonucleic acid construct of any of claims 1-33, the DNA construct of claim 25, the expression construct of any one of claims 26-28, or the host cell of any of claims 29-34.

36. The composition of claim 35, further comprising an agriculturally acceptable carrier.

37. The composition of claim 35, further comprising a herbicide compound, an insecticide, a fungicide, a nematocide, an agriculturally-acceptable carrier, and/or a bacteria, or combinations thereof.

38. The composition of claim 35 or 37, wherein the composition is in liquid form, solid form, or gel form.

39. The composition of claim 38, wherein the composition is solid form.

40. The composition of claim 39, wherein the solid form is a pellet, a powder, an aggregate, or a molded article.

41. A plant cell having stably incorporated into its genome a heterologous polynucleotide comprising at least one silencing element, wherein a silencing element comprises:

a) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment and is complementary to the nucleotide sequence of a target sequence from an insect species different from the species of the target sequence of segment 1, wherein the first and third segments form at least a partially double stranded region, and wherein a first or third segment may comprise

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; or,

b) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and

b1) wherein a first segment comprises:

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof; or

iii a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; and

b2) a third segment comprises

i) a nucleotide sequence comprising any one of SEQ ID NOs 15-24 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 15-24, or variants or fragments thereof, or complements thereof; or

iii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 15-24, or fragments or variants thereof, or complements thereof; or

c) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and wherein the RNA construct nucleotide sequence comprises SEQ ID NOs 10-14;

wherein the at least one silencing element, when ingested by a plant insect pest, reduces the level of a target sequence in the plant pest and thereby controls the plant pest.

42. The plant cell of claim 41, wherein the plant pest comprises a Coleoptera plant pest.

43. The plant cell of claim 41 or 42, wherein the Coleoptera plant pest comprises a *Diabrotica* plant pest.

44. The plant cell of claim 43, wherein the *Diabrotica* plant pest comprises *D. virgifera virgifera*, *D. virgifera zeae*, *D. speciosa, D. barberi*, *D. virgifera zeae,* or *D. undecimpunctata howardi*.

45. The plant cell of claim 41, wherein the plant pest comprises *Acyrthosiphon* spp., *Aedes* spp., *Anopheles* spp., *Bombyx* spp., *Chrysopa* spp., *Culex* spp., *Dendroctonus* spp., *Euschistus* spp., *Euschistus* spp., *Halyomorpha* spp., *Helicoverpa* spp., *Lygus* spp., *Manduca* spp., *Megachile* spp., *Megacopta* spp., *Nasonia* spp., *Nezara* spp., *Ostrinia* spp., *Pediculus* spp., *Phlebotomus* spp., *Phyllotreta* spp., *Phyllotreta* spp., *Piezodorus* spp., *Spodoptera* spp., *Spodoptera* spp., or *Tribolium* spp.

46. The plant cell of claim 41, wherein the plant cell comprises the expression cassette of claim 26.

47. The plant cell of claim 41, wherein the silencing element expresses a double stranded RNA.

48. The plant cell of claim 41, wherein the silencing element expresses a hairpin RNA.

49. The plant cell of claim 41, wherein the silencing element is operably linked to a heterologous promoter.

50. The plant cell of claim 41, wherein the plant cell is from a monocot.

51. The plant cell of claim 50, wherein the monocot is maize, barley, millet, wheat or rice.

52. The plant cell of claim 41, wherein the plant cell is from a dicot.

53. The plant cell of claim 52, wherein the dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

54. A plant or plant part comprising the plant cell of claim 41.

55. A transgenic seed from the plant of claim 54.

56. A method for controlling a plant insect pest comprising feeding to a plant insect pest a

composition comprising a silencing element, wherein the silencing element, when ingested by the plant pest, reduces the level of a target plant pest sequence and thereby controls the plant pest, wherein the silencing element comprises

a) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment and is complementary to the nucleotide sequence of a target sequence from an insect species different from the species of the target sequence of segment 1, wherein the first and third segments form at least a partially double stranded region, and wherein a first or third segment may comprise

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; or,

b) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and

b1) wherein a first segment comprises:

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof; or

iii a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; and

b2) a third segment comprises

i) a nucleotide sequence comprising any one of SEQ ID NOs 15-24 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 15-24, or variants or fragments thereof, or complements thereof; or

iii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 15-24, or fragments or variants thereof, or complements thereof; or

c) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and wherein the RNA construct nucleotide sequence comprises SEQ ID NOs 10-14.

57. The method of claim 56, wherein the plant pest comprises a Coleoptera plant pest.

58. The method of claim 57, wherein the Coleoptera plant pest comprises a *Diabrotica* plant pest.

59. The method of claim 58, wherein the Diabrotica plant pest comprises *D. virgifera virgifera*, *D. virgifera zeae*, *D. speciosa, D. barberi*, *D. virgifera zeae,* or *D. undecimpunctata howardi*.

60. The method of claim 56, wherein the plant pest comprises *Acyrthosiphon* spp., *Aedes* spp., *Anopheles* spp., *Bombyx* spp., *Chrysopa* spp., *Culex* spp., *Dendroctonus* spp., *Euschistus*

spp., *Euschistus* spp., *Halyomorpha* spp., *Helicoverpa* spp., *Lygus* spp., *Manduca* spp., *Megachile* spp., *Megacopta* spp., *Nasonia* spp., *Nezara* spp., *Ostrinia* spp., *Pediculus* spp., *Phlebotomus* spp., *Phyllotreta* spp., *Phyllotreta* spp., *Piezodorus* spp., *Spodoptera* spp., *Spodoptera* spp., or *Tribolium* spp.

61. The method of claim 56, wherein the composition comprises a plant or plant part having stably incorporated into its genome a polynucleotide comprising the silencing element.

62. The method of claim 56, wherein the silencing element expresses a double stranded RNA.

63. The method of claim 56, wherein the silencing element comprises a hairpin RNA.

64. The method of claim 56, wherein the silencing element is operably linked to a heterologous promoter.

65. The method of claim 64, wherein the 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.

66. The method of claim 56, wherein the plant is a monocot.

67. The method of claim 66, wherein the monocot is maize, barley, millet, wheat or rice.

68. The method of claim 56, wherein the plant is a dicot.

69. The method of claim 68, wherein the dicot is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

70. An isolated polynucleotide comprising:

a) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target

sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment and is complementary to the nucleotide sequence of a target sequence from an insect species different from the species of the target sequence of segment 1, wherein the first and third segments form at least a partially double stranded region, and wherein a first or third segment may comprise

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; or,

b) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and

b1) wherein a first segment comprises:

i) a nucleotide sequence comprising any one or SEQ ID NOs 1-9 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 1-9, or variants or fragments thereof, or complements thereof; or

iii a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 1-9, or fragments or variants thereof, or complements thereof; and

b2) a third segment comprises

i) a nucleotide sequence comprising any one of SEQ ID NOs 15-24 or variants or fragments thereof, or complements thereof;

ii) a nucleotide sequence comprising at least 85% sequence identity to any one of SEQ ID NOs 15-24, or variants or fragments thereof, or complements thereof; or

iii) a nucleotide sequence comprising at least 19 consecutive nucleotides of any one of SEQ ID NOs 15-24, or fragments or variants thereof, or complements thereof; or

c) a polynucleotide sequence, comprising at least three sequence segments comprising a first segment that is complementary to the nucleotide sequence of at least one target sequence, a second segment that is not complementary to the target or other segments of the construct, and a third segment that is at least partially complementary to the first segment, wherein the first and third segments form at least a partially double stranded region, and wherein the RNA construct nucleotide sequence comprises SEQ ID NOs 10-14;

wherein the polynucleotide encodes a silencing element having insecticidal activity against a plant insect pest.

71. The isolated polynucleotide of claim 70, wherein the plant pest comprises a Coleoptera plant pest.

72. The isolated polynucleotide of claim 71, wherein the Coleoptera plant pest is a *Diabrotica* plant pest.

73. The isolated polynucleotide of claim 72, wherein the *Diabrotica* plant pest comprises *D. virgifera virgifera*, *D. virgifera zeae*, *D. speciosa, D. barberi*, *D. virgifera zeae,* or *D. undecimpunctata howardi*.

74. The isolated polynucleotide of claim 70, wherein the plant pest comprises *Acyrthosiphon* spp., *Aedes* spp., *Anopheles* spp., *Bombyx* spp., *Chrysopa* spp., *Culex* spp., *Dendroctonus* spp., *Euschistus* spp., *Euschistus* spp., *Halyomorpha* spp., *Helicoverpa* spp., *Lygus* spp., *Manduca* spp., *Megachile* spp., *Megacopta* spp., *Nasonia* spp., *Nezara* spp., *Ostrinia* spp., *Pediculus* spp., *Phlebotomus* spp., *Phyllotreta* spp., *Phyllotreta* spp.,

Piezodorus spp., *Spodoptera* spp., *Spodoptera* spp., or *Tribolium* spp.

75. A kit comprising an ribonucleic acid construct of any of claims 1-33 and instructions for using the RNA construct as an insecticide against an insect pest organism.

76. The kit of claim 75, which comprises two or more ribonucleic acid constructs of any of claims 1-33.

77. The kit of any one of claims 75 - 76, wherein the instructions provide for sequential application of one or more ribonucleic acid constructs to reduce the incidence of the insect pest organism developing resistance to the one or more ribonucleic acid constructs.

78. The kit of any one of claims 75-76, wherein the instructions provide for concurrent application of one or more ribonucleic acid constructs to reduce the incidence of the insect pest organism developing resistance to the one or more ribonucleic acid constructs.

79. The kit of any one of claims 75-78, wherein the insect pest organism comprises a Coleoptera plant pest.

80. The kit of claim 79, wherein the Coleoptera insect pest organism is a *Diabrotica* plant pest.

81. The kit of claim 80, wherein the *Diabrotica* insect pest organism comprises *D. virgifera virgifera*, *D. virgifera zeae*, *D. speciosa, D. barberi*, *D. virgifera zeae,* or *D. undecimpunctata howardi*.

82. The kit of any one of claims 75-78, wherein the insect pest organism comprises *Acyrthosiphon* spp., *Aedes* spp., *Anopheles* spp., *Bombyx* spp., *Chrysopa* spp., *Culex* spp., *Dendroctonus* spp., *Euschistus* spp., *Euschistus* spp., *Halyomorpha* spp., *Helicoverpa* spp., *Lygus* spp., *Manduca* spp., *Megachile* spp., *Megacopta* spp., *Nasonia* spp., *Nezara* spp., *Ostrinia* spp., *Pediculus* spp., *Phlebotomus* spp., *Phyllotreta* spp., *Phyllotreta* spp., *Piezodorus* spp., *Spodoptera* spp., *Spodoptera* spp., or *Tribolium* spp.

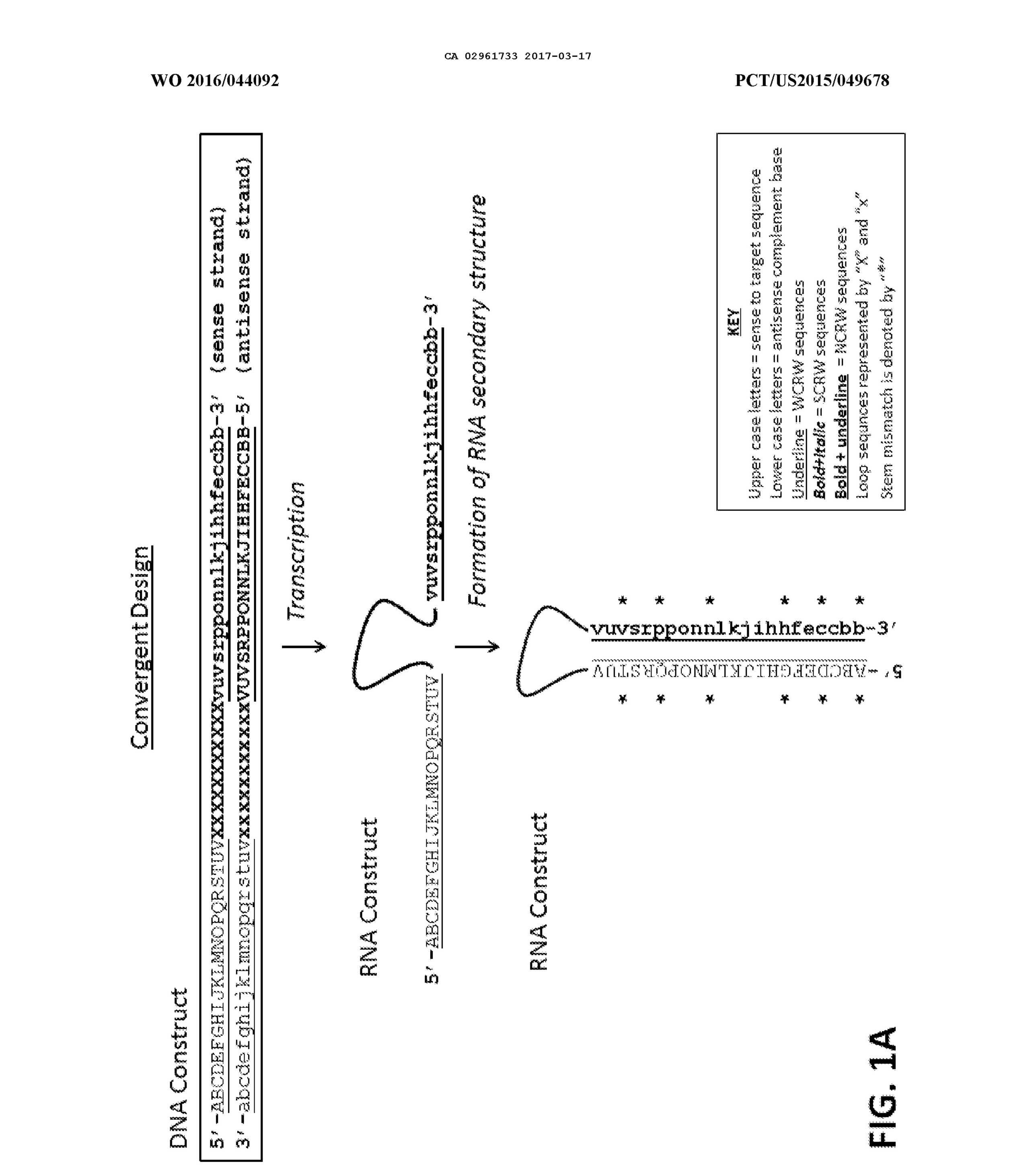

Convergent Design
DNA Construct
5′ -ABCDEFGHIJKLMNOPQRSTUVxxxxxxxxxvuvsrpponnlkjihhfeccbb-3′
3′ -abcdefghijklmnopqrstuvxxxxxxxxxVUVSRPPONNLKJIHHFECCBB-5′
(sense strand)
(antisense strand)
Transcription
RNA Construct
5′ -ABCDEFGHIJKLMNOPQRSTUV vuvsrpponnlkjihhfeccbb-3′
Formation of RNA secondary structure
RNA Construct
vuvsrpponnlkjihhfeccbb-3′
5′ -ABCDEFGHIJKLMNOPQRSTUV
KEY
Upper case letters = sense to target sequence
Lower case letters = antisense complement base
Underline = WCRW sequences
Bold+Italic = SCRW sequences
Bold + underline = NCRW sequences
Loop sequences represented by "X" and "x"
Stem mismatch is denoted by "*"

FIG. 1A

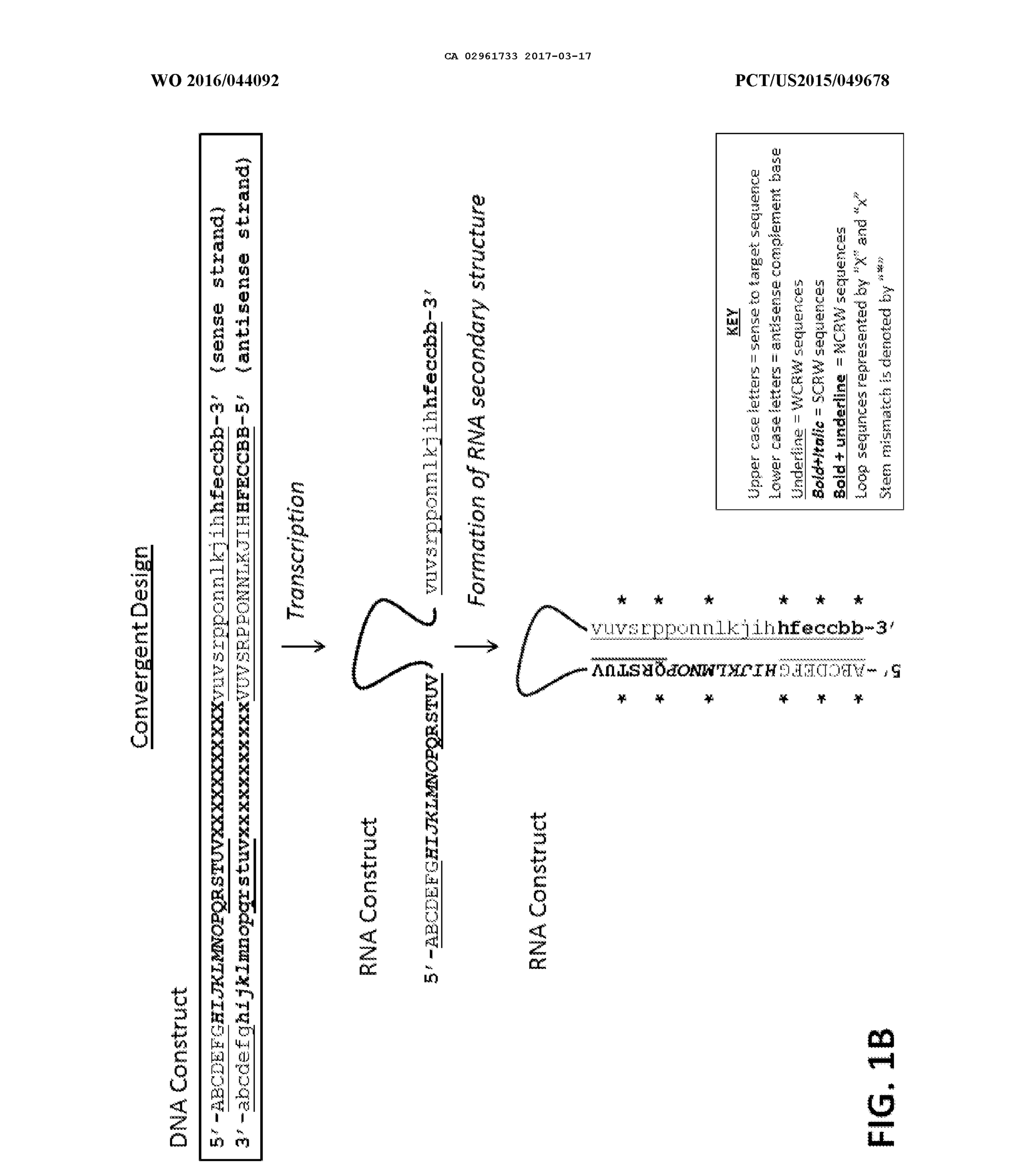

Convergent Design
DNA Construct
5′ -ABCDEFGHIJKLMNOPQRSTUVXXXXXXXvuvsrpponnlkjihhfeccbb-3′
3′ -abcdefghijklmnopqrstuvwxxxxxxxVUVSRPPONMLKJIHHFECCBB-5′
Transcription
vuvsrpponnlkjihhfeccbb-3′
Formation of RNA secondary structure
RNA Construct
5′ -ABCDEFGHIJKLMNOPQRSTUV
vuvsrpponnlkjihhfeccbb-3′
RNA Construct
KEY
Upper case letters = sense to target sequence
Lower case letters = antisense complement base
Underline = WCRW sequences
Bold+Italic = SCRW sequences
Bold + underline = WCRW sequences
Loop sequences represented by "X" and "x"
Stem mismatch is denoted by "*"

FIG. 1B

Divergent Design

DNA Construct

5′-vuvsrpponnlkjihhfeccbb**XXXXXXXXXXXABCDEFGHIJKLMNOPQRSTUV**-3′ (sense strand)
3′-VUVSRPPONNLKJIHHFECCBB**xxxxxxxxxxxabcdefghijklmnopqrstuv**-5′ (antisense strand)

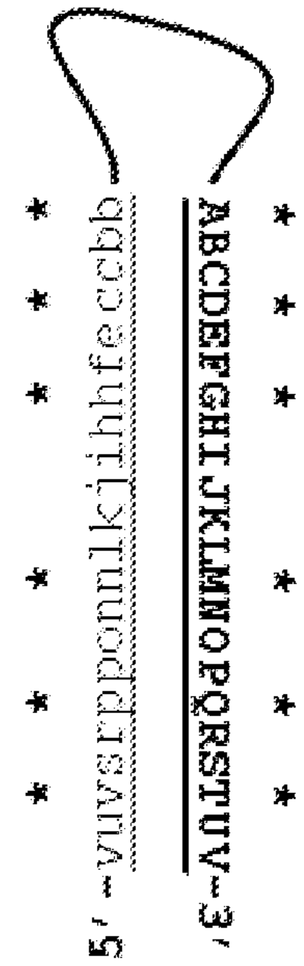
Transcription

RNA Construct

5′-vuvsrpponnlkjihhfeccbb **ABCDEFGHIJKLMNOPQRSTUV**-3′

Formation of RNA secondary structure

RNA Construct



KEY

Upper case letters = sense to target sequence
Lower case letters = antisense complement base
Underline = WCRW sequences
Bold+Italic = SCRW sequences
Bold + underline = NCRW sequences
Loop sequnces represented by "X" and "x"
Stem mismatch is denoted by "*"

FIG. 2A

CA 02961733 2017-03-17

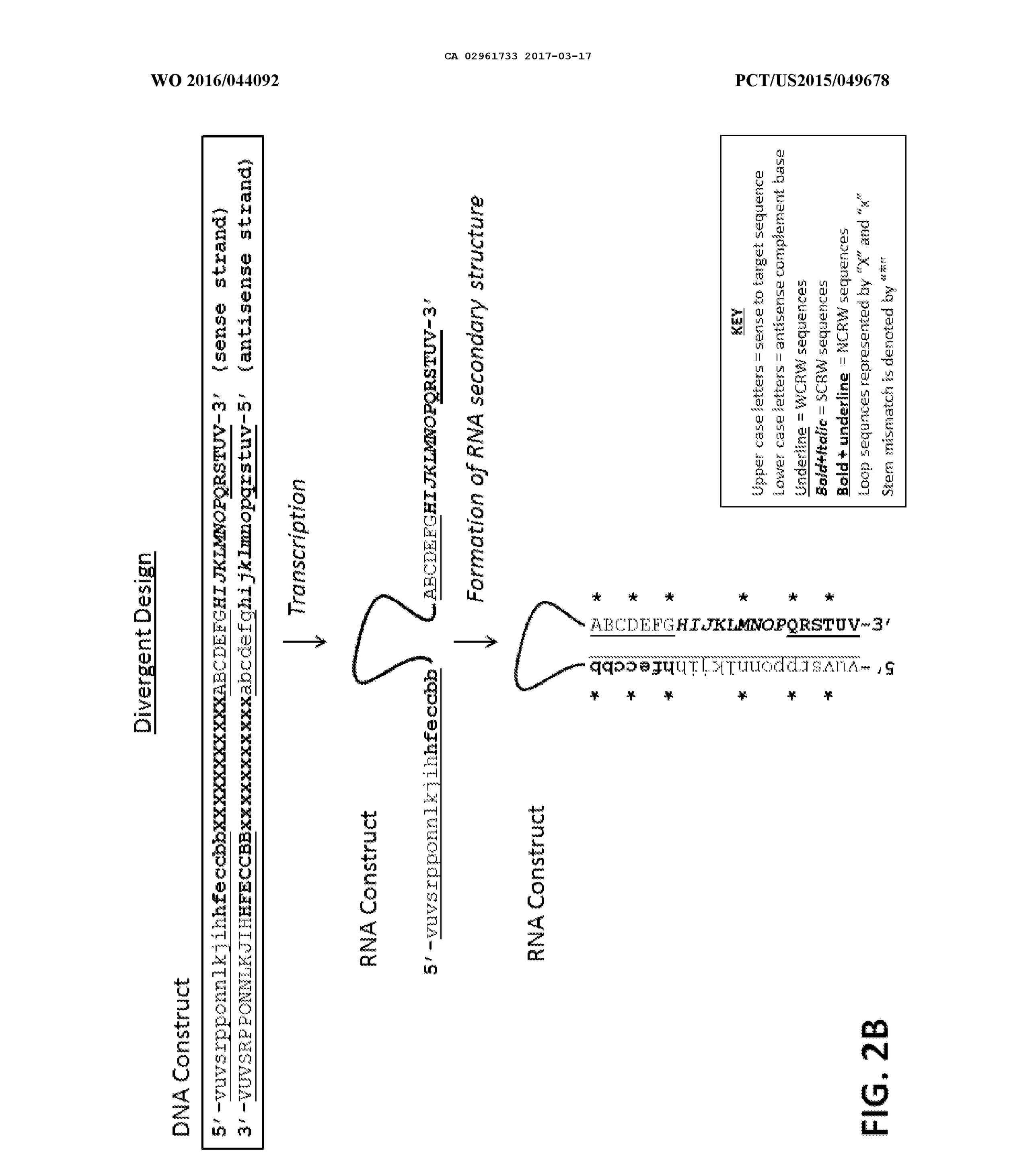

Divergent Design
DNA Construct
5′ –vuvsrppomlkjihhfeccbbXXXXXXXXXXABCDEFGHIJKLMNOPQRSTUV–3′
3′ –VUVSRPPOMMLKJIHHFECCBBxxxxxxxxxxabcdefghijklmnopqrstuv–5′
Transcription
RNA Construct
5′ –vuvsrppomlkjihhfeccbb
ABCDEFGHIJKLMNOPQRSTUV–3′
Formation of RNA secondary structure
RNA Construct
ABCDEFGHIJKLMNOPQRSTUV–3′
5′ –vuvsrppomlkjihhfeccbb
KEY
Upper case letters = sense to target sequence
Lower case letters = antisense complement base
Underline = WCRW sequences
Bold+Italic = SCRW sequences
Bold + underline = WCRW sequences
Loop sequences represented by "X" and "x"
Stem mismatch is denoted by "*"

FIG. 2B

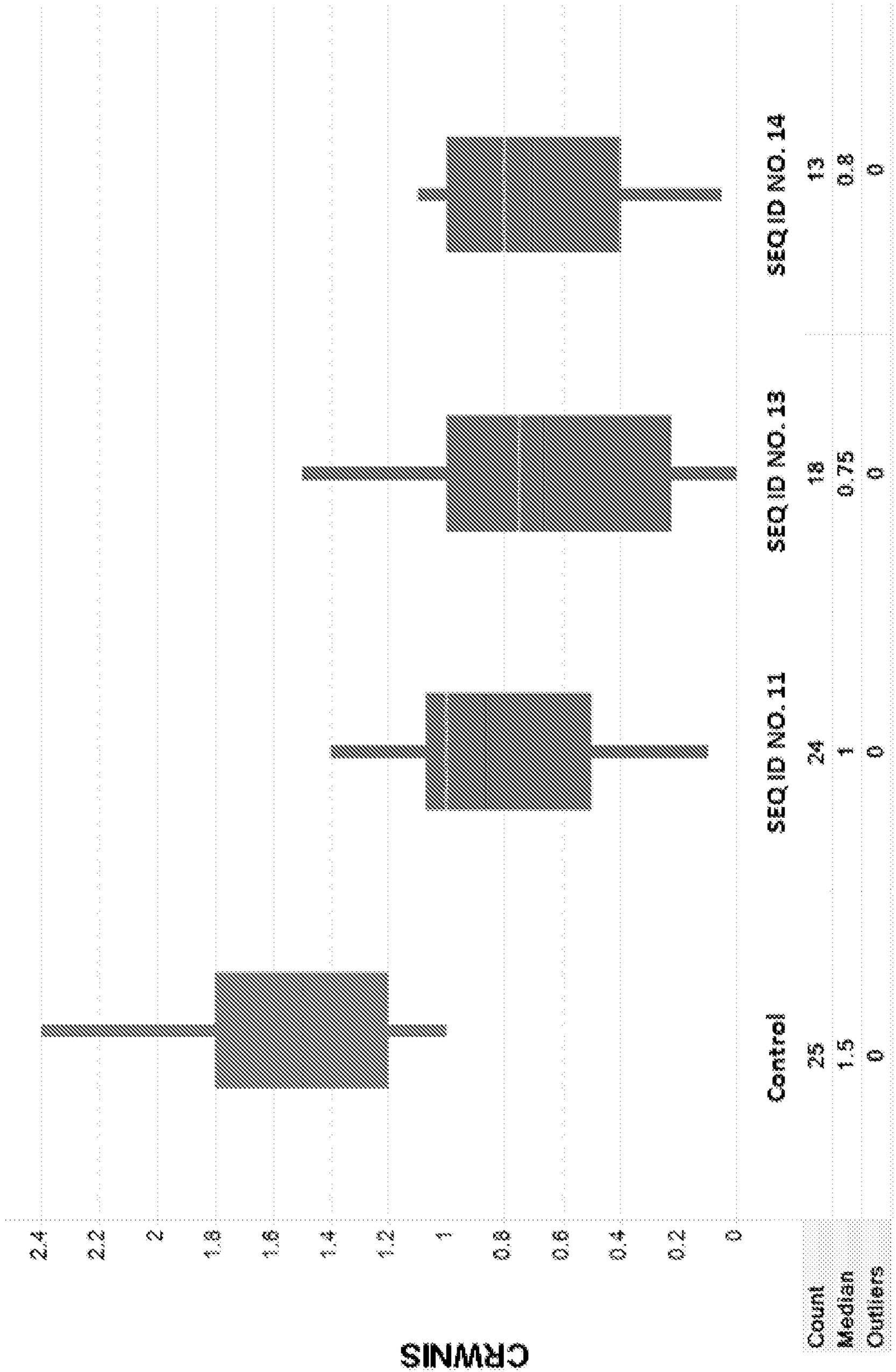

CRWNIS
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Count
Median
Outliers
Control
25
1.5
0
SEQ ID NO. 11
24
1
0
SEQ ID NO. 13
18
0.75
0
SEQ ID NO. 14
13
0.2
0

FIG. 3A

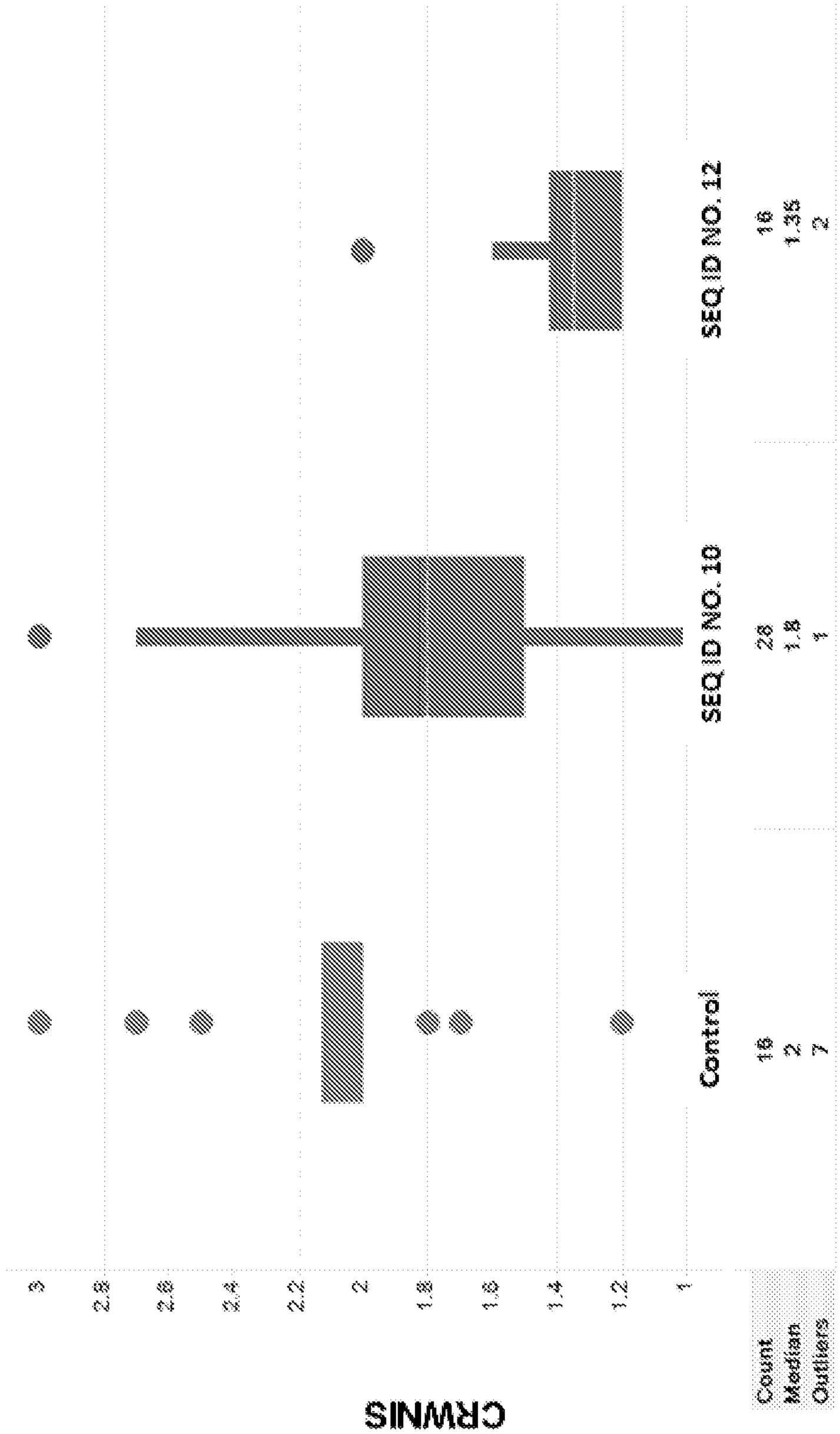

CRWNIS
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
Count
Median
Outliers
16
2
7
Control
SEQ ID NO. 10
26
1.8
1
SEQ ID NO. 12
16
1.35
2

FIG. 3B

Convergent Design

DNA Construct

5′-ABCDEFGHIJKLMNOPQRSTUV**XXXXXXXXXXX**vuvsrpponnlkjihhfeccbb-3′ (sense strand)
3′-abcdefghijklmnopqrstuv**xxxxxxxxxxx**VUVSRPPONNLKJIHHFECCBB-5′ (antisense strand)

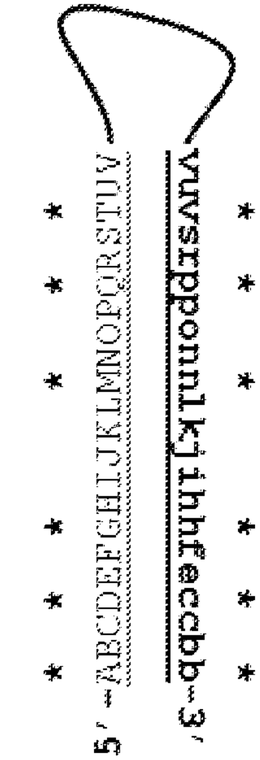
↓ *Transcription*

RNA Construct

5′-ABCDEFGHIJKLMNOPQRSTUV **vuvsrpponnlkjihhfeccbb**-3′

↓ *Formation of RNA secondary structure*

RNA Construct



KEY

Upper case letters = sense to target sequence
Lower case letters = antisense complement base
Underline = WCRW sequences
Bold+Italic = SCRW sequences
Bold + underline = NCRW sequences
Loop sequnces represented by "X" and "x"
Stem mismatch is denoted by "*"

FIG. 1A