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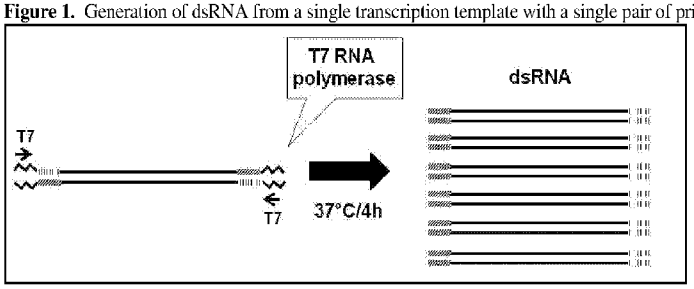
(54) Title: COPI COATOMER DELTA SUBUNIT NUCLEIC ACID MOLECULES THAT CONFER RESISTANCE TO COLEOPTERAN AND HEMIPTERAN PESTS

Figure 1. Generation of dsRNA from a single transcription template with a single pair of primers



(57) Abstract: This disclosure concerns nucleic acid molecules and methods of use thereof for control of insect pests through RNA interference-mediated inhibition of target coding and transcribed non-coding sequences in insect pests, including coleopteran and/or hemipteran pests. The disclosure also concerns methods for making transgenic plants that express nucleic acid molecules useful for the control of insect pests, and the plant cells and plants obtained thereby.

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COPI COATOMER DELTA SUBUNIT NUCLEIC ACID MOLECULES THAT CONFER RESISTANCE TO COLEOPTERAN AND HEMIPTERAN PESTS

PRIORITY CLAIMS

[0001] This application claims the benefit of the filing date of United States Provisional Patent Application Serial No. 62/063216, filed October 13, 2014, for "COPI Coatomer Delta Subunit Nucleic Acid Molecules that Confer Resistance to Coleopteran and Hemipteran Pests."

FIELD OF THE DISCLOSURE

[0002] The present invention relates generally to genetic control of plant damage caused by insect pests (*e.g.*, coleopteran pests and hemipteran pests). In particular embodiments, the present invention relates to identification of target coding and non-coding polynucleotides, and the use of recombinant DNA technologies for post-transcriptionally repressing or inhibiting expression of target coding and non-coding polynucleotides in the cells of an insect pest to provide a plant protective effect.

BACKGROUND

[0003] The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, is one of the most devastating corn rootworm species in North America and is a particular concern in corn-growing areas of the Midwestern United States. The northern corn rootworm (NCR), *Diabrotica barberi* Smith and Lawrence, is a closely-related species that co-inhabits much of the same range as WCR. There are several other related subspecies of *Diabrotica* that are significant pests in the Americas: the Mexican corn rootworm (MCR), *D. virgifera zeae* Krysan and Smith; the southern corn rootworm (SCR), *D. undecimpunctata howardi* Barber; *D. balteata* LeConte; *D. undecimpunctata tenella*; *D. speciosa* Germar; and *D. u. undecimpunctata* Mannerheim. The United States Department of Agriculture estimates that corn rootworms cause $1 billion in lost revenue each year, including $800 million in yield loss and $200 million in treatment costs.

[0004] Both WCR and NCR are deposited in the soil as eggs during the summer. The insects remain in the egg stage throughout the winter. The eggs are oblong, white, and less than 0.004 inches in length. The larvae hatch in late May or early June, with the precise timing of egg hatching varying from year to year due to temperature differences and location. The newly hatched larvae are white worms that are less than 0.125 inches in length. Once hatched, the larvae begin to feed on corn roots. Corn rootworms go through three larval instars. After feeding for several weeks, the larvae molt into the pupal stage. They pupate in the soil, and then they emerge from the soil as adults in July and August. Adult rootworms are about 0.25 inches in length.

[0005] Corn rootworm larvae complete development on corn and several other species of grasses. Larvae reared on yellow foxtail emerge later and have a smaller head capsule size as adults than larvae reared on corn (Ellsbury *et al.* (2005) Environ. Entomol. 34:627-634). WCR adults feed on corn silk, pollen, and kernels on exposed ear tips. If WCR adults emerge before corn reproductive tissues are present, they may feed on leaf tissue, thereby slowing plant growth and occasionally killing the host plant. However, the adults will quickly shift to preferred silks and pollen when they become available. NCR adults also feed on reproductive tissues of the corn plant, but in contrast rarely feed on corn leaves.

[0006] Most of the rootworm damage in corn is caused by larval feeding. Newly hatched rootworms initially feed on fine corn root hairs and burrow into root tips. As the larvae grow larger, they feed on and burrow into primary roots. When corn rootworms are abundant, larval feeding often results in the pruning of roots all the way to the base of the corn stalk. Severe root injury interferes with the roots' ability to transport water and nutrients into the plant, reduces plant growth, and results in reduced grain production, thereby often drastically reducing overall yield. Severe root injury also often results in lodging of corn plants, which makes harvest more difficult and further decreases yield. Furthermore, feeding by adults on the corn reproductive tissues can result in pruning of silks at the ear tip. If this "silk clipping" is severe enough during pollen shed, pollination may be disrupted.

[0007] Control of corn rootworms may be attempted by crop rotation, chemical insecticides, biopesticides (*e.g.*, the spore-forming gram-positive bacterium, *Bacillus thuringiensis*

(*Bt*)), transgenic plants that express *Bt* toxins, or a combination thereof. Crop rotation suffers from the significant disadvantage of placing unwanted restrictions upon the use of farmland. Moreover, oviposition of some rootworm species may occur in crop fields other than corn or extended diapauses results in egg hatching over multiple years, thereby mitigating the effectiveness of crop rotation practiced with corn and soybean.

[0008] Chemical insecticides are the most heavily relied upon strategy for achieving corn rootworm control. Chemical insecticide use, though, is an imperfect corn rootworm control strategy; over $1 billion may be lost in the United States each year due to corn rootworm when the costs of the chemical insecticides are added to the costs of the rootworm damage that may occur despite the use of the insecticides. High populations of larvae, heavy rains, and improper application of the insecticide(s) may all result in inadequate corn rootworm control. Furthermore, the continual use of insecticides may select for insecticide-resistant rootworm strains, as well as raise significant environmental concerns due to the toxicity of many of them to non-target species.

[0009] Stink bugs and other hemipteran insects (heteroptera) comprise another important agricultural pest complex. Worldwide over 50 closely related species of stink bugs are known to cause crop damage. McPherson & McPherson (2000) Stink bugs of economic importance in America north of Mexico, CRC Press. These insects are present in a large number of important crops including maize, soybean, fruit, vegetables, and cereals.

[0010] Stink bugs go through multiple nymph stages before reaching the adult stage. The time to develop from eggs to adults is about 30-40 days. Both nymphs and adults feed on sap from soft tissues into which they also inject digestive enzymes causing extra-oral tissue digestion and necrosis. Digested plant material and nutrients are then ingested. Depletion of water and nutrients from the plant vascular system results in plant tissue damage. Damage to developing grain and seeds is the most significant as yield and germination are significantly reduced. Multiple generations occur in warm climates resulting in significant insect pressure. Current management of stink bugs relies on insecticide treatment on an individual field basis. Therefore, alternative management strategies are urgently needed to minimize ongoing crop losses.

[0011] RNA interference (RNAi) is a process utilizing endogenous cellular pathways, whereby an interfering RNA (iRNA) molecule (*e.g.*, a double-stranded RNA (dsRNA) molecule) that is specific for all, or any portion of adequate size, of a target gene sequence results in the degradation of the mRNA encoded thereby. In recent years, RNAi has been used to perform gene "knockdown" in a number of species and experimental systems; for example, *Caenorhabditis elegans*, plants, insect embryos, and cells in tissue culture. *See*, *e.g.*, Fire *et al.* (1998) Nature 391:806-811; Martinez *et al.* (2002) Cell 110:563-574; McManus and Sharp (2002) Nature Rev. Genetics 3:737-747.

[0012] RNAi accomplishes degradation of mRNA through an endogenous pathway including the DICER protein complex. DICER cleaves long dsRNA molecules into short fragments of approximately 20 nucleotides, termed small interfering RNA (siRNA). The siRNA is unwound into two single-stranded RNAs: the passenger strand and the guide strand. The passenger strand is degraded, and the guide strand is incorporated into the RNA-induced silencing complex (RISC).

[0013] U.S. Patent 7,612,194 and U.S. Patent Publication Nos. 2007/0050860, 2010/0192265, and 2011/0154545 disclose a library of 9112 expressed sequence tag (EST) sequences isolated from *D. v. virgifera* LeConte pupae. It is suggested in U.S. Patent 7,612,194 and U.S. Patent Publication No. 2007/0050860 to operably link to a promoter a nucleic acid molecule that is complementary to one of several particular partial sequences of *D. v. virgifera* vacuolar-type H^+-ATPase (V-ATPase) disclosed therein for the expression of anti-sense RNA in plant cells. U.S. Patent Publication No. 2010/0192265 suggests operably linking a promoter to a nucleic acid molecule that is complementary to a particular partial sequence of a *D. v. virgifera* gene of unknown and undisclosed function (the partial sequence is stated to be 58% identical to C56C10.3 gene product in *C. elegans*) for the expression of anti-sense RNA in plant cells. U.S. Patent Publication No. 2011/0154545 suggests operably linking a promoter to a nucleic acid molecule that is complementary to two particular partial sequences of *D. v. virgifera* coatomer subunit genes for the expression of anti-sense RNA in plant cells. Further, U.S. Patent 7,943,819 discloses a library of 906 expressed sequence tag (EST) sequences isolated from *D. v. virgifera* LeConte larvae, pupae, and dissected midguts, and suggests operably linking a promoter to a nucleic acid molecule that is

complementary to a particular partial sequence of a *D. v. virgifera* charged multivesicular body protein 4b gene for the expression of double-stranded RNA in plant cells.

[0014] No further suggestion is provided in U.S. Patent 7,612,194, and U.S. Patent Publication Nos. 2007/0050860, 2010/0192265, and 2011/0154545 to use any particular sequence of the more than nine thousand sequences listed therein for RNA interference, other than the several particular partial sequences of V-ATPase and the particular partial sequences of genes of unknown function. Furthermore, none of U.S. Patent 7,612,194, and U.S. Patent Publication Nos. 2007/0050860 and 2010/0192265, and 2011/0154545 provides any guidance as to which other of the over nine thousand sequences provided would be lethal, or even otherwise useful, in species of corn rootworm when used as dsRNA or siRNA. U.S. Patent 7,943,819 provides no suggestion to use any particular sequence of the more than nine hundred sequences listed therein for RNA interference, other than the particular partial sequence of a charged multivesicular body protein 4b gene. Furthermore, U.S. Patent 7,943,819 provides no guidance as to which other of the over nine hundred sequences provided would be lethal, or even otherwise useful, in species of corn rootworm when used as dsRNA or siRNA. U.S. Patent Application Publication No. U.S. 2013/040173 and PCT Application Publication No. WO 2013/169923 describe the use of a sequence derived from a *Diabrotica virgifera* Snf7 gene for RNA interference in maize. (Also disclosed in Bolognesi *et al.* (2012) PLOS ONE 7(10): e47534. doi:10.1371/journal.pone.0047534).

[0015] The overwhelming majority of sequences complementary to corn rootworm DNAs (such as the foregoing) do not provide a plant protective effect from species of corn rootworm when used as dsRNA or siRNA. For example, Baum *et al.* (2007) Nature Biotechnology 25:1322-1326, describes the effects of inhibiting several WCR gene targets by RNAi. These authors reported that 8 of the 26 target genes they tested were not able to provide experimentally significant coleopteran pest mortality at a very high iRNA (*e.g.*, dsRNA) concentration of more than 520 ng/cm^2.

[0016] The authors of U.S. Patent 7,612,194 and U.S. Patent Publication No. 2007/0050860 made the first report of *in planta* RNAi in corn plants targeting the western corn rootworm. Baum *et al.* (2007) Nat. Biotechnol. 25(11):1322-6. These authors describe a high-throughput *in vivo* dietary RNAi system to screen potential target genes for developing transgenic

RNAi maize. Of an initial gene pool of 290 targets, only 14 exhibited larval control potential. One of the most effective double-stranded RNAs (dsRNA) targeted a gene encoding vacuolar ATPase subunit A (V-ATPase), resulting in a rapid suppression of corresponding endogenous mRNA and triggering a specific RNAi response with low concentrations of dsRNA. Thus, these authors documented for the first time the potential for *in planta* RNAi as a possible pest management tool, while simultaneously demonstrating that effective targets could not be accurately identified *a priori*, even from a relatively small set of candidate genes.

SUMMARY OF THE DISCLOSURE

[0017] Disclosed herein are nucleic acid molecules (*e.g.*, target genes, DNAs, dsRNAs, siRNAs, miRNAs, shRNAs, and hpRNAs), and methods of use thereof, for the control of insect pests, including, for example, coleopteran pests, such as *D. v. virgifera* LeConte (western corn rootworm, "WCR"); *D. barberi* Smith and Lawrence (northern corn rootworm, "NCR"); *D. u. howardi* Barber (southern corn rootworm, "SCR"); *D. v. zeae* Krysan and Smith (Mexican corn rootworm, "MCR"); *D. balteata* LeConte; *D. u. tenella*; *D. speciosa* Germar; *D. u. undecimpunctata* Mannerheim, and hemipteran pests, such as *Euschistus heros* (Fabr.) (Neotropical Brown Stink Bug, "BSB"); *E. servus* (Say) (Brown Stink Bug); *Nezara viridula* (L.) (Southern Green Stink Bug); *Piezodorus guildinii* (Westwood) (Red-banded Stink Bug); *Halyomorpha halys* (Stål) (Brown Marmorated Stink Bug); *Chinavia hilare* (Say) (Green Stink Bug); *C. marginatum* (Palisot de Beauvois); *Dichelops melacanthus* (Dallas); *D. furcatus* (F.); *Edessa meditabunda* (F.); *Thyanta perditor* (F.) (Neotropical Red Shouldered Stink Bug); *Horcias nobilellus* (Berg) (Cotton Bug); *Taedia stigmosa* (Berg); *Dysdercus peruvianus* (Guérin-Méneville); *Neomegalotomus parvus* (Westwood); *Leptoglossus zonatus* (Dallas); *Niesthrea sidae* (F.); *Lygus hesperus* (Knight) (Western Tarnished Plant Bug); and *L. lineolaris* (Palisot de Beauvois). In particular examples, exemplary nucleic acid molecules are disclosed that may be homologous to at least a portion of one or more native nucleic acids in an insect pest.

[0018] In these and further examples, the native nucleic acid may be a target gene, the product of which may be, for example and without limitation: involved in a metabolic process or

involved in larval/ nymph development. In some examples, post-translational inhibition of the expression of a target gene by a nucleic acid molecule comprising a polynucleotide homologous thereto may be lethal in coleopteran and/or hemipteran pests, or result in reduced growth and/or development thereof. In specific examples, a gene consisting of the coat protein complex delta subuit (referred to herein as *COPI delta* subunit and COPI DELTA) may be selected as a target gene for post-transcriptional silencing. In particular examples, a target gene useful for post-transcriptional inhibition is the novel gene referred to herein as COPI DELTA. An isolated nucleic acid molecule comprising a nucleotide sequence of *COPI delta* (SEQ ID NO:1 and SEQ ID NO:71); the complement of *COPI delta* (SEQ ID NO:1 and SEQ ID NO:71); and fragments of any of the foregoing is therefore disclosed herein.

[0019] Also disclosed are nucleic acid molecules comprising a polynucleotide that encodes a polypeptide that is at least about 85% identical to an amino acid sequence within a target gene product (for example, the product of a gene referred to as COPI DELTA). For example, a nucleic acid molecule may comprise a polynucleotide encoding a polypeptide that is at least 85% identical to SEQ ID NO:2 or SEQ ID NO:72 (COPI DELTA protein). In particular examples, a nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide that is at least 85% identical to an amino acid sequence within a product of COPI DELTA. Further disclosed are nucleic acid molecules comprising a polynucleotide that is the reverse complement of a polynucleotide that encodes a polypeptide at least 85% identical to an amino acid sequence within a target gene product.

[0020] Also disclosed are cDNA polynucleotides that may be used for the production of iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecules that are complementary to all or part of a coleopteran and/or hemipteran pest target gene, for example: *COPI delta*. In particular embodiments, dsRNAs, siRNAs, miRNAs, shRNAs, and/or hpRNAs may be produced *in vitro*, or *in vivo* by a genetically-modified organism, such as a plant or bacterium. In particular examples, cDNA molecules are disclosed that may be used to produce iRNA molecules that are complementary to all or part of *COPI delta* (SEQ ID NO:1 and SEQ ID NO:71).

[0021] Further disclosed are means for inhibiting expression of an essential gene in a coleopteran and/or hemipteran pest, and means for providing coleopteran and/or hemipteran pest resistance to a plant. A means for inhibiting expression of an essential gene in a coleopteran and/or hemipteran pest is a single- or double-stranded RNA molecule consisting of at least one of SEQ ID NO:3 (*Diabrotica COPI delta* region 1, herein sometimes referred to as *COPI delta* reg1)or SEQ ID NO:4 (*Diabrotica COPI delta* version 1, herein sometimes referred to as *COPI delta* v1), or SEQ ID NO:73 (*Euschistus heros COPI delta* region 1, herein sometimes referred to as BSB_*COPI delta*-1), or the complement thereof. Functional equivalents of means for inhibiting expression of an essential gene in a coleopteran and/or hemipteran pest include single- or double-stranded RNA molecules that are substantially homologous to all or part of a WCR or BSB gene comprising SEQ ID NO:1 or SEQ ID NO:71. A means for providing coleopteran and/or hemipteran pest resistance to a plant is a DNA molecule comprising a nucleic acid sequence encoding a means for inhibiting expression of an essential gene in a coleopteran and/or hemipteran pest operably linked to a promoter, wherein the DNA molecule is capable of being integrated into the genome of a maize or soybean plant.

[0022] Disclosed are methods for controlling a population of an insect pest (*e.g.*, a coleopteran or hemipteran pest), comprising providing to an insect pest (*e.g.*, a coleopteran or hemipteran pest) an iRNA (*e.g.*, dsRNA, siRNA, shRNA, miRNA, and hpRNA) molecule that functions upon being taken up by the pest to inhibit a biological function within the pest, wherein the iRNA molecule comprises all or part of a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73; the complement of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73; a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) or hemipteran organism (*e.g.* BSB) comprising all or part of any of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73; the complement of a native coding sequence of a *Diabrotica* organism or hemipteran organism comprising all or part of any of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73; a native non-coding sequence of a *Diabrotica* organism or hemipteran organism that is transcribed into a native RNA molecule comprising all or part of any

of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73; and the complement of a native non-coding sequence of a *Diabrotica* organism or hemipteran organism that is transcribed into a native RNA molecule comprising all or part of any of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71 and SEQ ID NO:73.

[0023] Also disclosed herein are methods wherein dsRNAs, siRNAs, miRNAs, shRNAs and/or hpRNAs may be provided to a coleopteran and/or hemipteran pest in a diet-based assay, or in genetically-modified plant cells expressing the dsRNAs, siRNAs, miRNAs, shRNAs and/or hpRNAs. In these and further examples, the dsRNAs, siRNAs, miRNAs, shRNAs and/or hpRNAs may be ingested by coleopteran larvae and/or hemipteran pest nymph. Ingestion of dsRNAs, siRNA, miRNAs, shRNAs and/or hpRNAs of the invention may then result in RNAi in the larvae/nymph, which in turn may result in silencing of a gene essential for viability of the coleopteran and/or hemipteran pest and leading ultimately to larval/nymph mortality. Thus, methods are disclosed wherein nucleic acid molecules comprising exemplary nucleic acid sequence(s) useful for control of coleopteran and/or hemipteran pests are provided to a coleopteran and/or hemipteran pest. In particular examples, the coleopteran and/or hemipteran pest controlled by use of nucleic acid molecules of the invention may be WCR, NCR, SCR, MCR, *D. balteata, D. u. tenella, D. speciosa, D. u. undecimpunctata, Euschistus heros, E. servus, Piezodorus guildinii, Halyomorpha halys, Nezara viridula, Chinavia hilare, C. marginatum, Dichelops melacanthus, D. furcatus, Edessa meditabunda, Thyanta perditor, Horcias nobilellus, Taedia stigmosa, Dysdercus peruvianus, Neomegalotomus parvus, Leptoglossus zonatus, Niesthrea sidae,* and/or *Lygus lineolaris*.

[0024] The foregoing and other features will become more apparent from the following Detailed Description of several embodiments, which proceeds with reference to the accompanying Figures.

BRIEF DESCRIPTION OF THE FIGURES

[0025] **FIG. 1** includes a depiction of the strategy used to generate dsRNA from a single transcription template with a single pair of primers.

[0026] **FIG. 2** includes a depiction of the strategy used to generate dsRNA from two transcription templates.

SEQUENCE LISTING

[0027] The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. § 1.822. The nucleic acid and amino acid sequences listed define molecules (*i.e.*, polynucleotides and polypeptides, respectively) having the nucleotide and amino acid monomers arranged in the manner described. The nucleic acid and amino acid sequences listed also each define a genus of polynucleotides or polypeptides that comprise the nucleotide and amino acid monomers arranged in the manner described. In view of the redundancy of the genetic code, it will be understood that a nucleotide sequence including a coding sequence also describes the genus of polynucleotides encoding the same polypeptide as a polynucleotide consisting of the reference sequence. It will further be understood that an amino acid sequence describes the genus of polynucleotide ORFs encoding that polypeptide.

[0028] Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. As the complement and reverse complement of a primary nucleic acid sequence are necessarily disclosed by the primary sequence, the complementary sequence and reverse complementary sequence of a nucleic acid sequence are included by any reference to the nucleic acid sequence, unless it is explicitly stated to be otherwise (or it is clear to be otherwise from the context in which the sequence appears). Furthermore, as it is understood in the art that the nucleotide sequence of an RNA strand is determined by the sequence of the DNA from which it was transcribed (but for the substitution of uracil (U) nucleobases for thymine (T)), an RNA sequence is included by any reference to the DNA sequence encoding it. In the accompanying sequence listing:

[0029] SEQ ID NO:1 shows a DNA sequence comprising *COPI delta* subunit from *Diabrotica virgifera*.

[0030] SEQ ID NO:2 shows an amino acid sequence of a *COPI* Delta protein from *Diabrotica virgifera*.

[0031] SEQ ID NO:3 shows a DNA sequence of *COPI delta* reg1 (region 1) from *Diabrotica virgifera* that was used for *in vitro* dsRNA synthesis (T7 promoter sequences at 5' and 3' ends not shown).

[0032] SEQ ID NO:4 shows a DNA sequence of *COPI delta* v1 (version 1) from *Diabrotica virgifera* that was used for *in vitro* dsRNA synthesis (T7 promoter sequences at 5' and 3' ends not shown).

[0033] SEQ ID NO:5 shows a DNA sequence of a T7 phage promoter.

[0034] SEQ ID NO:6 shows a DNA sequence of a YFP coding region segment that was used for *in vitro* dsRNA synthesis (T7 promoter sequences at 5' and 3' ends not shown).

[0035] SEQ ID NOs:7 to 10 show primers used to amplify portions of a *COPI delta* subunit sequence from *Diabrotica virgifera* comprising *COPI delta* reg1 and *COPI delta* reg2.

[0036] SEQ ID NO:11 presents a *COPI delta* hairpin v1-RNA-forming sequence from *Diabrotica virgifera* as found in pDAB117220. Upper case bases are *COPI delta* sense strand, underlined lower case bases comprise an ST-LS1 intron, non-underlined lower case bases are COPI delta antisense strand.

AATAGGTCGTGATGGTGGCGTACAACAATTCGAATTATTGGGACTTGCTACTTTACAC
ATTGGAGATGAGAGATGGGGTAGGATACGTGTGCAATTGGAAgactagtaccggttgggaaaggtat
gtttctgcttctacctttgatatatatataataattatcactaattagtagtaatatagtatttcaagtatttttttcaaaataaaagaatgtagtatatagctatt
gcttttctgtagtttataagtgtgtatattttaatttataacttttctaatatatgaccaaaacatggtgatgtgcaggttgatccgcggttattccaattgca
cacgtatcctaccccatctctcatctccaatgtgtaaagtagcaagtcccaataattcgaattgttgtacgccaccatcacgacctatt

[0037] SEQ ID NO:12 shows a YFP hairpin-RNA-forming sequence v2 as found in pDAB110853. Upper case bases are YFP sense strand, underlined bases comprise an ST-LS1 intron, lower case, non-underlined bases are YFP antisense strand.

ATGTCATCTGGAGCACTTCTCTTTCATGGGAAGATTCCTTACGTTGTGGAGATGGAAG
GGAATGTTGATGGCCACACCTTTAGCATACGTGGGAAAGGCTACGGAGATGCCTCAG
TGGGAAAGgactagtaccggttgggaaaggtatgtttctgcttctacctttgatatatatataataattatcactaattagtagtaatatagtattt
caagtatttttttcaaaataaaagaatgtagtatatagctattgcttttctgtagtttataagtgtgtatattttaatttataacttttctaatatatgaccaaaa
catggtgatgtgcaggttgatccgcggttactttcccactgaggcatctccgtagcctttcccacgtatgctaaaggtgtggccatcaacattccctt
ccatctccacaacgtaaggaatcttcccatgaaagagaagtgctccagatgacat

[0038] SEQ ID NO:13 shows a sequence comprising an ST-LS1 intron.

[0039] SEQ ID NO:14 shows a YFP protein coding sequence as found in pDAB101556.

[0040] SEQ ID NO:15 shows a DNA sequence of Annexin region 1.

[0041] SEQ ID NO:16 shows a DNA sequence of Annexin region 2.

[0042] SEQ ID NO:17 shows a DNA sequence of Beta spectrin 2 region 1.

[0043] SEQ ID NO:18 shows a DNA sequence of Beta spectrin 2 region 2.

[0044] SEQ ID NO:19 shows a DNA sequence of mtRP-L4 region 1.

[0045] SEQ ID NO:20 shows a DNA sequence of mtRP-L4 region 2.

[0046] SEQ ID NOs:21 to 48 show primers used to amplify gene regions of YFP, Annexin, Beta spectrin 2, and mtRP-L4 for dsRNA synthesis.

[0047] SEQ ID NO:49 shows a maize DNA sequence encoding a TIP41-like protein.

[0048] SEQ ID NO:50 shows a DNA sequence of oligonucleotide T20NV.

[0049] SEQ ID NOs:51 to 55 show sequences of primers and probes used to measure maize transcript levels.

[0050] SEQ ID NO:56 shows a DNA sequence of a portion of a SpecR coding region used for binary vector backbone detection.

[0051] SEQ ID NO:57 shows a DNA sequence of a portion of an AAD1 coding region used for genomic copy number analysis.

[0052] SEQ ID NO:58 shows a DNA sequence of a maize invertase gene.

[0053] SEQ ID NOs:59 to 67 show sequences of primers and probes used for gene copy number analyses.

[0054] SEQ ID NOs:68 to 70 show sequences of primers and probes used for maize expression analysis.

[0055] SEQ ID NO:71 shows an exemplary DNA sequence of BSB *COPI delta* transcript from a Neotropical Brown Stink Bug (*Euschistus heros*).

[0056] SEQ ID NO:72 shows an amino acid sequence of a from *Euschistus heros* COPI DELTA protein.

[0057] SEQ ID NO:73 shows a DNA sequence of BSB_*COPI delta*-1 from *Euschistus heros* that was used for *in vitro* dsRNA synthesis (T7 promoter sequences at 5' and 3' ends not shown).

[0058] SEQ ID NO:74-75 show primers used to amplify portions of a from *Euschistus heros COPI delta* sequence comprising BSB_*COPI delta*-1.

[0059] SEQ ID NO:76 is the sense strand of YFP-targeted dsRNA: YFPv2.

[0060] SEQ ID NO:77-78 show primers used to amplify portions of a YFP-targeted dsRNA: YFPv2.

DETAILED DESCRIPTION

I. Overview of several embodiments

[0061] Disclosed herein are methods and compositions for genetic control of insect (*e.g.*, coleopteran and/or hemipteran) pest infestations. Methods for identifying one or more gene(s) essential to the lifecycle of an insect pest for use as a target gene for RNAi-mediated control of an insect pest population are also provided. DNA plasmid vectors encoding an RNA molecule may be designed to suppress one or more target gene(s) essential for growth, survival, and/or development. In some embodiments, the RNA molecule may be capable of forming dsRNA molecules. In some embodiments, methods are provided for post-transcriptional repression of expression or inhibition of a target gene *via* nucleic acid molecules that are complementary to a coding or non-coding sequence of the target gene in an insect pest. In these and further embodiments, a pest may ingest one or more dsRNA, siRNA, shRNA, miRNA, and/or hpRNA molecules transcribed from all or a portion of a nucleic acid molecule that is complementary to a coding or non-coding sequence of a target gene, thereby providing a plant-protective effect.

[0062] Thus, some embodiments involve sequence-specific inhibition of expression of target gene products, using iRNA (*e.g.*, dsRNA, siRNA, shRNA, miRNA and/or hpRNA) that is complementary to coding and/or non-coding sequences of the target gene(s) to achieve at least partial control of an insect (*e.g.*, coleopteran and/or hemipteran) pest. Disclosed is a set of isolated and purified nucleic acid molecules comprising a polynucleotide, for example, as set forth in any of

SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:11, SEQ ID NO:71, SEQ ID NO:73, and fragments thereof. In some embodiments, a stabilized dsRNA molecule may be expressed from this sequence, fragments thereof, or a gene comprising one of these sequences, for the post-transcriptional silencing or inhibition of a target gene. In certain embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:1. In other embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:3. In yet other embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:4. In other embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:11. In still further embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:71. In other embodiments, isolated and purified nucleic acid molecules comprise all or part of SEQ ID NO:73.

[0063] Some embodiments involve a recombinant host cell (*e.g.*, a plant cell) having in its genome at least one recombinant DNA encoding at least one iRNA (*e.g.*, dsRNA) molecule(s). In particular embodiments, the dsRNA molecule(s) may be produced when ingested by a coleopteran and/or hemipteran pest to post-transcriptionally silence or inhibit the expression of a target gene in the pest. The recombinant DNA may comprise, for example, any of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71, or SEQ ID NO:73; fragments of any of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71, or SEQ ID NO:73; or a partial sequence of a gene comprising one or more of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:71, or SEQ ID NO:73; or complements thereof.

[0064] Some embodiments involve a recombinant host cell having in its genome a recombinant DNA encoding at least one iRNA (*e.g.*, dsRNA) molecule(s) comprising all or part of an RNA encoded by SEQ ID NO:1 and/or SEQ ID NO:71 and/or the complements thereof. When ingested by an insect (*e.g.*, coleopteran and/or hemipteran) pest, the iRNA molecule(s) may silence or inhibit the expression of a target gene comprising SEQ ID NO:1 and/or SEQ ID NO:71, in the coleopteran and/or hemipteran pest, and thereby result in cessation of growth, development, and/or feeding in the coleopteran and/or hemipteran pest.

[0065] In some embodiments, a recombinant host cell having in its genome at least one recombinant DNA encoding at least one RNA molecule capable of forming a dsRNA molecule may be a transformed plant cell. Some embodiments involve transgenic plants comprising such a transformed plant cell. In addition to such transgenic plants, progeny plants of any transgenic plant generation, transgenic seeds, and transgenic plant products, are all provided, each of which comprises recombinant DNA(s). In particular embodiments, an RNA molecule capable of forming a dsRNA molecule may be expressed in a transgenic plant cell. Therefore, in these and other embodiments, a dsRNA molecule may be isolated from a transgenic plant cell. In particular embodiments, the transgenic plant is a plant selected from the group comprising corn (*Zea mays*), soybean (*Glycine max*), and plants of the family *Poaceae*.

[0066] Some embodiments involve a method for modulating the expression of a target gene in an insect (*e.g.*, coleopteran and/or hemipteran) pest cell. In these and other embodiments, a nucleic acid molecule may be provided, wherein the nucleic acid molecule comprises a polynucleotide encoding an RNA molecule capable of forming a dsRNA molecule. In particular embodiments, a polynucleotide encoding an RNA molecule capable of forming a dsRNA molecule may be operatively linked to a promoter, and may also be operatively linked to a transcription termination sequence. In particular embodiments, a method for modulating the expression of a target gene in an insect pest cell may comprise: (a) transforming a plant cell with a vector comprising a polynucleotide encoding an RNA molecule capable of forming a dsRNA molecule; (b) culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture comprising a plurality of transformed plant cells; (c) selecting for a transformed plant cell that has integrated the vector into its genome; and (d) determining that the selected transformed plant cell comprises the RNA molecule capable of forming a dsRNA molecule encoded by the polynucleotide of the vector. A plant may be regenerated from a plant cell that has the vector integrated in its genome and comprises the dsRNA molecule encoded by the polynucleotide of the vector.

[0067] Thus, also disclosed is a transgenic plant comprising a vector having a polynucleotide encoding an RNA molecule capable of forming a dsRNA molecule integrated in its

genome, wherein the transgenic plant comprises the dsRNA molecule encoded by the polynucleotide of the vector. In particular embodiments, expression of an RNA molecule capable of forming a dsRNA molecule in the plant is sufficient to modulate the expression of a target gene in a cell of an insect (*e.g.*, coleopteran or hemipteran) pest that contacts the transformed plant or plant cell (for example, by feeding on the transformed plant, a part of the plant (*e.g.*, root) or plant cell), such that growth and/or survival of the pest is inhibited. Transgenic plants disclosed herein may display resistance and/or enhanced tolerance to insect pest infestations. Particular transgenic plants may display resistance and/or enhanced protection from one or more coleopteran and/or hemipteran pest(s) selected from the group consisting of: WCR; NCR; SCR; MCR; *D. balteata* LeConte; *D. u. tenella*; *D. speciosa* Germar; *D. u. undecimpunctata* Mannerheim; *Euschistus heros*; *Piezodorus guildinii*; *Halyomorpha halys*; *Nezara viridula*; *Chinavia hilare*; *Euschistus servus*; *Dichelops melacanthus*; *Dichelops furcatus*; *Edessa meditabunda*; *Thyanta perditor*; *Chinavia marginatum*; *Horcias nobilellus*; *Taedia stigmosa*; *Dysdercus peruvianus*; *Neomegalotomus parvus*; *Leptoglossus zonatus*; *Niesthrea sidae*; *Lygus hesperus*; and *Lygus lineolaris*.

[0068] Also disclosed herein are methods for delivery of control agents, such as an iRNA molecule, to an insect (*e.g.*, coleopteran and/or hemipteran) pest. Such control agents may cause, directly or indirectly, an impairment in the ability of an insect pest population to feed, grow or otherwise cause damage in a host. In some embodiments, a method is provided comprising delivery of a stabilized dsRNA molecule to an insect pest to suppress at least one target gene in the pest, thereby causing RNAi and reducing or eliminating plant damage in a pest host. In some embodiments, a method of inhibiting expression of a target gene in the insect pest may result in cessation of growth, survival, and/or development, in the pest.

[0069] In some embodiments, compositions (*e.g.*, a topical composition) are provided that comprise an iRNA (*e.g.*, dsRNA) molecule for use with plants, animals, and/or the environment of a plant or animal to achieve the elimination or reduction of an insect (*e.g.*, coleopteran and/or hemipteran) pest infestation. In particular embodiments, the composition may be a nutritional composition or food source to be fed to the insect pest. Some embodiments comprise making the nutritional composition or food source available to the pest. Ingestion of a composition comprising

iRNA molecules may result in the uptake of the molecules by one or more cells of the pest, which may in turn result in the inhibition of expression of at least one target gene in cell(s) of the pest. Ingestion of or damage to a plant or plant cell by an insect pest infestation may be limited or eliminated in or on any host tissue or environment in which the pest is present by providing one or more compositions comprising an iRNA molecule in the host of the pest.

[0070] The compositions and methods disclosed herein may be used together in combinations with other iRNA molecules directed to different targets (*e.g.*, RAS Opposite or ROP (U.S. Patent Application Publication No. 20150176025) and RNAPII (U.S. Patent Application Publication No. 20150176009). The potential to affect multiple target sequences in a pest, for example in larvae, may increase efficacy and also improve sustainable approaches to insect pest management involving iRNA technologies. The compositions and methods disclosed herein may also be used together in combinations with other methods and compositions for controlling damage by insect (*e.g.*, coleopteran and/or hemipteran) pests. For example, an iRNA molecule as described herein for protecting plants from insect pests may be used in a method comprising the additional use of one or more chemical agents effective against an insect pest, biopesticides effective against such a pest, crop rotation, recombinant genetic techniques that exhibit features different from the features of RNAi-mediated methods and RNAi compositions (*e.g.*, recombinant production of proteins in plants that are harmful to an insect pest (*e.g.*, *Bt* toxins)).

II. Abbreviations

[0071]	BSB	Neotropical brown stink bug (*Euschistus heros*)
[0072]	dsRNA	double-stranded ribonucleic acid
[0073]	EST	expressed sequence tag
[0074]	GI	growth inhibition
[0075]	NCBI	National Center for Biotechnology Information
[0076]	gDNA	genomic DNA
[0077]	iRNA	inhibitory ribonucleic acid
[0078]	ORF	open reading frame

[0079]	RNAi	ribonucleic acid interference
[0080]	miRNA	micro ribonucleic acid
[0081]	siRNA	small inhibitory ribonucleic acid
[0082]	shRNA	short hairpin ribonucleic acid
[0083]	hpRNA	hairpin ribonucleic acid
[0084]	UTR	untranslated region
[0085]	WCR	western corn rootworm (*Diabrotica virgifera virgifera* LeConte)
[0086]	NCR	northern corn rootworm (*Diabrotica barberi* Smith and Lawrence)
[0087]	MCR	Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith)
[0088]	PCR	Polymerase chain reaction
[0089]	qPCR	quantative polymerase chain reaction
[0090]	RISC	RNA-induced Silencing Complex
[0091]	SCR	southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber)
[0092]	SEM	standard error of the mean
[0093]	YFP	yellow fluorescent protein

III. Terms

[0094] In the description and tables which follow, a number of terms are used. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided:

[0095] Coleopteran pest: As used herein, the term "coleopteran pest" refers to insects of the order Coleoptera, including pest insects in the genus *Diabrotica*, which feed upon agricultural crops and crop products, including corn and other true grasses. In particular examples, a coleopteran pest is selected from a list comprising *D. v. virgifera* LeConte (WCR); *D. barberi* Smith

and Lawrence (NCR); *D. u. howardi* (SCR); *D. v. zeae* (MCR); *D. balteata* LeConte; *D. u. tenella*; *D. speciosa* Germar; and *D. u. undecimpunctata* Mannerheim.

[0096] Contact (with an organism): As used herein, the term "contact with" or "uptake by" an organism (*e.g.*, a coleopteran or hemipteran pest), with regard to a nucleic acid molecule, includes internalization of the nucleic acid molecule into the organism, for example and without limitation: ingestion of the molecule by the organism (*e.g.*, by feeding); contacting the organism with a composition comprising the nucleic acid molecule; and soaking of organisms with a solution comprising the nucleic acid molecule.

[0097] Contig: As used herein the term "contig" refers to a DNA sequence that is reconstructed from a set of overlapping DNA segments derived from a single genetic source.

[0098] Corn plant: As used herein, the term "corn plant" refers to a plant of the species, *Zea mays* (maize).

[0099] Expression: As used herein, "expression" of a coding polynucleotide (for example, a gene or a transgene) refers to the process by which the coded information of a nucleic acid transcriptional unit (including, *e.g.*, gDNA or cDNA) is converted into an operational, non-operational, or structural part of a cell, often including the synthesis of a protein. Gene expression can be influenced by external signals; for example, exposure of a cell, tissue, or organism to an agent that increases or decreases gene expression. Expression of a gene can also be regulated anywhere in the pathway from DNA to RNA to protein. Regulation of gene expression occurs, for example, through controls acting on transcription, translation, RNA transport and processing, degradation of intermediary molecules such as mRNA, or through activation, inactivation, compartmentalization, or degradation of specific protein molecules after they have been made, or by combinations thereof. Gene expression can be measured at the RNA level or the protein level by any method known in the art, including, without limitation, northern blot, RT-PCR, western blot, or *in vitro*, *in situ*, or *in vivo* protein activity assay(s).

[00100] Genetic material: As used herein, the term "genetic material" includes all genes, and nucleic acid molecules, such as DNA and RNA.

[00101] Hemipteran pest: As used herein, the term "hemipteran pest" refers to insects of the order Hemiptera, including, for example and without limitation, insects in the families Pentatomidae, Miridae, Pyrrhocoridae, Coreidae, Alydidae, and Rhopalidae, which feed on a wide range of host plants and have piercing and sucking mouth parts. In particular examples, a hemipteran pest is selected from the list comprising, *Euschistus heros* (Fabr.) (Neotropical Brown Stink Bug), *Nezara viridula* (L.) (Southern Green Stink Bug), *Piezodorus guildinii* (Westwood) (Red-banded Stink Bug), *Halyomorpha halys* (Stål) (Brown Marmorated Stink Bug), *Chinavia hilare* (Say) (Green Stink Bug), *Euschistus servus* (Say) (Brown Stink Bug), *Dichelops melacanthus* (Dallas), *Dichelops furcatus* (F.), *Edessa meditabunda* (F.), *Thyanta perditor* (F.) (Neotropical Red Shouldered Stink Bug), *Chinavia marginatum* (Palisot de Beauvois), *Horcias nobilellus* (Berg) (Cotton Bug), *Taedia stigmosa* (Berg), *Dysdercus peruvianus* (Guérin-Méneville), *Neomegalotomus parvus* (Westwood), *Leptoglossus zonatus* (Dallas), *Niesthrea sidae* (F.), *Lygus hesperus* (Knight) (Western Tarnished Plant Bug), and *Lygus lineolaris* (Palisot de Beauvois).

[00102] Inhibition: As used herein, the term "inhibition," when used to describe an effect on a coding polynucleotide (for example, a gene), refers to a measurable decrease in the cellular level of mRNA transcribed from the coding polynucleotide and/or peptide, polypeptide, or protein product of the coding polynucleotide. In some examples, expression of a coding polynucleotide may be inhibited such that expression is approximately eliminated. "Specific inhibition" refers to the inhibition of a target coding polynucleotide without consequently affecting expression of other coding polynucleotides (*e.g.*, genes) in the cell wherein the specific inhibition is being accomplished.

[00103] Insect: As used herein with regard to pests, the term "insect pest" specifically includes coleopteran insect pests and hemipteran insect pests. In some embodiments, the term also includes some other insect pests.

[00104] Isolated: An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs (*i.e.*, other chromosomal and extra-chromosomal DNA and RNA, and proteins), while effecting a chemical or

functional change in the component (*e.g.*, a nucleic acid may be isolated from a chromosome by breaking chemical bonds connecting the nucleic acid to the remaining DNA in the chromosome). Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically-synthesized nucleic acid molecules, proteins, and peptides.

[00105] Nucleic acid molecule: As used herein, the term "nucleic acid molecule" may refer to a polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, gDNA, and synthetic forms and mixed polymers of the above. A nucleotide or nucleobase may refer to a ribonucleotide, deoxyribonucleotide, or a modified form of either type of nucleotide. A "nucleic acid molecule" as used herein is synonymous with "nucleic acid" and "polynucleotide." A nucleic acid molecule is usually at least 10 bases in length, unless otherwise specified. By convention, the nucleotide sequence of a nucleic acid molecule is read from the 5' to the 3' end of the molecule. The "complement" of a nucleic acid molecule refers to a polynucleotide having nucleobases that may form base pairs with the nucleobases of the nucleic acid molecule (*i.e.*, A-T/U, and G-C).

[00106] Some embodiments include nucleic acids comprising a template DNA that is transcribed into an RNA molecule that is the complement of an mRNA molecule. In these embodiments, the complement of the nucleic acid transcribed into the mRNA molecule is present in the 5' to 3' orientation, such that RNA polymerase (which transcribes DNA in the 5' to 3' direction) will transcribe a nucleic acid from the complement that can hybridize to the mRNA molecule. Unless explicitly stated otherwise, or it is clear to be otherwise from the context, the term "complement" therefore refers to a polynucleotide having nucleobases, from 5' to 3', that may form base pairs with the nucleobases of a reference nucleic acid. Similarly, unless it is explicitly stated to be otherwise (or it is clear to be otherwise from the context), the "reverse complement" of a nucleic acid refers to the complement in reverse orientation. The foregoing is demonstrated in the following illustration:

5' ATGATGATG 3' polynucleotide

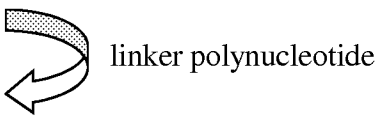
5' TACTACTAC 3' "complement" of the polynucleotide

5' CATCATCAT 3' "reverse complement" of the polynucleotide

[00107] Some embodiments of the invention include hairpin RNA-forming iRNA molecules. In these iRNAs, both the complement of a nucleic acid to be targeted by RNA interference and the reverse complement may be found in the same molecule, such that the single-stranded RNA molecule may "fold over" and hybridize to itself over region comprising the complementary and reverse complementary polynucleotides, as demonstrated in the following illustration:

5' AUGAUGAUG – linker polynucleotide – CAUCAUCAU 3',

which hybridizes to form:



[00108] "Nucleic acid molecules" include all polynucleotides, for example: single- and double-stranded forms of DNA; single-stranded forms of RNA; and double-stranded forms of RNA (dsRNA). The term "nucleotide sequence" or "nucleic acid sequence" refers to both the sense and antisense strands of a nucleic acid as either individual single strands or in the duplex. The term "ribonucleic acid" (RNA) is inclusive of iRNA (inhibitory RNA), dsRNA (double stranded RNA), siRNA (small interfering RNA), mRNA (messenger RNA), miRNA (micro-RNA), shRNA (small hairpin RNA), hpRNA (hairpin RNA), tRNA (transfer RNAs, whether charged or discharged with a corresponding acylated amino acid), and cRNA (complementary RNA). The term "deoxyribonucleic acid" (DNA) is inclusive of cDNA, gDNA, and DNA-RNA hybrids. The terms "polynucleotide," "nucleic acid," "segments" thereof, and "fragments" thereof will be understood by those in the art to include, for example, gDNAs; ribosomal RNAs; transfer RNAs; RNAs; messenger RNAs; operons; smaller engineered polynucleotides that encode or may be adapted to encode peptides, polypeptides, or proteins; and structural and/or functional elements within a nucleic acid molecule that are delineated by their corresponding nucleotide sequence.

[00109] Oligonucleotide: An oligonucleotide is a short nucleic acid polymer. Oligonucleotides may be formed by cleavage of longer nucleic acid segments, or by polymerizing

individual nucleotide precursors. Automated synthesizers allow the synthesis of oligonucleotides up to several hundred bases in length. Because oligonucleotides may bind to a complementary nucleic acid, they may be used as probes for detecting DNA or RNA. Oligonucleotides composed of DNA (oligodeoxyribonucleotides) may be used in PCR, a technique for the amplification of DNA and RNA (reverse transcribed into a cDNA) sequences. In PCR, the oligonucleotide is typically referred to as a "primer," which allows a DNA polymerase to extend the oligonucleotide and replicate the complementary strand.

[00110] A nucleic acid molecule may include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages. Nucleic acid molecules may be modified chemically or biochemically, or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications (*e.g.*, uncharged linkages: for example, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, *etc.*; charged linkages: for example, phosphorothioates, phosphorodithioates, *etc.;* pendent moieties: for example, peptides; intercalators: for example, acridine, psoralen, *etc.*; chelators; alkylators; and modified linkages: for example, alpha anomeric nucleic acids, *etc.*). The term "nucleic acid molecule" also includes any topological conformation, including single-stranded, double-stranded, partially duplexed, triplexed, hairpinned, circular, and padlocked conformations.

[00111] As used herein with respect to DNA, the term "coding sequence", "structural nucleotide sequence", or "structural nucleic acid molecule" refers to a nucleotide sequence that is ultimately translated into a polypeptide, *via* transcription and mRNA, when placed under the control of appropriate regulatory sequences. With respect to RNA, the term "coding sequence" refers to a nucleotide sequence that is translated into a peptide, polypeptide, or protein. The boundaries of a coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. Coding sequences include, but are not limited to: genomic DNA; cDNA; EST; and recombinant nucleotide sequences.

[00112] Genome: As used herein, the term "genome" refers to chromosomal DNA found within the nucleus of a cell, and also refers to organelle DNA found within subcellular components of the cell. In some embodiments of the invention, a DNA molecule may be introduced into a plant cell such that the DNA molecule is integrated into the genome of the plant cell. In these and further embodiments, the DNA molecule may be either integrated into the nuclear DNA of the plant cell, or integrated into the DNA of the chloroplast or mitochondrion of the plant cell. The term "genome" as it applies to bacteria refers to both the chromosome and plasmids within the bacterial cell. In some embodiments of the invention, a DNA molecule may be introduced into a bacterium such that the DNA molecule is integrated into the genome of the bacterium. In these and further embodiments, the DNA molecule may be either chromosomally-integrated or located as or in a stable plasmid.

[00113] Sequence identity: The term "sequence identity" or "identity", as used herein in the context of two nucleic acid or polypeptide sequences, refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

[00114] As used herein, the term "percentage of sequence identity" may refer to the value determined by comparing two optimally aligned sequences (*e.g.*, nucleic acid sequences or polypeptide sequences) over a comparison window, wherein the portion of the 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 nucleotide 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 comparison window, and multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be 100% identical to the reference sequence, and vice-versa.

[00115] Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in, for example: Smith and Waterman (1981) Adv. Appl. Math. 2:482; Needleman and Wunsch (1970) J. Mol. Biol. 48:443; Pearson and Lipman

(1988) Proc. Natl. Acad. Sci. U.S.A. 85:2444; Higgins and Sharp (1988) Gene 73:237-244; Higgins and Sharp (1989) CABIOS 5:151-153; Corpet et al. (1988) Nucleic Acids Res. 16:10881-10890; Huang et al. (1992) Comp. Appl. Biosci. 8:155-165; Pearson et al. (1994) Methods Mol. Biol. 24:307-331; Tatiana et al. (1999) FEMS Microbiol. Lett. 174:247-250. A detailed consideration of sequence alignment methods and homology calculations can be found in, e.g., Altschul et al. (1990) J. Mol. Biol. 215:403-410.

[00116] The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™; Altschul *et al.* (1990)) is available from several sources, including the National Center for Biotechnology Information (Bethesda, MD), and on the internet, for use in connection with several sequence analysis programs. A description of how to determine sequence identity using this program is available on the internet under the "help" section for BLAST™. For comparisons of nucleic acid sequences, the "Blast 2 sequences" function of the BLAST™ (Blastn) program may be employed using the default BLOSUM62 matrix set to default parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method.

[00117] Specifically hybridizable/Specifically complementary: As used herein, the terms "Specifically hybridizable" and "Specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the nucleic acid molecule and a target nucleic acid molecule. Hybridization between two nucleic acid molecules involves the formation of an anti-parallel alignment between the nucleic acid sequences of the two nucleic acid molecules. The two molecules are then able to form hydrogen bonds with corresponding bases on the opposite strand to form a duplex molecule that, if it is sufficiently stable, is detectable using methods well known in the art. A nucleic acid molecule need not be 100% complementary to its target sequence to be specifically hybridizable. However, the amount of sequence complementarity that must exist for hybridization to be specific is a function of the hybridization conditions used.

[00118] Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of

the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization will determine the stringency of hybridization. The ionic strength of the wash buffer and the wash temperature also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are known to those of ordinary skill in the art, and are discussed, for example, in Sambrook *et al.* (ed.) Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11, and updates; and Hames and Higgins (eds.) Nucleic Acid Hybridization, IRL Press, Oxford, 1985. Further detailed instruction and guidance with regard to the hybridization of nucleic acids may be found, for example, in Tijssen, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," in Laboratory Techniques in Biochemistry and Molecular Biology- Hybridization with Nucleic Acid Probes, Part I, Chapter 2, Elsevier, NY, 1993; and Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, Chapter 2, Greene Publishing and Wiley-Interscience, NY, 1995, and updates.

[00119] As used herein, "stringent conditions" encompass conditions under which hybridization will occur only if there is more than 80% sequence match between the hybridization molecule and a homologous sequence within the target nucleic acid molecule. "Stringent conditions" include further particular levels of stringency. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 80% sequence match (*i.e.* having less than 20% mismatch) will hybridize; conditions of "high stringency" are those under which sequences with more than 90% match (*i.e.* having less than 10% mismatch) will hybridize; and conditions of "very high stringency" are those under which sequences with more than 95% match (*i.e.* having less than 5% mismatch) will hybridize.

[00120] The following are representative, non-limiting hybridization conditions.

[00121] High Stringency condition (detects sequences that share at least 90% sequence identity): Hybridization in 5x SSC buffer at 65°C for 16 hours; wash twice in 2x SSC buffer at room temperature for 15 minutes each; and wash twice in 0.5x SSC buffer at 65°C for 20 minutes each.

[00122] Moderate Stringency condition (detects sequences that share at least 80% sequence identity): Hybridization in 5x-6x SSC buffer at 65-70°C for 16-20 hours; wash twice in 2x SSC buffer at room temperature for 5-20 minutes each; and wash twice in 1x SSC buffer at 55-70°C for 30 minutes each.

[00123] Non-stringent control condition (sequences that share at least 50% sequence identity will hybridize): Hybridization in 6x SSC buffer at room temperature to 55°C for 16-20 hours; wash at least twice in 2x-3x SSC buffer at room temperature to 55°C for 20-30 minutes each.

[00124] As used herein, the term "substantially homologous" or "substantial homology," with regard to a nucleic acid, refers to a polynucleotide having contiguous nucleobases that hybridize under stringent conditions to the reference nucleic acid. For example, nucleic acids that are substantially homologous to a reference nucleic acid sequence of SEQ ID NO:1 and/or SEQ ID NO:71 are those nucleic acids that hybridize under stringent conditions (e.g., the Moderate Stringency conditions set forth, supra) to the reference nucleic acid sequence of SEQ ID NO:1 and/or SEQ ID NO:71. Substantially homologous polynucleotides may have at least 80% sequence identity. For example, substantially homologous polynucleotides may have from about 80% to 100% sequence identity, such as 79%; 80%; about 81%; about 82%; about 83%; about 84%; about 85%; about 86%; about 87%; about 88%; about 89%; about 90%; about 91%; about 92%; about 93%; about 94% about 95%; about 96%; about 97%; about 98%; about 98.5%; about 99%; about 99.5%; and about 100%. The property of substantial homology is closely related to specific hybridization. For example, a nucleic acid molecule is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the nucleic acid to non-target polynucleotides under conditions where specific binding is desired, for example, under stringent hybridization conditions.

[00125] As used herein, the term "ortholog" refers to a gene in two or more species that has evolved from a common ancestral nucleic acid, and may retain the same function in the two or more species.

[00126] As used herein, two nucleic acid molecules are said to exhibit "complete complementarity" when every nucleotide of a polynucleotide read in the 5' to 3' direction is

complementary to every nucleotide of the other polynucleotide when read in the 3' to 5' direction. A polynucleotide that is complementary to a reference polynucleotide will exhibit a sequence identical to the reverse complement of the reference polynucleotide. These terms and descriptions are well defined in the art and are easily understood by those of ordinary skill in the art.

[00127] Operably linked: A first polynucleotide is operably linked with a second polynucleotide when the first polynucleotide is in a functional relationship with the second polynucleotide. When recombinantly produced, operably linked polynucleotides are generally contiguous, and, where necessary to join two protein-coding regions, in the same reading frame (*e.g.*, in a translationally fused ORF). However, nucleic acids need not be contiguous to be operably linked.

[00128] The term, "operably linked," when used in reference to a regulatory genetic element and a coding polynucleotide, means that the regulatory element affects the expression of the linked coding polynucleotide. "Regulatory elements," or "control elements," refer to polynucleotides that influence the timing and level/amount of transcription, RNA processing or stability, or translation of the associated coding polynucleotide. Regulatory elements may include promoters; translation leaders; introns; enhancers; stem-loop structures; repressor binding polynucleotides; polynucleotides with a termination sequence; polynucleotides with a polyadenylation recognition sequence; *etc*. Particular regulatory elements may be located upstream and/or downstream of a coding polynucleotide operably linked thereto. Also, particular regulatory elements operably linked to a coding polynucleotide may be located on the associated complementary strand of a double-stranded nucleic acid molecule.

[00129] Promoter: As used herein, the term "promoter" refers to a region of DNA that may be upstream from the start of transcription, and that may be involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A promoter may be operably linked to a coding polynucleotide for expression in a cell, or a promoter may be operably linked to a polynucleotide encoding a signal peptide which may be operably linked to a coding polynucleotide for expression in a cell. A "plant promoter" may be a promoter capable of initiating transcription in plant cells. Examples of promoters under developmental control include promoters that

preferentially initiate transcription in certain tissues, such as leaves, roots, seeds, fibers, xylem vessels, tracheids, or sclerenchyma. Such promoters are referred to as "tissue-preferred". Promoters which initiate transcription only in certain tissues are referred to as "tissue-specific". A "cell type-specific" promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" promoter may be a promoter which may be under environmental control. Examples of environmental conditions that may initiate transcription by inducible promoters include anaerobic conditions and the presence of light. Tissue-specific, tissue-preferred, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which may be active under most environmental conditions or in most tissue or cell types.

[00130] Any inducible promoter can be used in some embodiments of the invention. *See* Ward *et al.* (1993) Plant Mol. Biol. 22:361-366. With an inducible promoter, the rate of transcription increases in response to an inducing agent. Exemplary inducible promoters include, but are not limited to: Promoters from the ACEI system that respond to copper; *In2* gene from maize that responds to benzenesulfonamide herbicide safeners; Tet repressor from Tn10; and the inducible promoter from a steroid hormone gene, the transcriptional activity of which may be induced by a glucocorticosteroid hormone (Schena *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:0421).

[00131] Exemplary constitutive promoters include, but are not limited to: Promoters from plant viruses, such as the 35S promoter from Cauliflower Mosaic Virus (CaMV); promoters from rice actin genes; ubiquitin promoters; pEMU; MAS; maize H3 histone promoter; and the ALS promoter, *Xba1/NcoI* fragment 5' to the *Brassica napus ALS3* structural gene (or a polynucleotide similar to said *Xba1/NcoI* fragment) (International PCT Publication No. WO96/30530).

[00132] Additionally, any tissue-specific or tissue-preferred promoter may be utilized in some embodiments of the invention. Plants transformed with a nucleic acid molecule comprising a coding polynucleotide operably linked to a tissue-specific promoter may produce the product of the coding polynucleotide exclusively, or preferentially, in a specific tissue. Exemplary tissue-specific or tissue-preferred promoters include, but are not limited to: A seed-preferred promoter, such as

that from the phaseolin gene; a leaf-specific and light-induced promoter such as that from *cab* or *rubisco*; an anther-specific promoter such as that from *LAT52*; a pollen-specific promoter such as that from *Zm13*; and a microspore-preferred promoter such as that from *apg*.

[00133] Soybean plant: As used herein, the term "soybean plant" refers to a plant of the species *Glycine* sp.; for example, *G. max*.

[00134] Transformation: As used herein, the term "transformation" or "transduction" refers to the transfer of one or more nucleic acid molecule(s) into a cell. A cell is "transformed" by a nucleic acid molecule transduced into the cell when the nucleic acid molecule becomes stably replicated by the cell, either by incorporation of the nucleic acid molecule into the cellular genome, or by episomal replication. As used herein, the term "transformation" encompasses all techniques by which a nucleic acid molecule can be introduced into such a cell. Examples include, but are not limited to: transfection with viral vectors; transformation with plasmid vectors; electroporation (Fromm *et al.* (1986) Nature 319:791-3); lipofection (Felgner *et al.* (1987) Proc. Natl. Acad. Sci. USA 84:7413-7); microinjection (Mueller *et al.* (1978) Cell 15:579-85); *Agrobacterium*-mediated transfer (Fraley *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:4803-7); direct DNA uptake; and microprojectile bombardment (Klein *et al.* (1987) Nature 327:70).

[00135] Transgene: An exogenous nucleic acid. In some examples, a transgene may be a DNA that encodes one or both strand(s) of an RNA capable of forming a dsRNA molecule that comprises a polynucleotide that is complementary to a nucleic acid molecule found in a coleopteran and/or hemipteran pest. In further examples, a transgene may be a gene (*e.g.*, a herbicide-tolerance gene, a gene encoding an industrially or pharmaceutically useful compound, or a gene encoding a desirable agricultural trait). In these and other examples, a transgene may contain regulatory elements operably linked to a coding polynucleotide of the transgene (*e.g.*, a promoter).

[00136] Vector: A nucleic acid molecule as introduced into a cell, for example, to produce a transformed cell. A vector may include genetic elements that permit it to replicate in the host cell, such as an origin of replication. Examples of vectors include, but are not limited to: a plasmid; cosmid; bacteriophage; or virus that carries exogenous DNA into a cell. A vector may also include one or more genes, including ones that produce antisense molecules, and/or selectable marker genes

and other genetic elements known in the art. A vector may transduce, transform, or infect a cell, thereby causing the cell to express the nucleic acid molecules and/or proteins encoded by the vector. A vector optionally includes materials to aid in achieving entry of the nucleic acid molecule into the cell (*e.g.*, a liposome, protein coating, *etc.*).

[00137] Yield: A stabilized yield of about 100% or greater relative to the yield of check varieties in the same growing location growing at the same time and under the same conditions. In particular embodiments, "improved yield" or "improving yield" means a cultivar having a stabilized yield of 105% or greater relative to the yield of check varieties in the same growing location containing significant densities of the coleopteran and/or hemipteran pests that are injurious to that crop growing at the same time and under the same conditions, which pests are targeted by the compositions and methods herein.

[00138] Unless specifically indicated or implied, the terms "a," "an," and "the" signify "at least one," as used herein.

[00139] Unless otherwise specifically explained, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology can be found in, for example, Lewin's Genes X, Jones & Bartlett Publishers, 2009 (ISBN 10 0763766321); Krebs *et al.* (eds.), The Encyclopedia of Molecular Biology, Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Meyers R.A. (ed.), Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. All temperatures are in degrees Celsius.

IV. Nucleic Acid Molecules Comprising an Insect Pest Polynucleotide

A. Overview

[00140] Described herein are nucleic acid molecules useful for the control of insect pests. In some examples, the insect pest is a coleopteran or hemipteran insect pest. Described nucleic acid molecules include target polynucleotides (*e.g.*, native genes, and non-coding polynucleotides),

dsRNAs, siRNAs, shRNAs, hpRNAs, and miRNAs. For example, dsRNA, siRNA, miRNA, shRNA, and/or hpRNA molecules are described in some embodiments that may be specifically complementary to all or part of one or more native nucleic acids in a coleopteran and/or hemipteran pest. In these and further embodiments, the native nucleic acid(s) may be one or more target gene(s), the product of which may be, for example and without limitation: involved in in larval/nymph development. Nucleic acid molecules described herein, when introduced into a cell comprising at least one native nucleic acid(s) to which the nucleic acid molecules are specifically complementary, may initiate RNAi in the cell, and consequently reduce or eliminate expression of the native nucleic acid(s). In some examples, reduction or elimination of the expression of a target gene by a nucleic acid molecule specifically complementary thereto may result in reduction or cessation of growth, development, and/or feeding of the pest.

[00141] In some embodiments, at least one target gene in an insect pest may be selected, wherein the target gene comprises a *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71). In particular examples, a target gene in a coleopteran and/or hemipteran pest is selected, wherein the target gene comprises a novel nucleotide sequence comprising *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71).

[00142] In some embodiments, a target gene may be a nucleic acid molecule comprising a polynucleotide that can be translated *in silico* to a polypeptide comprising a contiguous amino acid sequence that is at least about 85% identical (*e.g.*, at least 84%, 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, or 100% identical) to the amino acid sequence of a protein product of *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71). A target gene may be any nucleic acid in an insect pest, the post-transcriptional inhibition of which has a deleterious effect on the growth and/or survival of the pest, for example, to provide a protective benefit against the pest to a plant. In particular examples, a target gene is a nucleic acid molecule comprising a polynucleotide that can be reverse translated *in silico* to a polypeptide comprising a contiguous amino acid sequence that is at least about 85% identical, about 90% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, about 100% identical, or 100% identical to the amino acid sequence of a protein product of novel nucleotide sequence SEQ ID NO:1 or SEQ ID NO:71.

[00143] Provided in some embodiments are DNAs, the expression of which results in an RNA molecule comprising a polynucleotide that is specifically complementary to all or part of a native RNA molecule that is encoded by a coding polynucleotide in an insect (*e.g.*, coleopteran and/or hemipteran) pest. In some embodiments, after ingestion of the expressed RNA molecule by an insect pest, down-regulation of the coding polynucleotide in cells of the pest may be obtained. In particular embodiments, down-regulation of the coding sequence in cells of the insect pest may result in a deleterious effect on the growth, development, and/or survival of the pest.

[00144] In some embodiments, target polynucleotides include transcribed non-coding RNAs, such as 5'UTRs; 3'UTRs; spliced leaders; introns; outrons (*e.g.*, 5'UTR RNA subsequently modified in trans splicing); donatrons (*e.g.*, non-coding RNA required to provide donor sequences for *trans* splicing); and other non-coding transcribed RNA of target insect pest genes. Such polynucleotides may be derived from both mono-cistronic and poly-cistronic genes.

[00145] Thus, also described herein in connection with some embodiments are iRNA molecules (*e.g.*, dsRNAs, siRNAs, miRNAs, shRNAs, and hpRNAs) that comprise at least one polynucleotide that is specifically complementary to all or part of a target nucleic acid in an insect (*e.g.*, coleopteran and/or hemipteran) pest. In some embodiments an iRNA molecule may comprise polynucleotide(s) that are complementary to all or part of a plurality of target nucleic acids; for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more target nucleic acids. In particular embodiments, an iRNA molecule may be produced *in vitro*, or *in vivo* by a genetically-modified organism, such as a plant or bacterium. Also disclosed are cDNAs that may be used for the production of dsRNA molecules, siRNA molecules, miRNA molecules, shRNA molecules, and/or hpRNA molecules that are specifically complementary to all or part of a target nucleic acid in an insect pest. Further described are recombinant DNA constructs for use in achieving stable transformation of particular host targets. Transformed host targets may express effective levels of dsRNA, siRNA, miRNA, shRNA, and/or hpRNA molecules from the recombinant DNA constructs. Therefore, also described is a plant transformation vector comprising at least one polynucleotide operably linked to a heterologous promoter functional in a plant cell, wherein expression of the polynucleotide(s) results in an RNA

molecule comprising a string of contiguous nucleobases that is specifically complementary to all or part of a target nucleic acid in an insect pest.

[00146] In particular examples, nucleic acid molecules useful for the control of insect (*e.g.*, coleopteran and/or hemipteran) pests may include: all or part of a native nucleic acid isolated from *Diabrotica* or hemipteran organism comprising *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71); nucleotide sequences that when expressed result in an RNA molecule comprising a nucleotide sequence that is specifically complementary to all or part of a native RNA molecule that is encoded by *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71); iRNA molecules (*e.g.*, dsRNAs, siRNAs, shRNAs, and hpRNAs) that comprise at least one polynucleotide that is specifically complementary to all or part of *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71); cDNA sequences that may be used for the production of dsRNA molecules, siRNA molecules, miRNA molecules, shRNA molecules, and/or hpRNA molecules that are specifically complementary to all or part of *COPI delta* (SEQ ID NO:1 or SEQ ID NO:71); and recombinant DNA constructs for use in achieving stable transformation of particular host targets, wherein a transformed host target comprises one or more of the foregoing nucleic acid molecules.

B. Nucleic Acid Molecules

[00147] The present invention provides, *inter alia*, iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecules that inhibit target gene expression in a cell, tissue, or organ of an insect (*e.g.*, coleopteran and/or hemipteran) pest; and DNA molecules capable of being expressed as an iRNA molecule in a cell or microorganism to inhibit target gene expression in a cell, tissue, or organ of an insect pest.

[00148] Some embodiments of the invention provide an isolated nucleic acid molecule comprising at least one (*e.g.*, one, two, three, or more) polynucleotide(s) selected from the group consisting of: any ofSEQ ID NO:1 or SEQ ID NO:71; the complement of any of SEQ ID NO:1 or SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of any of SEQ ID NO:1 or SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of any of SEQ ID NO:1 or SEQ ID NO:71; a native coding polynucleotide of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; a native coding sequence of a hemipteran organism comprising SEQ ID

NO:71; the complement of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71. In particular embodiments, contact with or uptake by a coleopteran and/or hemipteran pest of the isolated polynucleotide inhibits the growth, development and/or feeding of the pest.

[00149] In some embodiments, a nucleic acid molecule of the invention may comprise at least one (*e.g.*, one, two, three, or more) DNA(s) capable of being expressed as an iRNA molecule in a cell or microorganism to inhibit target gene expression in a cell, tissue, or organ of a coleopteran and/or hemipteran pest. Such DNA(s) may be operably linked to a promoter that

functions in a cell comprising the DNA molecule to initiate or enhance the transcription of the encoded RNA capable of forming a dsRNA molecule(s). In one embodiment, the at least one (*e.g.*, one, two, three, or more) DNA(s) may be derived from a polynucleotide selected from SEQ ID NO:1 or SEQ ID NO:71. Derivatives of SEQ ID NO:1 or SEQ ID NO:71 include fragments of SEQ ID NO:1 or SEQ ID NO:71. In some embodiments, such a fragment may comprise, for example, at least about 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71, or a complement thereof. Thus, such a fragment may comprise, for example, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 or more contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71, or a complement thereof. In some examples, such a fragment may comprise, for example, at least 19 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71, or a complement thereof. Thus, a fragment of SEQ ID NO:1 or SEQ ID NO:71 may comprise, for example, 15, 16, 17, 18, 19, 20, 21, about 25,(*e.g.*, 22, 23, 24, 25, 26, 27, 28, and 29), about 30, about 40, (*e.g.*, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,and 45), about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200 or more contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71, or a complement thereof.

[00150] Some embodiments comprise introducing partially- or fully-stabilized dsRNA molecules into a coleopteran and/or hemipteran pest to inhibit expression of a target gene in a cell, tissue, or organ of the coleopteran and/or hemipteran pest. When expressed as an iRNA molecule (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) and taken up by a coleopteran and/or hemipteran pest, polynucleotides comprising one or more fragments of any of SEQ ID NO:1 or SEQ ID NO:71and the complements thereof, may cause one or more of death, developmental arrest, growth inhibition, change in sex ratio, reduction in brood size, cessation of infection, and/or cessation of feeding by a coleopteran and/or hemipteran pest. For example, in some embodiments, a dsRNA molecule comprising a nucleotide sequence including about 15 to about 300 or about 19 to about 300 nucleotides that are substantially homologous to a coleopteran and/or hemipteran pest target gene sequence and comprising one or more fragments of a nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:71 is provided. Expression of such a dsRNA molecule may, for

example, lead to mortality and/or growth inhibition in a coleopteran and/or hemipteran pest that takes up the dsRNA molecule.

[00151] In certain embodiments, dsRNA molecules provided by the invention comprise polynucleotides complementary to a transcript from a target gene comprising SEQ ID NO:1 or SEQ ID NO:71 and/or nucleotide sequences complementary to a fragment of SEQ ID NO:1 or SEQ ID NO:71, the inhibition of which target gene in an insect pest results in the reduction or removal of a polypeptide or polynucleotide agent that is essential for the pest's growth, development, or other biological function. A selected polynucleotide may exhibit from about 80% to about 100% sequence identity to any of SEQ ID NO:1 or SEQ ID NO:71, a contiguous fragment of the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:71, or the complement of either of the foregoing. For example, a selected polynucleotide may exhibit 79%; 80%; about 81%; about 82%; about 83%; about 84%; about 85%; about 86%; about 87%; about 88%; about 89%; about 90%; about 91%; about 92%; about 93%; about 94% about 95%; about 96%; about 97%; about 98%; about 98.5%; about 99%; about 99.5%; or about 100% sequence identity to any of SEQ ID NO:1 or SEQ ID NO:71, a contiguous fragment of the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:71, or the complement of either of the foregoing.

[00152] In some embodiments, a DNA molecule capable of being expressed as an iRNA molecule in a cell or microorganism to inhibit target gene expression may comprise a single polynucleotide that is specifically complementary to all or part of a native polynucleotide found in one or more target insect pest species (*e.g.*, a coleopteran or hemipteran pest species), or the DNA molecule can be constructed as a chimera from a plurality of such specifically complementary polynucleotides.

[00153] In some embodiments, a nucleic acid molecule may comprise a first and a second polynucleotide separated by a "spacer." A spacer may be a region comprising any sequence of nucleotides that facilitates secondary structure formation between the first and second polynucleotides, where this is desired. In one embodiment, the spacer is part of a sense or antisense coding polynucleotide for mRNA. The spacer may alternatively comprise any combination of nucleotides or homologues thereof that are capable of being linked covalently to a nucleic acid

molecule. In some examples, the spacer may be an intron (*e.g.*, an ST-LS1 intron or a RTM1 intron).

[00154] For example, in some embodiments, the DNA molecule may comprise a polynucleotide coding for one or more different iRNA molecules, wherein each of the different iRNA molecules comprises a first polynucleotide and a second polynucleotide, wherein the first and second polynucleotides are complementary to each other. The first and second polynucleotides may be connected within an RNA molecule by a spacer. The spacer may constitute part of the first polynucleotide or the second polynucleotide. Expression of an RNA molecule comprising the first and second nucleotide polynucleotides may lead to the formation of a dsRNA molecule, by specific intramolecular base-pairing of the first and second nucleotide polynucleotides. The first polynucleotide or the second polynucleotide may be substantially identical to a polynucleotide (*e.g.*, a target gene, or transcribed non-coding polynucleotide) native to an insect pest (*e.g.*, a coleopteran or hemipteran pest), a derivative thereof, or a complementary polynucleotide thereto.

[00155] dsRNA nucleic acid molecules comprise double strands of polymerized ribonucleotides, and may include modifications to either the phosphate-sugar backbone or the nucleoside. Modifications in RNA structure may be tailored to allow specific inhibition. In one embodiment, dsRNA molecules may be modified through a ubiquitous enzymatic process so that siRNA molecules may be generated. This enzymatic process may utilize an RNase III enzyme, such as DICER in eukaryotes, either *in vitro* or *in vivo*. *See* Elbashir *et al.* (2001) Nature 411:494-8; and Hamilton and Baulcombe (1999) Science 286(5441):950-2. DICER or functionally-equivalent RNase III enzymes cleave larger dsRNA strands and/or hpRNA molecules into smaller oligonucleotides (*e.g.*, siRNAs), each of which is about 19-25 nucleotides in length. The siRNA molecules produced by these enzymes have 2 to 3 nucleotide 3' overhangs, and 5' phosphate and 3' hydroxyl termini. The siRNA molecules generated by RNase III enzymes are unwound and separated into single-stranded RNA in the cell. The siRNA molecules then specifically hybridize with RNAs transcribed from a target gene, and both RNA molecules are subsequently degraded by an inherent cellular RNA-degrading mechanism. This process may result in the effective degradation or removal of the RNA encoded by the target gene in the target organism. The outcome

is the post-transcriptional silencing of the targeted gene. In some embodiments, siRNA molecules produced by endogenous RNase III enzymes from heterologous nucleic acid molecules may efficiently mediate the down-regulation of target genes in coleopteran and/or hemipteran pests.

[00156] In some embodiments, a nucleic acid molecule may include at least one non-naturally occurring polynucleotide that can be transcribed into a single-stranded RNA molecule capable of forming a dsRNA molecule *in vivo* through intermolecular hybridization. Such dsRNAs typically self-assemble, and can be provided in the nutrition source of an insect (*e.g.*, coleopteran or hemipteran) pest to achieve the post-transcriptional inhibition of a target gene. In these and further embodiments, a nucleic acid molecule may comprise two different non-naturally occurring polynucleotides, each of which is specifically complementary to a different target gene in an insect pest. When such a nucleic acid molecule is provided as a dsRNA molecule to, for example, a coleopteran and/or hemipteran pest, the dsRNA molecule inhibits the expression of at least two different target genes in the pest.

C. Obtaining Nucleic Acid Molecules

[00157] A variety of polynucleotides in insect (*e.g.*, coleopteran and hemipteran) pests may be used as targets for the design of nucleic acid molecules, such as iRNAs and DNA molecules encoding iRNAs. Selection of native polynucleotides is not, however, a straight-forward process. For example, only a small number of native polynucleotides in a coleopteran or hemipteran pest will be effective targets. It cannot be predicted with certainty whether a particular native polynucleotide can be effectively down-regulated by nucleic acid molecules of the invention, or whether down-regulation of a particular native polynucleotide will have a detrimental effect on the growth, development and/or survival of an insect pest. The vast majority of native coleopteran and hemipteran pest polynucleotides, such as ESTs isolated therefrom (for example, the coleopteran pest polynucleotides listed in U.S. Patent 7,612,194), do not have a detrimental effect on the growth, development, and/or survival of the pest. Neither is it predictable which of the native polynucleotides that may have a detrimental effect on an insect pest are able to be used in recombinant techniques for expressing nucleic acid molecules complementary to such native

polynucleotides in a host plant and providing the detrimental effect on the pest upon feeding without causing harm to the host plant.

[00158] In some embodiments, nucleic acid molecules (*e.g.*, dsRNA molecules to be provided in the host plant of an insect (*e.g.*, coleopteran or hemipteran) pest) are selected to target cDNAs that encode proteins or parts of proteins essential for pest development, such as polypeptides involved in metabolic or catabolic biochemical pathways, cell division, energy metabolism, digestion, host plant recognition, and the like. As described herein, ingestion of compositions by a target pest organism containing one or more dsRNAs, at least one segment of which is specifically complementary to at least a substantially identical segment of RNA produced in the cells of the target pest organism, can result in the death or other inhibition of the target. A polynucleotide, either DNA or RNA, derived from an insect pest can be used to construct plant cells resistant to infestation by the pests. The host plant of the coleopteran and/or hemipteran pest (*e.g.*, *Z. mays* or *G. max*), for example, can be transformed to contain one or more polynucleotides derived from the coleopteran and/or hemipteran pest as provided herein. The polynucleotide transformed into the host may encode one or more RNAs that form into a dsRNA structure in the cells or biological fluids within the transformed host, thus making the dsRNA available if/when the pest forms a nutritional relationship with the transgenic host. This may result in the suppression of expression of one or more genes in the cells of the pest, and ultimately death or inhibition of its growth or development.

[00159] Thus, in some embodiments, a gene is targeted that is essentially involved in the growth and development of an insect (*e.g.*, coleopteran or hemipteran) pest. Other target genes for use in the present invention may include, for example, those that play important roles in pest movement, migration, growth, development, infectivity, and establishment of feeding sites. A target gene may therefore be a housekeeping gene or a transcription factor. Additionally, a native insect pest polynucleotide for use in the present invention may also be derived from a homolog (*e.g.*, an ortholog), of a plant, viral, bacterial or insect gene, the function of which is known to those of skill in the art, and the polynucleotide of which is specifically hybridizable with a target gene in

the genome of the target pest. Methods of identifying a homolog of a gene with a known nucleotide sequence by hybridization are known to those of skill in the art.

[00160] In some embodiments, the invention provides methods for obtaining a nucleic acid molecule comprising a polynucleotide for producing an iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecule. One such embodiment comprises: (a) analyzing one or more target gene(s) for their expression, function, and phenotype upon dsRNA-mediated gene suppression in an insect (*e.g.*, coleopteran or hemipteran) pest; (b) probing a cDNA or gDNA library with a probe comprising all or a portion of a polynucleotide or a homolog thereof from a targeted pest that displays an altered (*e.g.*, reduced) growth or development phenotype in a dsRNA-mediated suppression analysis; (c) identifying a DNA clone that specifically hybridizes with the probe; (d) isolating the DNA clone identified in step (b); (e) sequencing the cDNA or gDNA fragment that comprises the clone isolated in step (d), wherein the sequenced nucleic acid molecule comprises all or a substantial portion of the RNA or a homolog thereof; and (f) chemically synthesizing all or a substantial portion of a gene, or an siRNA, miRNA, hpRNA, mRNA, shRNA, or dsRNA.

[00161] In further embodiments, a method for obtaining a nucleic acid fragment comprising a polynucleotide for producing a substantial portion of an iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecule includes: (a) synthesizing first and second oligonucleotide primers specifically complementary to a portion of a native polynucleotide from a targeted insect (*e.g.*, coleopteran or hemipteran) pest; and (b) amplifying a cDNA or gDNA insert present in a cloning vector using the first and second oligonucleotide primers of step (a), wherein the amplified nucleic acid molecule comprises a substantial portion of a siRNA, miRNA, hpRNA, mRNA, shRNA, or dsRNA molecule.

[00162] Nucleic acids can be isolated, amplified, or produced by a number of approaches. For example, an iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecule may be obtained by PCR amplification of a target polynucleotide (*e.g.*, a target gene or a target transcribed non-coding polynucleotide) derived from a gDNA or cDNA library, or portions thereof. DNA or RNA may be extracted from a target organism, and nucleic acid libraries may be prepared therefrom

using methods known to those ordinarily skilled in the art. gDNA or cDNA libraries generated from a target organism may be used for PCR amplification and sequencing of target genes. A confirmed PCR product may be used as a template for *in vitro* transcription to generate sense and antisense RNA with minimal promoters. Alternatively, nucleic acid molecules may be synthesized by any of a number of techniques (*See*, *e.g.*, Ozaki *et al.* (1992) Nucleic Acids Research, 20: 5205-5214; and Agrawal *et al.* (1990) Nucleic Acids Research, 18: 5419-5423), including use of an automated DNA synthesizer (for example, a P.E. Biosystems, Inc. (Foster City, Calif.) model 392 or 394 DNA/RNA Synthesizer), using standard chemistries, such as phosphoramidite chemistry. *See*, *e.g.*, Beaucage *et al.* (1992) Tetrahedron, 48: 2223-2311; U.S. Patents 4,980,460, 4,725,677, 4,415,732, 4,458,066, and 4,973,679. Alternative chemistries resulting in non-natural backbone groups, such as phosphorothioate, phosphoramidate, and the like, can also be employed.

[00163] An RNA, dsRNA, siRNA, miRNA, shRNA, or hpRNA molecule of the present invention may be produced chemically or enzymatically by one skilled in the art through manual or automated reactions, or *in vivo* in a cell comprising a nucleic acid molecule comprising a polynucleotide encoding the RNA, dsRNA, siRNA, miRNA, shRNA, or hpRNA molecule. RNA may also be produced by partial or total organic synthesis- any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis. An RNA molecule may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3 RNA polymerase, T7 RNA polymerase, and SP6 RNA polymerase). Expression constructs useful for the cloning and expression of polynucleotides are known in the art. *See*, *e.g.*, International PCT Publication No. WO97/32016; and U.S. Patents 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693. RNA molecules that are synthesized chemically or by *in vitro* enzymatic synthesis may be purified prior to introduction into a cell. For example, RNA molecules can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, RNA molecules that are synthesized chemically or by *in vitro* enzymatic synthesis may be used with no or a minimum of purification, for example, to avoid losses due to sample processing. The RNA molecules may be dried for storage or dissolved in an aqueous

solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of dsRNA molecule duplex strands.

[00164] In embodiments, a dsRNA molecule may be formed by a single self-complementary RNA strand or from two complementary RNA strands. dsRNA molecules may be synthesized either *in vivo* or *in vitro*. An endogenous RNA polymerase of the cell may mediate transcription of the one or two RNA strands *in vivo*, or cloned RNA polymerase may be used to mediate transcription *in vivo* or *in vitro*. Post-transcriptional inhibition of a target gene in an insect pest may be host-targeted by specific transcription in an organ, tissue, or cell type of the host (*e.g.*, by using a tissue-specific promoter); stimulation of an environmental condition in the host (*e.g.*, by using an inducible promoter that is responsive to infection, stress, temperature, and/or chemical inducers); and/or engineering transcription at a developmental stage or age of the host (*e.g.*, by using a developmental stage-specific promoter). RNA strands that form a dsRNA molecule, whether transcribed *in vitro* or *in vivo*, may or may not be polyadenylated, and may or may not be capable of being translated into a polypeptide by a cell's translational apparatus.

D. Recombinant Vectors and Host Cell Transformation

[00165] In some embodiments, the invention also provides a DNA molecule for introduction into a cell (*e.g.*, a bacterial cell, a yeast cell, or a plant cell), wherein the DNA molecule comprises a polynucleotide that, upon expression to RNA and ingestion by an insect (*e.g.*, coleopteran and/or hemipteran) pest, achieves suppression of a target gene in a cell, tissue, or organ of the pest. Thus, some embodiments provide a recombinant nucleic acid molecule comprising a polynucleotide capable of being expressed as an iRNA (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) molecule in a plant cell to inhibit target gene expression in an insect pest. In order to initiate or enhance expression, such recombinant nucleic acid molecules may comprise one or more regulatory elements, which regulatory elements may be operably linked to the polynucleotide capable of being expressed as an iRNA. Methods to express a gene suppression molecule in plants are known, and may be used to express a polynucleotide of the present invention. *See*, *e.g.*, International PCT Publication No. WO06/073727; and U.S. Patent Publication No. 2006/0200878 Al)

[00166] In specific embodiments, a recombinant DNA molecule of the invention may comprise a polynucleotide encoding an RNA that may form a dsRNA molecule. Such recombinant DNA molecules may encode RNAs that may form dsRNA molecules capable of inhibiting the expression of endogenous target gene(s) in an insect (*e.g.*, coleopteran and/or hemipteran) pest cell upon ingestion. In many embodiments, a transcribed RNA may form a dsRNA molecule that may be provided in a stabilized form; *e.g.*, as a hairpin and stem and loop structure.

[00167] In some embodiments, one strand of a dsRNA molecule may be formed by transcription from a polynucleotide which is substantially homologous to a polynucleotide of any of SEQ ID NO:1; the complement of SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; the complement of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; the complement of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1.

[00168] In some embodiments, one strand of a dsRNA molecule may be formed by transcription from a polynucleotide that is substantially homologous to a polynucleotide selected from the group consisting of SEQ ID NO:71; the complement of SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; the complement of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a native non-coding sequence of a hemipteran organism that is

transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71.

[00169] In particular embodiments, a recombinant DNA molecule encoding an RNA that may form a dsRNA molecule may comprise a coding region wherein at least two polynucleotides are arranged such that one polynucleotide is in a sense orientation, and the other polynucleotide is in an antisense orientation, relative to at least one promoter, wherein the sense polynucleotide and the antisense polynucleotide are linked or connected by a spacer of, for example, from about five (~5) to about one thousand (~1000) nucleotides. The spacer may form a loop between the sense and antisense polynucleotides. The sense polynucleotide or the antisense polynucleotide may be substantially homologous to a target gene (*e.g.*, a gene comprising SEQ ID NO:1 or SEQ ID NO:71) or fragment thereof. In some embodiments, however, a recombinant DNA molecule may encode an RNA that may form a dsRNA molecule without a spacer. In embodiments, a sense coding polynucleotide and an antisense coding polynucleotide may be different lengths.

[00170] Polynucleotides identified as having a deleterious effect on an insect pest or a plant-protective effect with regard to the pest may be readily incorporated into expressed dsRNA molecules through the creation of appropriate expression cassettes in a recombinant nucleic acid molecule of the invention. For example, such polynucleotides may be expressed as a hairpin with stem and loop structure by taking a first segment corresponding to a target gene polynucleotide (*e.g.,* SEQ ID NO:1 or SEQ ID NO:71 and fragments thereof); linking this polynucleotide to a second segment spacer region that is not homologous or complementary to the first segment; and

linking this to a third segment, wherein at least a portion of the third segment is substantially complementary to the first segment. Such a construct forms a stem and loop structure by intramolecular base-pairing of the first segment with the third segment, wherein the loop structure forms comprising the second segment. *See*, *e.g.*, U.S. Patent Publication Nos. 2002/0048814 and 2003/0018993; and International PCT Publication Nos. WO94/01550 and WO98/05770. A dsRNA molecule may be generated, for example, in the form of a double-stranded structure such as a stem-loop structure (*e.g.*, hairpin), whereby production of siRNA targeted for a native insect (*e.g.*, coleopteran and/or hemipteran) pest polynucleotide is enhanced by co-expression of a fragment of the targeted gene, for instance on an additional plant expressible cassette, that leads to enhanced siRNA production, or reduces methylation to prevent transcriptional gene silencing of the dsRNA hairpin promoter.

[00171] Embodiments of the invention include introduction of a recombinant nucleic acid molecule of the present invention into a plant (*i.e.*, transformation) to achieve insect (*e.g.*, coleopteran and/or hemipteran) pest-inhibitory levels of expression of one or more iRNA molecules. A recombinant DNA molecule may, for example, be a vector, such as a linear or a closed circular plasmid. The vector system may be a single vector or plasmid, or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of a host. In addition, a vector may be an expression vector. Nucleic acids of the invention can, for example, be suitably inserted into a vector under the control of a suitable promoter that functions in one or more hosts to drive expression of a linked coding polynucleotide or other DNA element. Many vectors are available for this purpose, and selection of the appropriate vector will depend mainly on the size of the nucleic acid to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components depending on its function (*e.g.*, amplification of DNA or expression of DNA) and the particular host cell with which it is compatible.

[00172] To impart protection from insect (*e.g.*, coleopteran and/or hemipteran) pests to a transgenic plant, a recombinant DNA may, for example, be transcribed into an iRNA molecule (*e.g.*, a RNA molecule that forms a dsRNA molecule) within the tissues or fluids of the recombinant

plant. An iRNA molecule may comprise a polynucleotide that is substantially homologous and specifically hybridizable to a corresponding transcribed polynucleotide within an insect pest that may cause damage to the host plant species. The pest may contact the iRNA molecule that is transcribed in cells of the transgenic host plant, for example, by ingesting cells or fluids of the transgenic host plant that comprise the iRNA molecule. Thus, in particular examples, expression of a target gene is suppressed by the iRNA molecule within coleopteran and/or hemipteran pests that infest the transgenic host plant. In some embodiments, suppression of expression of the target gene in a target coleopteran and/or hemipteran pest may result in the plant being protected from attack by the pest.

[00173] In order to enable delivery of iRNA molecules to an insect pest in a nutritional relationship with a plant cell that has been transformed with a recombinant nucleic acid molecule of the invention, expression (*i.e.*, transcription) of iRNA molecules in the plant cell is required. Thus, a recombinant nucleic acid molecule may comprise a polynucleotide of the invention operably linked to one or more regulatory elements, such as a heterologous promoter element that functions in a host cell, such as a bacterial cell wherein the nucleic acid molecule is to be amplified, and a plant cell wherein the nucleic acid molecule is to be expressed.

[00174] Promoters suitable for use in nucleic acid molecules of the invention include those that are inducible, viral, synthetic, or constitutive, all of which are well known in the art. Non-limiting examples describing such promoters include U.S. Patents 6,437,217 (maize RS81 promoter); 5,641,876 (rice actin promoter); 6,426,446 (maize RS324 promoter); 6,429,362 (maize PR-1 promoter); 6,232,526 (maize A3 promoter); 6,177,611 (constitutive maize promoters); 5,322,938, 5,352,605, 5,359,142, and 5,530,196 (CaMV 35S promoter); 6,433,252 (maize L3 oleosin promoter); 6,429,357 (rice actin 2 promoter, and rice actin 2 intron); 6,294,714 (light-inducible promoters); 6,140,078 (salt-inducible promoters); 6,252,138 (pathogen-inducible promoters); 6,175,060 (phosphorous deficiency-inducible promoters); 6,388,170 (bidirectional promoters); 6,635,806 (gamma-coixin promoter); and U.S. Patent Publication No. 2009/757,089 (maize chloroplast aldolase promoter). Additional promoters include the nopaline synthase (NOS) promoter (Ebert *et al.* (1987) Proc. Natl. Acad. Sci. USA 84(16):5745-9) and the octopine synthase

(OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*); the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S promoter (Lawton *et al.* (1987) Plant Mol. Biol. 9:315-24); the CaMV 35S promoter (Odell *et al.* (1985) Nature 313:810-2; the figwort mosaic virus 35S-promoter (Walker *et al.* (1987) Proc. Natl. Acad. Sci. USA 84(19):6624-8); the sucrose synthase promoter (Yang and Russell (1990) Proc. Natl. Acad. Sci. USA 87:4144-8); the R gene complex promoter (Chandler *et al.* (1989) Plant Cell 1:1175-83); the chlorophyll a/b binding protein gene promoter; CaMV 35S (U.S. Patents 5,322,938, 5,352,605, 5,359,142, and 5,530,196); FMV 35S (U.S. Patents 6,051,753, and 5,378,619); a PC1SV promoter (U.S. Patent 5,850,019); the SCP1 promoter (U.S. Patent 6,677,503); and AGRtu.nos promoters (GenBank™ Accession No. V00087; Depicker *et al.* (1982) J. Mol. Appl. Genet. 1:561-73; Bevan *et al.* (1983) Nature 304:184-7).

[00175] In particular embodiments, nucleic acid molecules of the invention comprise a tissue-specific promoter, such as a root-specific promoter. Root-specific promoters drive expression of operably-linked coding polynucleotides exclusively or preferentially in root tissue. Examples of root-specific promoters are known in the art. *See, e.g.*, U.S. Patents 5,110,732; 5,459,252 and 5,837,848; and Opperman *et al.* (1994) Science 263:221-3; and Hirel *et al.* (1992) Plant Mol. Biol. 20:207-18. In some embodiments, a polynucleotide or fragment for coleopteran and/or hemipteran pest control according to the invention may be cloned between two root-specific promoters oriented in opposite transcriptional directions relative to the polynucleotide or fragment, and which are operable in a transgenic plant cell and expressed therein to produce RNA molecules in the transgenic plant cell that subsequently may form dsRNA molecules, as described, *supra*. The iRNA molecules expressed in plant tissues may be ingested by an insect pest so that suppression of target gene expression is achieved.

[00176] Additional regulatory elements that may optionally be operably linked to a nucleic acid include 5'UTRs located between a promoter element and a coding polynucleotide that function as a translation leader element. The translation leader element is present in fully-processed mRNA, and it may affect processing of the primary transcript, and/or RNA stability. Examples of translation leader elements include maize and petunia heat shock protein leaders (U.S. Patent

5,362,865), plant virus coat protein leaders, plant rubisco leaders, and others. *See*, *e.g.*, Turner and Foster (1995) Molecular Biotech. 3(3):225-36. Non-limiting examples of 5'UTRs include GmHsp (U.S. Patent 5,659,122); PhDnaK (U.S. Patent 5,362,865); AtAnt1; TEV (Carrington and Freed (1990) J. Virol. 64:1590-7); and AGRtunos (GenBank™ Accession No. V00087; and Bevan *et al.* (1983) Nature 304:184-7).

[00177] Additional regulatory elements that may optionally be operably linked to a nucleic acid also include 3' non-translated elements, 3' transcription termination regions, or polyadenylation regions. These are genetic elements located downstream of a polynucleotide, and include polynucleotides that provide polyadenylation signal, and/or other regulatory signals capable of affecting transcription or mRNA processing. The polyadenylation signal functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the mRNA precursor. The polyadenylation element can be derived from a variety of plant genes, or from T-DNA genes. A non-limiting example of a 3' transcription termination region is the nopaline synthase 3' region (nos 3'; Fraley *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:4803-7). An example of the use of different 3' non-translated regions is provided in Ingelbrecht *et al.*, (1989) Plant Cell 1:671-80. Non-limiting examples of polyadenylation signals include one from a *Pisum sativum* RbcS2 gene (Ps.RbcS2-E9; Coruzzi *et al.* (1984) EMBO J. 3:1671-9) and AGRtu.nos (GenBank™ Accession No. E01312).

[00178] Some embodiments may include a plant transformation vector that comprises an isolated and purified DNA molecule comprising at least one of the above-described regulatory elements operatively linked to one or more polynucleotides of the present invention. When expressed, the one or more polynucleotides result in one or more iRNA molecule(s) comprising a polynucleotide that is specifically complementary to all or part of a native RNA molecule in an insect (*e.g.*, coleopteran and/or hemipteran) pest. Thus, the polynucleotide(s) may comprise a segment encoding all or part of a polyribonucleotide present within a targeted coleopteran and/or hemipteran pest RNA transcript, and may comprise inverted repeats of all or a part of a targeted pest transcript. A plant transformation vector may contain polynucleotides specifically complementary to more than one target polynucleotide, thus allowing production of more than one dsRNA for inhibiting expression of two or more genes in cells of one or more populations or species of target

insect pests. Segments of polynucleotides specifically complementary to polynucleotides present in different genes can be combined into a single composite nucleic acid molecule for expression in a transgenic plant. Such segments may be contiguous or separated by a spacer.

[00179] In some embodiments, a plasmid of the present invention already containing at least one polynucleotide(s) of the invention can be modified by the sequential insertion of additional polynucleotide(s) in the same plasmid, wherein the additional polynucleotide(s) are operably linked to the same regulatory elements as the original at least one polynucleotide(s). In some embodiments, a nucleic acid molecule may be designed for the inhibition of multiple target genes. In some embodiments, the multiple genes to be inhibited can be obtained from the same insect (*e.g.*, coleopteran or hemipteran) pest species, which may enhance the effectiveness of the nucleic acid molecule. In other embodiments, the genes can be derived from different insect pests, which may broaden the range of pests against which the agent(s) is/are effective. When multiple genes are targeted for suppression or a combination of expression and suppression, a polycistronic DNA element can be engineered.

[00180] A recombinant nucleic acid molecule or vector of the present invention may comprise a selectable marker that confers a selectable phenotype on a transformed cell, such as a plant cell. Selectable markers may also be used to select for plants or plant cells that comprise a recombinant nucleic acid molecule of the invention. The marker may encode biocide resistance, antibiotic resistance (*e.g.*, kanamycin, Geneticin (G418), bleomycin, hygromycin, *etc.*), or herbicide tolerance (*e.g.*, glyphosate, *etc.*). Examples of selectable markers include, but are not limited to: a *neo* gene which codes for kanamycin resistance and can be selected for using kanamycin, G418, *etc.*; a *bar* gene which codes for bialaphos resistance; a mutant EPSP synthase gene which encodes glyphosate tolerance; a *nitrilase* gene which confers resistance to bromoxynil; a mutant acetolactate synthase (*ALS*) gene which confers imidazolinone or sulfonylurea tolerance; and a methotrexate resistant *DHFR* gene. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, spectinomycin, rifampicin, streptomycin and tetracycline, and the

like. Examples of such selectable markers are illustrated in, *e.g.*, U.S. Patents 5,550,318; 5,633,435; 5,780,708 and 6,118,047.

[00181] A recombinant nucleic acid molecule or vector of the present invention may also include a screenable marker. Screenable markers may be used to monitor expression. Exemplary screenable markers include a β-glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known (Jefferson *et al.* (1987) Plant Mol. Biol. Rep. 5:387-405); an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.* (1988) "Molecular cloning of the maize *R-nj* allele by transposon tagging with *Ac*." In 18th Stadler Genetics Symposium, P. Gustafson and R. Appels, eds. (New York: Plenum), pp. 263-82); a β-lactamase gene (Sutcliffe *et al.* (1978) Proc. Natl. Acad. Sci. USA 75:3737-41); a gene which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a luciferase gene (Ow *et al.* (1986) Science 234:856-9); an *xylE* gene that encodes a catechol dioxygenase that can convert chromogenic catechols (Zukowski *et al.* (1983) Gene 46(2-3):247-55); an amylase gene (Ikatu *et al.* (1990) Bio/Technol. 8:241-2); a tyrosinase gene which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to melanin (Katz *et al.* (1983) J. Gen. Microbiol. 129:2703-14); and an α-galactosidase.

[00182] In some embodiments, recombinant nucleic acid molecules, as described, *supra*, may be used in methods for the creation of transgenic plants and expression of heterologous nucleic acids in plants to prepare transgenic plants that exhibit reduced susceptibility to insect (*e.g.*, coleopteran and/or hemipteran) pests. Plant transformation vectors can be prepared, for example, by inserting nucleic acid molecules encoding iRNA molecules into plant transformation vectors and introducing these into plants.

[00183] Suitable methods for transformation of host cells include any method by which DNA can be introduced into a cell, such as by transformation of protoplasts (*See*, *e.g.*, U.S. Patent 5,508,184), by desiccation/inhibition-mediated DNA uptake (*See*, *e.g.*, Potrykus *et al.* (1985) Mol. Gen. Genet. 199:183-8), by electroporation (*See*, *e.g.*, U.S. Patent 5,384,253), by agitation with silicon carbide fibers (*See*, *e.g.*, U.S. Patents 5,302,523 and 5,464,765), by *Agrobacterium*-mediated

transformation (*See*, *e.g.*, U.S. Patents 5,563,055; 5,591,616; 5,693,512; 5,824,877; 5,981,840; and 6,384,301) and by acceleration of DNA-coated particles (*See*, *e.g.*, U.S. Patents 5,015,580; 5,550,318; 5,538,880; 6,160,208; 6,399,861; and 6,403,865), *etc*. Techniques that are particularly useful for transforming corn are described, for example, in U.S. Patents 7,060,876 and 5,591,616; and International PCT Publication WO95/06722. Through the application of techniques such as these, the cells of virtually any species may be stably transformed. In some embodiments, transforming DNA is integrated into the genome of the host cell. In the case of multicellular species, transgenic cells may be regenerated into a transgenic organism. Any of these techniques may be used to produce a transgenic plant, for example, comprising one or more nucleic acids encoding one or more iRNA molecules in the genome of the transgenic plant.

[00184] The most widely utilized method for introducing an expression vector into plants is based on the natural transformation system of *Agrobacterium*. *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria which genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of the plant. The Ti (tumor-inducing)-plasmids contain a large segment, known as T-DNA, which is transferred to transformed plants. Another segment of the Ti plasmid, the Vir region, is responsible for T-DNA transfer. The T-DNA region is bordered by terminal repeats. In modified binary vectors, the tumor-inducing genes have been deleted, and the functions of the Vir region are utilized to transfer foreign DNA bordered by the T-DNA border elements. The T-region may also contain a selectable marker for efficient recovery of transgenic cells and plants, and a multiple cloning site for inserting polynucleotides for transfer such as a dsRNA encoding nucleic acid.

[00185] Thus, in some embodiments, a plant transformation vector is derived from a Ti plasmid of *A. tumefaciens* (*See*, *e.g.*, U.S. Patents 4,536,475, 4,693,977, 4,886,937, and 5,501,967; and European Patent No. EP 0 122 791) or a Ri plasmid of *A. rhizogenes*. Additional plant transformation vectors include, for example and without limitation, those described by Herrera-Estrella *et al.* (1983) Nature 303:209-13; Bevan *et al.* (1983) Nature 304:184-7; Klee *et al.* (1985) Bio/Technol. 3:637-42; and in European Patent No. EP 0 120 516, and those derived from any of the foregoing. Other bacteria such as *Sinorhizobium*, *Rhizobium*, and *Mesorhizobium* that interact

with plants naturally can be modified to mediate gene transfer to a number of diverse plants. These plant-associated symbiotic bacteria can be made competent for gene transfer by acquisition of both a disarmed Ti plasmid and a suitable binary vector.

[00186] After providing exogenous DNA to recipient cells, transformed cells are generally identified for further culturing and plant regeneration. In order to improve the ability to identify transformed cells, one may desire to employ a selectable or screenable marker gene, as previously set forth, with the transformation vector used to generate the transformant. In the case where a selectable marker is used, transformed cells are identified within the potentially transformed cell population by exposing the cells to a selective agent or agents. In the case where a screenable marker is used, cells may be screened for the desired marker gene trait.

[00187] Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In some embodiments, any suitable plant tissue culture media (*e.g.*, MS and N6 media) may be modified by including further substances, such as growth regulators. Tissue may be maintained on a basic medium with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration (*e.g.*, at least 2 weeks), then transferred to media conducive to shoot formation. Cultures are transferred periodically until sufficient shoot formation has occurred. Once shoots are formed, they are transferred to media conducive to root formation. Once sufficient roots are formed, plants can be transferred to soil for further growth and maturation.

[00188] To confirm the presence of a nucleic acid molecule of interest (for example, a DNA encoding one or more iRNA molecules that inhibit target gene expression in a coleopteran and/or hemipteran pest) in the regenerating plants, a variety of assays may be performed. Such assays include, for example: molecular biological assays, such as Southern and northern blotting, PCR, and nucleic acid sequencing; biochemical assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISA and/or western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and analysis of the phenotype of the whole regenerated plant.

[00189] Integration events may be analyzed, for example, by PCR amplification using, *e.g.*, oligonucleotide primers specific for a nucleic acid molecule of interest. PCR genotyping is understood to include, but not be limited to, polymerase-chain reaction (PCR) amplification of gDNA derived from isolated host plant callus tissue predicted to contain a nucleic acid molecule of interest integrated into the genome, followed by standard cloning and sequence analysis of PCR amplification products. Methods of PCR genotyping have been well described (for example, Rios, G. *et al.* (2002) Plant J. 32:243-53) and may be applied to gDNA derived from any plant species (*e.g.*, *Z. mays* or *G. max*) or tissue type, including cell cultures.

[00190] A transgenic plant formed using *Agrobacterium*-dependent transformation methods typically contains a single recombinant DNA inserted into one chromosome. The polynucleotide of the single recombinant DNA is referred to as a "transgenic event" or "integration event". Such transgenic plants are heterozygous for the inserted exogenous polynucleotide. In some embodiments, a transgenic plant homozygous with respect to a transgene may be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single exogenous gene to itself, for example a T_0 plant, to produce T_1 seed. One fourth of the T_1 seed produced will be homozygous with respect to the transgene. Germinating T_1 seed results in plants that can be tested for heterozygosity, typically using an SNP assay or a thermal amplification assay that allows for the distinction between heterozygotes and homozygotes (*i.e.*, a zygosity assay).

[00191] In particular embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 or more different iRNA molecules are produced in a plant cell that have an insect (*e.g.*, coleopteran and/or hemipteran) pest-inhibitory effect. The iRNA molecules (*e.g.*, dsRNA molecules) may be expressed from multiple nucleic acids introduced in different transformation events, or from a single nucleic acid introduced in a single transformation event. In some embodiments, a plurality of iRNA molecules are expressed under the control of a single promoter. In other embodiments, a plurality of iRNA molecules are expressed under the control of multiple promoters. Single iRNA molecules may be expressed that comprise multiple polynucleotides that are each homologous to different loci within one or more insect pests (for example, the loci defined by SEQ ID NO:1 or SEQ ID NO:71), both in different populations of the same species of insect pest, or in different species of insect pests.

[00192] In addition to direct transformation of a plant with a recombinant nucleic acid molecule, transgenic plants can be prepared by crossing a first plant having at least one transgenic event with a second plant lacking such an event. For example, a recombinant nucleic acid molecule comprising a polynucleotide that encodes an iRNA molecule may be introduced into a first plant line that is amenable to transformation to produce a transgenic plant, which transgenic plant may be crossed with a second plant line to introgress the polynucleotide that encodes the iRNA molecule into the second plant line.

[00193] The invention also includes commodity products containing one or more of the sequences of the present invention. Particular embodiments include commodity products produced from a recombinant plant or seed containing one or more of the nucleotide sequences of the present invention. A commodity product containing one or more of the sequences of the present invention is intended to include, but not be limited to, meals, oils, crushed or whole grains or seeds of a plant, or any food or animal feed product comprising any meal, oil, or crushed or whole grain of a recombinant plant or seed containing one or more of the sequences of the present invention. The detection of one or more of the sequences of the present invention in one or more commodity or commodity products contemplated herein is *de facto* evidence that the commodity or commodity product is produced from a transgenic plant designed to express one or more of the nucleotides sequences of the present invention for the purpose of controlling coleopteran and/or hemipteran plant pests using dsRNA-mediated gene suppression methods.

[00194] In some aspects, seeds and commodity products produced by transgenic plants derived from transformed plant cells are included, wherein the seeds or commodity products comprise a detectable amount of a nucleic acid of the invention. In some embodiments, such commodity products may be produced, for example, by obtaining transgenic plants and preparing food or feed from them. Commodity products comprising one or more of the polynucleotides of the invention includes, for example and without limitation: meals, oils, crushed or whole grains or seeds of a plant, and any food product comprising any meal, oil, or crushed or whole grain of a recombinant plant or seed comprising one or more of the nucleic acids of the invention. The detection of one or more of the polynucleotides of the invention in one or more commodity or

commodity products is *de facto* evidence that the commodity or commodity product is produced from a transgenic plant designed to express one or more of the iRNA molecules of the invention for the purpose of controlling insect (*e.g.*, coleopteran and/or hemipteran) pests.

[00195] In some embodiments, a transgenic plant or seed comprising a nucleic acid molecule of the invention also may comprise at least one other transgenic event in its genome, including without limitation: a transgenic event from which is transcribed an iRNA molecule targeting a locus in an insect pest other than the one defined by SEQ ID NO:1 or SEQ ID NO:71, such as, for example, one or more loci selected from the group consisting of Caf1-180 (U.S. Patent Application Publication No. 2012/0174258), VatpaseC (U.S. Patent Application Publication No. 2012/0174259), Rho1 (U.S. Patent Application Publication No. 2012/0174260), VatpaseH (U.S. Patent Application Publication No. 2012/0198586), PPI-87B (U.S. Patent Application Publication No. 2013/0091600), RPA70 (U.S. Patent Application Publication No. 2013/0091601), and RPS6 (U.S. Patent Application Publication No. 2013/0097730); a transgenic event from which is transcribed an iRNA molecule targeting a gene in an organism other than a coleopteran and/or hemipteran pest (*e.g.*, a plant-parasitic nematode); a gene encoding an insecticidal protein (*e.g.*, a *Bacillus thuringiensis, Alcaligenes* spp. (*e.g.*, U.S. Patent Application Publication No. 2014/0033361) or *Pseudomonas* spp. (*e.g.*, PCT Application Publication No. WO2015038734) insecticidal protein); an herbicide tolerance gene (*e.g.*, a gene providing tolerance to glyphosate); and a gene contributing to a desirable phenotype in the transgenic plant, such as increased yield, altered fatty acid metabolism, or restoration of cytoplasmic male sterility). In particular embodiments, polynucleotides encoding iRNA molecules of the invention may be combined with other insect control and disease traits in a plant to achieve desired traits for enhanced control of plant disease and insect damage. Combining insect control traits that employ distinct modes-of-action may provide protected transgenic plants with superior durability over plants harboring a single control trait, for example, because of the reduced probability that resistance to the trait(s) will develop in the field.

V. Target Gene Suppression in a Coleopteran and/or Hemipteran Pest

A. Overview

[00196] In some embodiments of the invention, at least one nucleic acid molecule useful for the control of coleopteran and/or hemipteran pests may be provided to a coleopteran and/or hemipteran pest, wherein the nucleic acid molecule leads to RNAi-mediated gene silencing in the pest(s). In particular embodiments, an iRNA molecule (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) may be provided to the coleopteran and/or hemipteran host. In some embodiments, a nucleic acid molecule useful for the control of coleopteran and/or hemipteran pests may be provided to a pest by contacting the nucleic acid molecule with the pest. In these and further embodiments, a nucleic acid molecule useful for the control of coleopteran and/or hemipteran pests may be provided in a feeding substrate of the pest, for example, a nutritional composition. In these and further embodiments, a nucleic acid molecule useful for the control of a coleopteran and/or hemipteran pest may be provided through ingestion of plant material comprising the nucleic acid molecule that is ingested by the pest. In certain embodiments, the nucleic acid molecule is present in plant material through expression of a recombinant nucleic acid introduced into the plant material, for example, by transformation of a plant cell with a vector comprising the recombinant nucleic acid and regeneration of a plant material or whole plant from the transformed plant cell.

B. RNAi-mediated Target Gene Suppression

[00197] In embodiments, the invention provides iRNA molecules (*e.g.*, dsRNA, siRNA, miRNA, shRNA, and hpRNA) that may be designed to target essential native polynucleotides (*e.g.*, essential genes) in the transcriptome of an insect pest (for example, a coleopteran (*e.g.*, WCR or NCR) or hemipteran (*e.g.*, BSB) pest), for example by designing an iRNA molecule that comprises at least one strand comprising a polynucleotide that is specifically complementary to the target polynucleotide. The sequence of an iRNA molecule so designed may be identical to that of the target polynucleotide, or may incorporate mismatches that do not prevent specific hybridization between the iRNA molecule and its target polynucleotide.

[00198] iRNA molecules of the invention may be used in methods for gene suppression in an insect (*e.g.*, coleopteran and/or hemipteran) pest, thereby reducing the level or incidence of damage caused by the pest on a plant (for example, a protected transformed plant comprising an

iRNA molecule). As used herein the term "gene suppression" refers to any of the well-known methods for reducing the levels of protein produced as a result of gene transcription to mRNA and subsequent translation of the mRNA, including the reduction of protein expression from a gene or a coding polynucleotide including post-transcriptional inhibition of expression and transcriptional suppression. Post-transcriptional inhibition is mediated by specific homology between all or a part of an mRNA transcribed from a gene targeted for suppression and the corresponding iRNA molecule used for suppression. Additionally, post-transcriptional inhibition refers to the substantial and measurable reduction of the amount of mRNA available in the cell for binding by ribosomes.

[00199] In embodiments wherein an iRNA molecule is a dsRNA molecule, the dsRNA molecule may be cleaved by the enzyme, DICER, into short siRNA molecules (approximately 20 nucleotides in length). The double-stranded siRNA molecule generated by DICER activity upon the dsRNA molecule may be separated into two single-stranded siRNAs; the "passenger strand" and the "guide strand". The passenger strand may be degraded, and the guide strand may be incorporated into RISC. Post-transcriptional inhibition occurs by specific hybridization of the guide strand with a specifically complementary polynucleotide of an mRNA molecule, and subsequent cleavage by the enzyme, Argonaute (catalytic component of the RISC complex).

[00200] In embodiments of the invention, any form of iRNA molecule may be used. Those of skill in the art will understand that dsRNA molecules typically are more stable during preparation and during the step of providing the iRNA molecule to a cell than are single-stranded RNA molecules, and are typically also more stable in a cell. Thus, while siRNA and miRNA molecules, for example, may be equally effective in some embodiments, a dsRNA molecule may be chosen due to its stability.

[00201] In particular embodiments, a nucleic acid molecule is provided that comprises a polynucleotide, which polynucleotide may be expressed *in vitro* to produce an iRNA molecule that is substantially homologous to a nucleic acid molecule encoded by a polynucleotide within the genome of an insect (*e.g.*, coleopteran and/or hemipteran) pest. In certain embodiments, the *in vitro* transcribed iRNA molecule may be a stabilized dsRNA molecule that comprises a stem-loop

structure. After an insect pest contacts the *in vitro* transcribed iRNA molecule, post-transcriptional inhibition of a target gene in the pest (for example, an essential gene) may occur.

[00202] In some embodiments of the invention, expression of a nucleic acid molecule comprising at least 15 contiguous nucleotides (*e.g.*, at least 19 contiguous nucleotides) of a polynucleotide are used in a method for post-transcriptional inhibition of a target gene in an insect (*e.g.*, coleopteran and/or hemipteran) pest, wherein the polynucleotide is selected from the group consisting of: SEQ ID NO:1; the complement of SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of an RNA expressed from a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of an RNA expressed from a native coding polynucleotide of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of an RNA expressed from a native coding polynucleotide of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1. In certain embodiments, expression of a nucleic acid molecule that is at least about 80% identical (*e.g.*, 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, and 100%) with any of the foregoing may be used.

[00203] In certain embodiments of the invention, expression of a nucleic acid molecule comprising at least 15 contiguous nucleotides of a nucleotide sequence is used in a method for post-

transcriptional inhibition of a target gene in a hemipteran pest, wherein the nucleotide sequence is selected from the group consisting of: SEQ ID NO:71; the complement of SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; a native coding sequence of a hemipteran organism SEQ ID NO:71; the complement of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71. In certain embodiments, expression of a nucleic acid molecule that is at least 80% identical (e.g., 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, and 100%) with any of the foregoing may be used. In these and further embodiments, a nucleic acid molecule may be expressed that specifically hybridizes to an RNA molecule present in at least one cell of an insect (*e.g.*, coleopteran and/or hemipteran) pest.

[00204] In some embodiments, expression of at least one nucleic acid molecule comprising at least 15 contiguous nucleotides of a nucleotide sequence may be used in a method for post-transcriptional inhibition of a target gene in a coleopteran pest, wherein the nucleotide sequence is selected from the group consisting of: SEQ ID NO:1; the complement of SEQ ID NO:1; a fragment

of at least 15 contiguous nucleotides of SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; the complement of a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; the complement of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism (*e.g.*, WCR) comprising SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a *Diabrotica* organism that is transcribed into a native RNA molecule comprising SEQ ID NO:1. In certain embodiments, expression of a nucleic acid molecule that is at least 80% identical (*e.g.*, 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, and 100%) with any of the foregoing may be used. In these and further embodiments, a nucleic acid molecule may be expressed that specifically hybridizes to an RNA molecule present in at least one cell of a coleopteran pest. In particular examples, such a nucleic acid molecule may comprise a nucleotide sequence comprising SEQ ID NO:1.

[00205] In particular embodiments of the invention, expression of a nucleic acid molecule comprising at least 15 contiguous nucleotides of a nucleotide sequence is used in a method for post-transcriptional inhibition of a target gene in a hemipteran pest, wherein the nucleotide sequence is selected from the group consisting of: SEQ ID NO:71; the complement of SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:71; a native coding sequence of a hemipteran organism SEQ ID NO:71; the complement of a native coding sequence of a hemipteran organism

comprising SEQ ID NO:71; a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; the complement of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a hemipteran organism comprising SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71; and the complement of a fragment of at least 15 contiguous nucleotides of a native non-coding sequence of a hemipteran organism that is transcribed into a native RNA molecule comprising SEQ ID NO:71. In certain embodiments, expression of a nucleic acid molecule that is at least 80% identical (e.g., 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 100%, and 100%) with any of the foregoing may be used. In these and further embodiments, a nucleic acid molecule may be expressed that specifically hybridizes to an RNA molecule present in at least one cell of a hemipteran pest. In particular examples, such a nucleic acid molecule may comprise a nucleotide sequence comprising SEQ ID NO:71.

[00206] It is an important feature of some embodiments herein that the RNAi post-transcriptional inhibition system is able to tolerate sequence variations among target genes that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence. The introduced nucleic acid molecule may not need to be absolutely homologous to either a primary transcription product or a fully-processed mRNA of a target gene, so long as the introduced nucleic acid molecule is specifically hybridizable to either a primary transcription product or a fully-processed mRNA of the target gene. Moreover, the introduced nucleic acid molecule may not need

to be full-length, relative to either a primary transcription product or a fully processed mRNA of the target gene.

[00207] Inhibition of a target gene using the iRNA technology of the present invention is sequence-specific; *i.e.*, polynucleotides substantially homologous to the iRNA molecule(s) are targeted for genetic inhibition. In some embodiments, an RNA molecule comprising a polynucleotide with a nucleotide sequence that is identical to that of a portion of a target gene may be used for inhibition. In these and further embodiments, an RNA molecule comprising a polynucleotide with one or more insertion, deletion, and/or point mutations relative to a target polynucleotide may be used. In particular embodiments, an iRNA molecule and a portion of a target gene may share, for example, at least from about 80%, at least from about 81%, at least from about 82%, at least from about 83%, at least from about 84%, at least from about 85%, at least from about 86%, at least from about 87%, at least from about 88%, at least from about 89%, at least from about 90%, at least from about 91%, at least from about 92%, at least from about 93%, at least from about 94%, at least from about 95%, at least from about 96%, at least from about 97%, at least from about 98%, at least from about 99%, at least from about 100%, and 100% sequence identity. Alternatively, the duplex region of a dsRNA molecule may be specifically hybridizable with a portion of a target gene transcript. In specifically hybridizable molecules, a less than full length polynucleotide exhibiting a greater homology compensates for a longer, less homologous polynucleotide. The length of the polynucleotide of a duplex region of a dsRNA molecule that is identical to a portion of a target gene transcript may be at least about 25, 50, 100, 200, 300, 400, 500, or at least about 1000 bases. In some embodiments, a polynucleotide of greater than 20-100 nucleotides may be used. In particular embodiments, a polynucleotide of greater than about 200-300 nucleotides may be used. In particular embodiments, a polynucleotide of greater than about 500-1000 nucleotides may be used, depending on the size of the target gene.

[00208] In certain embodiments, expression of a target gene in a pest (*e.g.*, coleopteran or hemipteran) pest may be inhibited by at least 10%; at least 33%; at least 50%; or at least 80% within a cell of the pest, such that a significant inhibition takes place. Significant inhibition refers to inhibition over a threshold that results in a detectable phenotype (*e.g.*, cessation of growth, cessation

of feeding, cessation of development, induced mortality, *etc.*), or a detectable decrease in RNA and/or gene product corresponding to the target gene being inhibited. Although, in certain embodiments of the invention, inhibition occurs in substantially all cells of the pest, in other embodiments inhibition occurs only in a subset of cells expressing the target gene.

[00209] In some embodiments, transcriptional suppression is mediated by the presence in a cell of a dsRNA molecule exhibiting substantial sequence identity to a promoter DNA or the complement thereof to effect what is referred to as "promoter trans suppression." Gene suppression may be effective against target genes in an insect pest that may ingest or contact such dsRNA molecules, for example, by ingesting or contacting plant material containing the dsRNA molecules. dsRNA molecules for use in promoter trans suppression may be specifically designed to inhibit or suppress the expression of one or more homologous or complementary polynucleotides in the cells of the insect pest. Post-transcriptional gene suppression by antisense or sense oriented RNA to regulate gene expression in plant cells is disclosed in U.S. Patents 5,107,065; 5,759,829; 5,283,184; and 5,231,020.

C. Expression of iRNA Molecules Provided to an Insect Pest

[00210] Expression of iRNA molecules for RNAi-mediated gene inhibition in an insect (*e.g.*, coleopteran and/or hemipteran) pest may be carried out in any one of many *in vitro* or *in vivo* formats. The iRNA molecules may then be provided to an insect pest, for example, by contacting the iRNA molecules with the pest, or by causing the pest to ingest or otherwise internalize the iRNA molecules. Some embodiments include transformed host plants of a coleopteran and/or hemipteran pest, transformed plant cells, and progeny of transformed plants. The transformed plant cells and transformed plants may be engineered to express one or more of the iRNA molecules, for example, under the control of a heterologous promoter, to provide a pest-protective effect. Thus, when a transgenic plant or plant cell is consumed by an insect pest during feeding, the pest may ingest iRNA molecules expressed in the transgenic plants or cells. The polynucleotides of the present invention may also be introduced into a wide variety of prokaryotic and eukaryotic microorganism hosts to produce iRNA molecules. The term "microorganism" includes prokaryotic and eukaryotic species, such as bacteria and fungi.

[00211] Modulation of gene expression may include partial or complete suppression of such expression. In another embodiment, a method for suppression of gene expression in an insect (*e.g.*, coleopteran and/or hemipteran) pest comprises providing in the tissue of the host of the pest a gene-suppressive amount of at least one dsRNA molecule formed following transcription of a polynucleotide as described herein, at least one segment of which is complementary to an mRNA within the cells of the insect pest. A dsRNA molecule, including its modified form such as an siRNA, miRNA, shRNA, or hpRNA molecule, ingested by an insect pest may be at least from about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100% identical to an RNA molecule transcribed from a molecule comprising a nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:71. Isolated and substantially purified nucleic acid molecules including, but not limited to, non-naturally occurring polynucleotides and recombinant DNA constructs for providing dsRNA molecules are therefore provided, which suppress or inhibit the expression of an endogenous coding polynucleotide or a target coding polynucleotide in an insect pest when introduced thereto.

[00212] Particular embodiments provide a delivery system for the delivery of iRNA molecules for the post-transcriptional inhibition of one or more target gene(s) in an insect (*e.g.*, coleopteran and/or hemipteran) plant pest and control of a population of the plant pest. In some embodiments, the delivery system comprises ingestion of a host transgenic plant cell or contents of the host cell comprising RNA molecules transcribed in the host cell. In these and further embodiments, a transgenic plant cell or a transgenic plant is created that contains a recombinant DNA construct providing a stabilized dsRNA molecule of the invention. Transgenic plant cells and transgenic plants comprising nucleic acids encoding a particular iRNA molecule may be produced by employing recombinant DNA technologies (which basic technologies are well-known in the art) to construct a plant transformation vector comprising a polynucleotide encoding an iRNA molecule of the invention (*e.g.*, a stabilized dsRNA molecule); to transform a plant cell or plant; and to generate the transgenic plant cell or the transgenic plant that contains the transcribed iRNA molecule.

[00213] To impart protection from insect (*e.g.*, coleopteran and/or hemipteran) pests to a transgenic plant, a recombinant DNA molecule may, for example, be transcribed into an iRNA molecule, such as a dsRNA molecule, an siRNA molecule, an miRNA molecule, an shRNA molecule, or an hpRNA molecule. In some embodiments, an RNA molecule transcribed from a recombinant DNA molecule may form a dsRNA molecule within the tissues or fluids of the recombinant plant. Such a dsRNA molecule may be comprised in part of a polynucleotide that is identical to a corresponding polynucleotide transcribed from a DNA within an insect pest of a type that may infest the host plant. Expression of a target gene within the pest is suppressed by the dsRNA molecule, and the suppression of expression of the target gene in the pest results in the transgenic plant being resistant to the pest. The modulatory effects of dsRNA molecules have been shown to be applicable to a variety of genes expressed in pests, including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including house-keeping genes; transcription factors; molting-related genes; and other genes which encode polypeptides involved in cellular metabolism or normal growth and development.

[00214] For transcription from a transgene *in vivo* or an expression construct, a regulatory region (*e.g.*, promoter, enhancer, silencer, and polyadenylation signal) may be used in some embodiments to transcribe the RNA strand (or strands). Therefore, in some embodiments, as set forth, *supra*, a polynucleotide for use in producing iRNA molecules may be operably linked to one or more promoter elements functional in a plant host cell. The promoter may be an endogenous promoter, normally resident in the host genome. The polynucleotide of the present invention, under the control of an operably linked promoter element, may further be flanked by additional elements that advantageously affect its transcription and/or the stability of a resulting transcript. Such elements may be located upstream of the operably linked promoter, downstream of the 3' end of the expression construct, and may occur both upstream of the promoter and downstream of the 3' end of the expression construct.

[00215] Some embodiments provide methods for reducing the damage to a host plant (*e.g.*, a corn plant) caused by an insect (*e.g.*, coleopteran and/or hemipteran) pest that feeds on the plant, wherein the method comprises providing in the host plant a transformed plant cell expressing at

least one nucleic acid molecule of the invention, wherein the nucleic acid molecule(s) functions upon being taken up by the pest(s) to inhibit the expression of a target polynucleotide within the pest(s), which inhibition of expression results in mortality and/or reduced growth of the pest(s), thereby reducing the damage to the host plant caused by the pest(s). In some embodiments, the nucleic acid molecule(s) comprise dsRNA molecules. In these and further embodiments, the nucleic acid molecule(s) comprise dsRNA molecules that each comprise more than one polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in a coleopteran and/or hemipteran pest cell. In some embodiments, the nucleic acid molecule(s) consist of one polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in an insect pest cell.

[00216] In some embodiments, a method for increasing the yield of a corn crop is provided, wherein the method comprises introducing into a corn plant at least one nucleic acid molecule of the invention; cultivating the corn plant to allow the expression of an iRNA molecule comprising the nucleic acid, wherein expression of an iRNA molecule comprising the nucleic acid inhibits insect (*e.g.*, coleopteran and/or hemipteran) pest damage and/or growth, thereby reducing or eliminating a loss of yield due to pest infestation. In some embodiments, the iRNA molecule is a dsRNA molecule. In these and further embodiments, the nucleic acid molecule(s) comprise dsRNA molecules that each comprise more than one polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in an insect pest cell. In some examples, the nucleic acid molecule(s) comprises a polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in a coleopteran and/or hemipteran pest cell.

[00217] In some embodiments, a method for modulating the expression of a target gene in an insect (*e.g.*, coleopteran and/or hemipteran) pest is provided, the method comprising: transforming a plant cell with a vector comprising a polynucleotide encoding at least one iRNA molecule of the invention, wherein the polynucleotide is operatively-linked to a promoter and a transcription termination element; culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture including a plurality of transformed plant cells; selecting for transformed plant cells that have integrated the polynucleotide into their genomes;

screening the transformed plant cells for expression of an iRNA molecule encoded by the integrated polynucleotide; selecting a transgenic plant cell that expresses the iRNA molecule; and feeding the selected transgenic plant cell to the insect pest. Plants may also be regenerated from transformed plant cells that express an iRNA molecule encoded by the integrated nucleic acid molecule. In some embodiments, the iRNA molecule is a dsRNA molecule. In these and further embodiments, the nucleic acid molecule(s) comprise dsRNA molecules that each comprise more than one polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in an insect pest cell. In some examples, the nucleic acid molecule(s) comprises a polynucleotide that is specifically hybridizable to a nucleic acid molecule expressed in a coleopteran and/or hemipteran pest cell.

[00218] iRNA molecules of the invention can be incorporated within the seeds of a plant species (*e.g.*, corn), either as a product of expression from a recombinant gene incorporated into a genome of the plant cells, or as incorporated into a coating or seed treatment that is applied to the seed before planting. A plant cell comprising a recombinant gene is considered to be a transgenic event. Also included in embodiments of the invention are delivery systems for the delivery of iRNA molecules to insect (*e.g.*, coleopteran and/or hemipteran) pests. For example, the iRNA molecules of the invention may be directly introduced into the cells of a pest(s). Methods for introduction may include direct mixing of iRNA with plant tissue from a host for the insect pest(s), as well as application of compositions comprising iRNA molecules of the invention to host plant tissue. For example, iRNA molecules may be sprayed onto a plant surface. Alternatively, an iRNA molecule may be expressed by a microorganism, and the microorganism may be applied onto the plant surface, or introduced into a root or stem by a physical means such as an injection. As discussed, *supra*, a transgenic plant may also be genetically engineered to express at least one iRNA molecule in an amount sufficient to kill the insect pests known to infest the plant. iRNA molecules produced by chemical or enzymatic synthesis may also be formulated in a manner consistent with common agricultural practices, and used as spray-on products for controlling plant damage by an insect pest. The formulations may include the appropriate adjuvants (*e.g.,* stickers and wetters) required for efficient foliar coverage, as well as UV protectants to protect iRNA molecules (*e.g.*, dsRNA molecules) from UV damage. Such additives are commonly used in the bioinsecticide

industry, and are well known to those skilled in the art. Such applications may be combined with other spray-on insecticide applications (biologically based or otherwise) to enhance plant protection from the pests.

[00219] All references, including publications, patents, and patent applications, cited herein are hereby incorporated by reference to the extent they are not inconsistent with the explicit details of this disclosure, and are so incorporated to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

[00220] The following EXAMPLES are provided to illustrate certain particular features and/or aspects. These EXAMPLES should not be construed to limit the disclosure to the particular features or aspects described.

EXAMPLES

EXAMPLE 1
Insect Diet Bioassays

[00221] Sample preparation and bioassays A number of dsRNA molecules (including those corresponding to *COPI delta* reg1 (SEQ ID NO:3) and *COPI delta* v1 (SEQ ID NO:4) were synthesized and purified using a MEGASCRIPT® RNAi kit. The purified dsRNA molecules were prepared in TE buffer, and all bioassays contained a control treatment consisting of this buffer, which served as a background check for mortality or growth inhibition of WCR (*Diabrotica virgifera virgifera* LeConte). The concentrations of dsRNA molecules in the bioassay buffer were measured using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE).

[00222] Samples were tested for insect activity in bioassays conducted with neonate insect larvae on artificial insect diet. WCR eggs were obtained from CROP CHARACTERISTICS, INC. (Farmington, MN).

[00223] The bioassays were conducted in 128-well plastic trays specifically designed for insect bioassays (C-D INTERNATIONAL, Pitman, NJ). Each well contained approximately 1.0 mL of an artificial diet designed for growth of coleopteran insects. A 60 μL aliquot of dsRNA sample was delivered by pipette onto the surface of the diet of each well (40 $\mu L/cm^2$). dsRNA sample concentrations were calculated as the amount of dsRNA per square centimeter (ng/cm^2) of surface area (1.5 cm^2) in the well. The treated trays were held in a fume hood until the liquid on the diet surface evaporated or was absorbed into the diet.

[00224] Within a few hours of eclosion, individual larvae were picked up with a moistened camel hair brush and deposited on the treated diet (one or two larvae per well). The infested wells of the 128-well plastic trays were then sealed with adhesive sheets of clear plastic, and vented to allow gas exchange. Bioassay trays were held under controlled environmental conditions (28°C, ~40% Relative Humidity, 16:8 (Light:Dark)) for 9 days, after which time the total number of insects exposed to each sample, the number of dead insects, and the weight of surviving insects were recorded. Average percent mortality and average growth inhibition were calculated for each treatment. Growth inhibition (GI) was calculated as follows:

GI = [1 – (TWIT/TNIT)/(TWIBC/TNIBC)]
where TWIT is the Total Weight of live Insects in the Treatment;
TNIT is the Total Number of Insects in the Treatment;
TWIBC is the Total Weight of live Insects in the Background Check (Buffer control); and
TNIBC is the Total Number of Insects in the Background Check (Buffer control).

[00225] Statistical analysis was done using JMP™ software (SAS, Cary, NC).

[00226] LC_{50} (Lethal Concentration) is defined as the dosage at which 50% of the test insects are killed. GI_{50} (Growth Inhibition) is defined as the dosage at which the mean growth (*e.g.* live weight) of the test insects is 50% of the mean value seen in Background Check samples.

[00227] Replicated bioassays demonstrated that ingestion of particular samples resulted in a surprising and unexpected mortality and growth inhibition of corn rootworm larvae.

EXAMPLE 2
Identification of Candidate Target Genes

[00228] Multiple stages of WCR (*Diabrotica virgifera virgifera* LeConte) development were selected for pooled transcriptome analysis to provide candidate target gene sequences for control by RNAi transgenic plant insect resistance technology.

[00229] In one exemplification, total RNA was isolated from about 0.9 gm whole first-instar WCR larvae; (4 to 5 days post-hatch; held at 16°C), and purified using the following phenol/TRI REAGENT®-based method (MOLECULAR RESEARCH CENTER, Cincinnati, OH):

[00230] Larvae were homogenized at room temperature in a 15 mL homogenizer with 10 mL of TRI REAGENT® until a homogenous suspension was obtained. Following 5 min. incubation at room temperature, the homogenate was dispensed into 1.5 mL microfuge tubes (1 mL per tube), 200 µL of chloroform was added, and the mixture was vigorously shaken for 15 seconds. After allowing the extraction to sit at room temperature for 10 min, the phases were separated by centrifugation at 12,000 x g at 4°C. The upper phase (comprising about 0.6 mL) was carefully transferred into another sterile 1.5 mL tube, and an equal volume of room temperature isopropanol was added. After incubation at room temperature for 5 to 10 min, the mixture was centrifuged 8 min at 12,000 x g (4°C or 25°C).

[00231] The supernatant was carefully removed and discarded, and the RNA pellet was washed twice by vortexing with 75% ethanol, with recovery by centrifugation for 5 min at 7,500 x g (4°C or 25°C) after each wash. The ethanol was carefully removed, the pellet was allowed to air-dry for 3 to 5 min, and then was dissolved in nuclease-free sterile water. RNA concentration was determined by measuring the absorbance (A) at 260 nm and 280 nm. A typical extraction from about 0.9 gm of larvae yielded over 1 mg of total RNA, with an A_{260}/A_{280} ratio of 1.9. The RNA thus extracted was stored at -80°C until further processed.

[00232] RNA quality was determined by running an aliquot through a 1% agarose gel. The agarose gel solution was made using autoclaved 10x TAE buffer (Tris-acetate EDTA; 1x concentration is 0.04 M Tris-acetate, 1 mM EDTA (ethylenediamine tetra-acetic acid sodium salt), pH 8.0) diluted with DEPC (diethyl pyrocarbonate)-treated water in an autoclaved container. 1x TAE was used as the running buffer. Before use, the electrophoresis tank and the well-forming comb were cleaned with RNaseAway™ (INVITROGEN INC., Carlsbad, CA). Two µL of RNA

sample were mixed with 8 µL of TE buffer (10 mM Tris HCl pH 7.0; 1 mM EDTA) and 10 µL of RNA sample buffer (NOVAGEN® Catalog No 70606; EMD4 Bioscience, Gibbstown, NJ). The sample was heated at 70°C for 3 min, cooled to room temperature, and 5 µL (containing 1 µg to 2 µg RNA) were loaded per well. Commercially available RNA molecular weight markers were simultaneously run in separate wells for molecular size comparison. The gel was run at 60 volts for 2 hr.

[00233] A normalized cDNA library was prepared from the larval total RNA by a commercial service provider (EUROFINS MWG Operon, Huntsville, AL), using random priming. The normalized larval cDNA library was sequenced at 1/2 plate scale by GS FLX 454 Titanium™ series chemistry at EUROFINS MWG Operon, which resulted in over 600,000 reads with an average read length of 348 bp. 350,000 reads were assembled into over 50,000 contigs. Both the unassembled reads and the contigs were converted into BLASTable databases using the publicly available program, FORMATDB (available from NCBI).

[00234] Total RNA and normalized cDNA libraries were similarly prepared from materials harvested at other WCR developmental stages. A pooled transcriptome library for target gene screening was constructed by combining cDNA library members representing the various developmental stages.

[00235] Candidate genes for RNAi targeting were selected using information regarding lethal RNAi effects of particular genes in other insects such as *Drosophila* and *Tribolium*. These genes were hypothesized to be essential for survival and growth in coleopteran insects. Selected target gene homologs were identified in the transcriptome sequence database as described below. Full-length or partial sequences of the target genes were amplified by PCR to prepare templates for double-stranded RNA (dsRNA) production.

[00236] TBLASTN searches using candidate protein coding sequences were run against BLASTable databases containing the unassembled *Diabrotica* sequence reads or the assembled contigs. Significant hits to a *Diabrotica* sequence (defined as better than e^{-20} for contigs homologies and better than e^{-10} for unassembled sequence reads homologies) were confirmed using BLASTX against the NCBI non-redundant database. The results of this BLASTX search confirmed that the

Diabrotica homolog candidate gene sequences identified in the TBLASTN search indeed comprised *Diabrotica* genes, or were the best hit to the non-*Diabrotica* candidate gene sequence present in the *Diabrotica* sequences. In most cases, *Tribolium* candidate genes which were annotated as encoding a protein gave an unambiguous sequence homology to a sequence or sequences in the *Diabrotica* transcriptome sequences. In a few cases, it was clear that some of the *Diabrotica* contigs or unassembled sequence reads selected by homology to a non-*Diabrotica* candidate gene overlapped, and that the assembly of the contigs had failed to join these overlaps. In those cases, Sequencher™ v4.9 (GENE CODES CORPORATION, Ann Arbor, MI) was used to assemble the sequences into longer contigs.

[00237] A candidate target gene encoding *Diabrotica COPI delta* (SEQ ID NO:1) was identified as a gene that may lead to coleopteran pest mortality, inhibition of growth, inhibition of development, or inhibition of reproduction in WCR.

Genes with Homology to WCR *COPI delta*

[00238] COPI refers to the specific coat protein complex that inhibits the budding process on the cis-Golgi membrane (Nickel,et al. 2002. Journal of Cell Science 115, 3235-3240). The COPI coatomer complex consists of seven subunits. COPI coatomer delta is one of the subunits. The function of the complex is to transport vesicles from the cis-end of the Golgi complex back to the rough endoplasmic reticulum, where they were originally synthesized. Other *Diabrotica virgifera* proteins that also contain this domain may share structural and/or functional properties, and thus a gene that encodes one of these proteins may comprise a candidate target gene that may lead to coleopteran pest mortality, inhibition of growth, inhibition of development, or inhibition of reproduction in WCR.

[00239] The sequence of SEQ ID NO:1 is novel. The sequence is not provided in public databases and is not disclosed in WO/2011/025860; U.S. Patent Application No. 20070124836; U.S. Patent Application No. 20090306189; U.S. Patent Application No. US20070050860; U.S. Patent Application No.20100192265;or U.S. Patent No.7,612,194. There was no significant homologous nucleotide sequence found with a search in GENBANK. The closest homolog of the *Diabrotica COPI delta* amino acid sequence (SEQ ID NO:2) is a *Tribolium casetanum* protein

having GENBANK Accession No. XP_967725.2 (94% similar; 85% identical over the homology region).

[00240] *COPI delta* dsRNA transgenes can be combined with other dsRNA molecules to provide redundant RNAi targeting and synergistic RNAi effects. Transgenic corn events expressing dsRNA that targets *COPI delta* are useful for preventing root feeding damage by corn rootworm. *COPI delta* dsRNA transgenes represent new modes of action for combining with *Bacillus thuringiensis*, *Alcaligenes* spp., or *Pseudomonas* spp. insecticidal protein technology in Insect Resistance Management gene pyramids to mitigate against the development of rootworm populations resistant to either of these rootworm control technologies.

[00241] Full-length or partial clones of sequences of a *Diabrotica* candidate gene, herein referred to as *COPI delta*, were used to generate PCR amplicons for dsRNA synthesis.

[00242] SEQ ID NO:1 shows a 1539 bp DNA sequence of *Diabrotica COPI delta*.

[00243] SEQ ID NO:3 shows a 672 bp DNA sequence of *COPI delta* reg1.

[00244] SEQ ID NO:4 shows a 100 bp DNA sequence of *COPI delta* v1.

EXAMPLE 3
Amplification of Target Genes to produce dsRNA

[00245] Primers were designed to amplify portions of coding regions of each target gene by PCR. *See* **Table 1**. Where appropriate, a T7 phage promoter sequence (TTAATACGACTCACTATAGGGAGA; SEQ ID NO:5) was incorporated into the 5' ends of the amplified sense or antisense strands. *See* **Table 1**. Total RNA was extracted from WCR, and first-strand cDNA was used as template for PCR reactions using opposing primers positioned to amplify all or part of the native target gene sequence. dsRNA was also amplified from a DNA clone comprising the coding region for a yellow fluorescent protein (YFP) (SEQ ID NO:6; Shagin *et al.* (2004) Mol. Biol. Evol. 21(5):841-50).

Table 1. Primers and Primer Pairs used to amplify portions of coding regions of exemplary *COPI delta* target gene and YFP negative control gene.

	Gene ID	Primer ID	SEQ ID NO:	Sequence

Pair 1	*COPI delta* reg1	COPI DELTA-FT7	7	TTAATACGACTCACTATAGGGAGA CGATGATGTTCATTTAAGATTGGA
		COPI DELTA–RT7	8	TTAATACGACTCACTATAGGGAGA GGAGAATCATCATCAACTAGGACA A
Pair 2	*COPI delta* v1	*COPI delta*v1-FT7	9	TTAATACGACTCACTATAGGGAGA AATAGGTCGTGATGGTGGC
		*COPI delta*v1–RT7	10	TTAATACGACTCACTATAGGGAGA TTCCAATTGCACACGTATCCT
Pair 3	YFP	YFP-F_T7	21	TTAATACGACTCACTATAGGGAGA CACCATGGGCTCCAGCGGCGCCC
		YFP-R_T7	24	TTAATACGACTCACTATAGGGAGA AGATCTTGAAGGCGCTCTTCAGG

EXAMPLE 4
RNAi Constructs

[00246] Template preparation by PCR and dsRNA synthesis.

[00247] A strategy used to provide specific templates for *COPI delta* and YFP dsRNA production is shown in **Figure 1**. Template DNAs intended for use in *COPI delta* dsRNA synthesis were prepared by PCR using the primer pairs in **Table 1** and (as PCR template) first-strand cDNA prepared from total RNA isolated from WCR first-instar larvae. For each selected *COPI delta* and YFP target gene region, PCR amplifications introduced a T7 promoter sequence at the 5' ends of the amplified sense and antisense strands (the YFP segment was amplified from a DNA clone of the YFP coding region). The PCR products having a T7 promoter sequence at their 5' ends of both sense and antisense strands for each region of a given gene were used for dsRNA generation. *See* **Figure 1**. The sequences of the dsRNA templates amplified with the particular primer pairs were: SEQ ID NO:3 (*COPI delta* reg1), SEQ ID NO:4 (*COPI delta* v1) and YFP (SEQ ID NO:6). Double-stranded RNA for insect bioassay was synthesized and purified using an AMBION® MEGASCRIPT® RNAi kit following the manufacturer's instructions (INVITROGEN). The concentrations of dsRNAs were measured using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE).

[00248] Construction of plant transformation vectors

[00249] An entry vector (pDAB117214) harboring a target gene construct for hairpin formation comprising segments of *COPI delta* (SEQ ID NO:1) was assembled using a combination of chemically synthesized fragments (DNA2.0, Menlo Park, CA) and standard molecular cloning methods. Intramolecular hairpin formation by RNA primary transcripts was facilitated by arranging (within a single transcription unit) two copies of a segment of *COPI delta*target gene sequence in opposite orientation to one another, the two segments being separated by an ST-LS1 intron sequence (SEQ ID NO:13; Vancanneyt *et al.* (1990) Mol. Gen. Genet. 220(2):245-50). Thus, the primary mRNA transcript contains the two *COPI delta* gene segment sequences as large inverted repeats of one another, separated by the intron sequence. A copy of a maize ubiquitin 1 promoter (U.S. Patent No. 5,510,474) was used to drive production of the primary mRNA hairpin transcript, and a fragment comprising a 3' untranslated region from a maize peroxidase 5 gene (ZmPer5 3'UTR v2; U.S. Patent No. 6,699,984) was used to terminate transcription of the hairpin-RNA-expressing gene.

[00250] Entry vector pDAB117214 comprises a *COPI delta* hairpin v1-RNA construct (SEQ ID NO:11) that comprises a segment of *COPI delta* (SEQ ID NO:1)

[00251] Entry vector pDAB117214 described above was used in standard GATEWAY® recombination reactions with a typical binary destination vector (pDAB109805) to produce *COPI delta* hairpin RNA expression transformation vectors for *Agrobacterium*-mediated maize embryo transformations (pDAB114515 and pDAB115770, respectively).

[00252] A negative control binary vector, pDAB110853, which comprises a gene that expresses a YFP hairpin dsRNA, was constructed by means of standard GATEWAY® recombination reactions with a typical binary destination vector (pDAB109805) and entry vector pDAB101670. Entry Vector pDAB101670 comprises a YFP hairpin sequence (SEQ ID NO:12) under the expression control of a maize ubiquitin 1 promoter (as above) and a fragment comprising a 3' untranslated region from a maize peroxidase 5 gene (as above).

[00253] Binary destination vector pDAB109805 comprises a herbicide resistance gene (aryloxyalknoate dioxygenase; AAD-1 v3) (U.S. Patent No. 7838733(B2), and Wright *et al.* (2010)

Proc. Natl. Acad. Sci. U.S.A. 107:20240-5) under the regulation of a sugarcane bacilliform badnavirus (ScBV) promoter (Schenk *et al.* (1999) Plant Molec. Biol. 39:1221-30). A synthetic 5'UTR sequence, comprised of sequences from a Maize Streak Virus (MSV) coat protein gene 5'UTR and intron 6 from a maize Alcohol Dehydrogenase 1 (ADH1) gene, is positioned between the 3' end of the SCBV promoter segment and the start codon of the AAD-1 coding region. A fragment comprising a 3' untranslated region from a maize lipase gene (ZmLip 3'UTR; U.S. Patent No. 7,179,902) was used to terminate transcription of the AAD-1 mRNA.

[00254] A further negative control binary vector, pDAB101556, which comprises a gene that expresses a YFP protein, was constructed by means of standard GATEWAY® recombination reactions with a typical binary destination vector (pDAB9989) and entry vector pDAB100287. Binary destination vector pDAB9989 comprises a herbicide resistance gene (aryloxyalknoate dioxygenase; AAD-1 v3) (as above) under the expression regulation of a maize ubiquitin 1 promoter (as above) and a fragment comprising a 3' untranslated region from a maize lipase gene (ZmLip 3'UTR; as above). Entry Vector pDAB100287 comprises a YFP coding region (SEQ ID NO:14) under the expression control of a maize ubiquitin 1 promoter (as above) and a fragment comprising a 3' untranslated region from a maize peroxidase 5 gene (as above).

[00255] SEQ ID NO:11 presents a *COPI delta* hairpin v1-RNA-forming sequence as found in pDAB117220.

EXAMPLE 5
Screening of Candidate Target Genes

[00256] Synthetic dsRNA designed to inhibit target gene sequences identified in EXAMPLE 2 caused mortality and growth inhibition when administered to WCR in diet-based assays. *COPI delta* reg1 and *COPI delta* v1 were observed to exhibit greatly increased efficacy in this assay over other dsRNAs screened.

[00257] Replicated bioassays demonstrated that ingestion of dsRNA preparations derived from *COPI delta* reg1 and *COPI delta* v1 each resulted in mortality and/or growth inhibition of western corn rootworm larvae. **Table 2** and **Table 3** show the results of diet-based feeding bioassays of WCR larvae following 9-day exposure to these dsRNAs, as well as the results

obtained with a negative control sample of dsRNA prepared from a yellow fluorescent protein (YFP) coding region (SEQ ID NO:6). It should be appreciated by one having ordinary skill in the art that, for the quantitative characteristics identified in **Table 3**, the values presented are typical values. These values may vary due to the environment and accordingly, other values that are substantially equivalent are also within the scope of the embodiments of the disclosure.

Table 2. Results of *COPI delta* dsRNA diet feeding assays obtained with western corn rootworm larvae after 9 days of feeding. ANOVA analysis found significance differences in Mean % Mortality and Mean % Growth Inhibition (GI). Means were separated using the Tukey-Kramer test.

Gene Name	Dose (ng/cm^2)	No. Rows	Mean (%Mortality) ± SEM*	Mean (GI) ± SEM
***COPI delta* reg1**	500	4	52.65 ± 10.58 (A)	0.77 ± 0.06 (A)
***COPI delta* v1**	500	12	49.83 ± 5.62 (A)	0.83 ± 0.04 (A)
TE**	0	14	15.09 ± 2.73 (B)	0.00 ± 0.03 (B)
WATER	0	14	10.92 ± 2.12 (B)	-0.06 ± 0.09 (B)
YFP***	500	14	13.18 ± 2.33 (B)	-0.14 ± 0.20 (B)

*SEM =Standard Error of the Mean. Letters in parentheses designate statistical levels. Levels not connected by same letter are significantly different ($P<0.05$).
**TE = Tris HCl (1 mM) plus EDTA (1 mM) buffer, pH7.2.
***YFP = Yellow Fluorescent Protein

Table 3. Summary of oral potency of *COPI delta* dsRNA on WCR larvae (ng/cm^2).

Gene Name	LC_{50} (ng/cm^2)	Range	GI_{50} (ng/cm^2)	Range
***COPI delta* v1**	12.24	5.30 - 3484804	0.05	0.001- 3.47

[00258] It has previously been suggested that certain genes of *Diabrotica* spp. may be exploited for RNAi-mediated insect control. *See* U.S. Patent Publication No. 2007/0124836, which discloses 906 sequences, and U.S. Patent No. 7,612,194, which discloses 9,112 sequences. However, it was determined that many genes suggested to have utility for RNAi-mediated insect control are not efficacious in controlling *Diabrotica*. It was also determined that sequences *COPI delta* reg1 and *COPI delta* v1 each provide surprising and unexpected superior control of *Diabrotica*, compared to other genes suggested to have utility for RNAi-mediated insect control.

[00259] For example, Annexin, Beta spectrin 2, and mtRP-L4 were each suggested in U.S. Patent No. 7,612,194 to be efficacious in RNAi-mediated insect control. SEQ ID NO:15 is the DNA sequence of Annexin region 1 (Reg 1), and SEQ ID NO:16 is the DNA sequence of Annexin region 2 (Reg 2). SEQ ID NO:17 is the DNA sequence of Beta spectrin 2 region 1 (Reg 1), and SEQ ID NO:18 is the DNA sequence of Beta spectrin 2 region 2 (Reg2). SEQ ID NO:19 is the DNA sequence of mtRP-L4 region 1 (Reg 1), and SEQ ID NO:20 is the DNA sequence of mtRP-L4 region 2 (Reg 2). A YFP sequence (SEQ ID NO:6) was also used to produce dsRNA as a negative control.

[00260] Each of the aforementioned sequences was used to produce dsRNA by the methods of EXAMPLE 3. The strategy used to provide specific templates for dsRNA production is shown in **Figure 2**. Template DNAs intended for use in dsRNA synthesis were prepared by PCR using the primer pairs in **Table 4** and (as PCR template) first-strand cDNA prepared from total RNA isolated from WCR first-instar larvae. (YFP was amplified from a DNA clone.) For each selected target gene region, two separate PCR amplifications were performed. The first PCR amplification introduced a T7 promoter sequence at the 5' end of the amplified sense strands. The second reaction incorporated the T7 promoter sequence at the 5' ends of the antisense strands. The two PCR amplified fragments for each region of the target genes were then mixed in approximately equal amounts, and the mixture was used as transcription template for dsRNA production. *See* **Figure 2**. Double-stranded RNA was synthesized and purified using an AMBION® MEGAscript® RNAi kit following the manufacturer's instructions (INVITROGEN). The concentrations of dsRNAs were measured using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE). and the dsRNAs were each tested by the same diet-based bioassay methods described above. **Table 4** lists the sequences of the primers used to produce the YFP, Annexin Reg1, Annexin Reg2, Beta spectrin 2 Reg1, Beta spectrin 2 Reg2, mtRP-L4 Reg1, and mtRP-L4 Reg2 dsRNA molecules. YFP primer sequences for use in the method depicted in **Figure 2** are also listed in **Table 4**. **Table 5** presents the results of diet-based feeding bioassays of WCR larvae following 9-day exposure to these dsRNA molecules. Replicated bioassays demonstrated

that ingestion of these dsRNAs resulted in no mortality or growth inhibition of western corn rootworm larvae above that seen with control samples of TE buffer, Water, or YFP protein.

Table 4 . Primers and Primer Pairs used to amplify portions of coding regions of genes.

	Gene (Region)	Primer ID	SEQ ID NO:	Sequence
Pair 4	YFP	YFP-F_T7	21	TTAATACGACTCACTATAGGGAGAC ACCATGGGCTCCAGCGGCGCCC
		YFP-R	22	AGATCTTGAAGGCGCTCTTCAGG
Pair 5	YFP	YFP-F	23	CACCATGGGCTCCAGCGGCGCCC
		YFP-R_T7	24	TTAATACGACTCACTATAGGGAGAA GATCTTGAAGGCGCTCTTCAGG
Pair 6	Annexin (Reg 1)	Ann-F1_T7	25	TTAATACGACTCACTATAGGGAGAG CTCCAACAGTGGTTCCTTATC
	Annexin (Reg 1)	Ann-R1	26	CTAATAATTCTTTTTTAATGTTCCTG AGG
Pair 7	Annexin (Reg 1)	Ann-F1	27	GCTCCAACAGTGGTTCCTTATC
	Annexin (Reg 1)	Ann-R1_T7	28	TTAATACGACTCACTATAGGGAGAC TAATAATTCTTTTTTAATGTTCCTGA GG
Pair 8	Annexin (Reg 2)	Ann-F2_T7	29	TTAATACGACTCACTATAGGGAGAT TGTTACAAGCTGGAGAACTTCTC
	Annexin (Reg 2)	Ann-R2	30	CTTAACCAACAACGGCTAATAAGG
Pair 9	Annexin (Reg 2)	Ann-F2	31	TTGTTACAAGCTGGAGAACTTCTC
	Annexin (Reg 2)	Ann-R2T7	32	TTAATACGACTCACTATAGGGAGAC TTAACCAACAACGGCTAATAAGG
Pair 10	Beta-spect2 (Reg 1)	Betasp2-F1_T7	33	TTAATACGACTCACTATAGGGAGAA GATGTTGGCTGCATCTAGAGAA
	Beta-spect2 (Reg 1)	Betasp2-R1	34	GTCCATTCGTCCATCCACTGCA
Pair 11	Beta-spect2 (Reg 1)	Betasp2-F1	35	AGATGTTGGCTGCATCTAGAGAA
	Beta-spect2 (Reg 1)	Betasp2-R1_T7	36	TTAATACGACTCACTATAGGGAGAG TCCATTCGTCCATCCACTGCA

Pair 12	Beta-spect2 (Reg 2)	Betasp2-F2_T7	37	TTAATACGACTCACTATAGGGAGAG CAGATGAACACCAGCGAGAAA
	Beta-spect2 (Reg 2)	Betasp2-R2	38	CTGGGCAGCTTCTTGTTTCCTC
Pair 13	Beta-spect2 (Reg 2)	Betasp2-F2	39	GCAGATGAACACCAGCGAGAAA
	Beta-spect2 (Reg 2)	Betasp2-R2_T7	40	TTAATACGACTCACTATAGGGAGAC TGGGCAGCTTCTTGTTTCCTC
Pair 14	mtRP-L4 (Reg 1)	L4-F1_T7	41	TTAATACGACTCACTATAGGGAGAA GTGAAATGTTAGCAAATATAACATC C
	mtRP-L4 (Reg 1)	L4-R1	42	ACCTCTCACTTCAAATCTTGACTTTG
Pair 15	mtRP-L4 (Reg 1)	L4-F1	43	AGTGAAATGTTAGCAAATATAACAT CC
	mtRP-L4 (Reg 1)	L4-R1_T7	44	TTAATACGACTCACTATAGGGAGAA CCTCTCACTTCAAATCTTGACTTTG
Pair 16	mtRP-L4 (Reg 2)	L4-F2_T7	45	TTAATACGACTCACTATAGGGAGAC AAAGTCAAGATTTGAAGTGAGAGGT
	mtRP-L4 (Reg 2)	L4-R2	46	CTACAAATAAAACAAGAAGGACCC C
Pair 17	mtRP-L4 (Reg 2)	L4-F2	47	CAAAGTCAAGATTTGAAGTGAGAGG T
	mtRP-L4 (Reg 2)	L4-R2_T7	48	TTAATACGACTCACTATAGGGAGAC TACAAATAAAACAAGAAGGACCCC

Table 5. Results of diet feeding assays obtained with western corn rootworm larvae after 9 days.

Gene Name	Dose (ng/cm^2)	Mean Live Larval Weight (mg)	Mean % Mortality	Mean Growth Inhibition
Annexin-Reg 1	1000	0.545	0	-0.262
Annexin-Reg 2	1000	0.565	0	-0.301
Beta spectrin2 Reg 1	1000	0.340	12	-0.014
Beta spectrin2 Reg 2	1000	0.465	18	-0.367
mtRP-L4 Reg 1	1000	0.305	4	-0.168
mtRP-L4 Reg 2	1000	0.305	7	-0.180
TE buffer*	0	0.430	13	0.000
Water	0	0.535	12	0.000

YFP**	1000	0.480	9	-0.386

*TE = Tris HCl (10 mM) plus EDTA (1 mM) buffer, pH8.
**YFP = Yellow Fluorescent Protein

EXAMPLE 6
Production of Transgenic Maize Tissues Comprising Insecticidal Hairpin dsRNAs

[00261] *Agrobacterium*-mediated Transformation Transgenic maize cells, tissues, and plants that produce one or more insecticidal dsRNA molecules (for example, at least one dsRNA molecule including a dsRNA molecule targeting a gene comprising *COPI delta*; SEQ ID NO:1) through expression of a chimeric gene stably-integrated into the plant genome were produced following *Agrobacterium*-mediated transformation. Maize transformation methods employing superbinary or binary transformation vectors are known in the art, as described, for example, in U.S. Patent No. 8,304,604, which is herein incorporated by reference in its entirety. Transformed tissues were selected by their ability to grow on Haloxyfop-containing medium and were screened for dsRNA production, as appropriate. Portions of such transformed tissue cultures may be presented to neonate corn rootworm larvae for bioassay, essentially as described in EXAMPLE 1.

[00262] *Agrobacterium* Culture Initiation Glycerol stocks of *Agrobacterium* strain DAt13192 cells (WO 2012/016222A2) harboring a binary transformation vector pDAB114515, pDAB115770, pDAB110853 or pDAB101556 described above (EXAMPLE 4) were streaked on AB minimal medium plates (Watson, *et al.*, (1975) J. Bacteriol. 123:255-264) containing appropriate antibiotics and were grown at 20°C for 3 days. The cultures were then streaked onto YEP plates (gm/L: yeast extract, 10; Peptone, 10; NaCl 5) containing the same antibiotics and were incubated at 20°C for 1 day.

[00263] *Agrobacterium* culture On the day of an experiment, a stock solution of Inoculation Medium and acetosyringone was prepared in a volume appropriate to the number of constructs in the experiment and pipetted into a sterile, disposable, 250 mL flask. Inoculation Medium (Frame *et al.* (2011) *Genetic Transformation Using Maize Immature Zygotic Embryos*. IN Plant Embryo Culture Methods and Protocols: Methods in Molecular Biology. T. A. Thorpe and E. C. Yeung, (Eds), Springer Science and Business Media, LLC. pp 327-341) contained: 2.2 gm/L MS salts; 1X ISU Modified MS Vitamins (Frame *et al.*, *ibid.*) 68.4 gm/L sucrose; 36 gm/L glucose; 115

mg/L L-proline; and 100 mg/L myo-inositol; at pH 5.4.) Acetosyringone was added to the flask containing Inoculation Medium to a final concentration of 200 μM from a 1 M stock solution in 100% dimethyl sulfoxide and the solution was thoroughly mixed.

[00264] For each construct, 1 or 2 inoculating loops-full of *Agrobacterium* from the YEP plate were suspended in 15 mL of the Inoculation Medium/acetosyringone stock solution in a sterile, disposable, 50 mL centrifuge tube, and the optical density of the solution at 550 nm (OD_{550}) was measured in a spectrophotometer. The suspension was then diluted to OD_{550} of 0.3 to 0.4 using additional Inoculation Medium/acetosyringone mixture. The tube of *Agrobacterium* suspension was then placed horizontally on a platform shaker set at about 75 rpm at room temperature and shaken for 1 to 4 hours while embryo dissection was performed.

[00265] Ear sterilization and embryo isolation Maize immature embryos were obtained from plants of *Zea mays* inbred line B104 (Hallauer *et al.* (1997) Crop Science 37:1405-1406) grown in the greenhouse and self- or sib-pollinated to produce ears. The ears were harvested approximately 10 to 12 days post-pollination. On the experimental day, de-husked ears were surface-sterilized by immersion in a 20% solution of commercial bleach (ULTRA CLOROX® Germicidal Bleach, 6.15% sodium hypochlorite; with two drops of TWEEN 20) and shaken for 20 to 30 min, followed by three rinses in sterile deionized water in a laminar flow hood. Immature zygotic embryos (1.8 to 2.2 mm long) were aseptically dissected from each ear and randomly distributed into microcentrifuge tubes containing 2.0 mL of a suspension of appropriate *Agrobacterium* cells in liquid Inoculation Medium with 200 μM acetosyringone, into which 2 μL of 10% BREAK-THRU® S233 surfactant (EVONIK INDUSTRIES; Essen, Germany) had been added. For a given set of experiments, embryos from pooled ears were used for each transformation.

[00266] *Agrobacterium* co-cultivation Following isolation, the embryos were placed on a rocker platform for 5 minutes. The contents of the tube were then poured onto a plate of Co-cultivation Medium, which contained 4.33 gm/L MS salts; 1X ISU Modified MS Vitamins; 30 gm/L sucrose; 700 mg/L L-proline; 3.3 mg/L Dicamba in KOH (3,6-dichloro-o-anisic acid or 3,6-dichloro-2-methoxybenzoic acid); 100 mg/L myo-inositol; 100 mg/L Casein Enzymatic

Hydrolysate; 15 mg/L $AgNO_3$; 200 µM acetosyringone in DMSO; and 3 gm/L GELZAN™, at pH 5.8. The liquid *Agrobacterium* suspension was removed with a sterile, disposable, transfer pipette. The embryos were then oriented with the scutellum facing up using sterile forceps with the aid of a microscope. The plate was closed, sealed with 3M™ MICROPORE™ medical tape, and placed in an incubator at 25°C with continuous light at approximately 60 µmol $m^{-2}s^{-1}$ of Photosynthetically Active Radiation (PAR).

[00267] Callus Selection and Regeneration of Transgenic Events Following the Co-Cultivation period, embryos were transferred to Resting Medium, which was composed of 4.33 gm/L MS salts; 1X ISU Modified MS Vitamins; 30 gm/L sucrose; 700 mg/L L-proline; 3.3 mg/L Dicamba in KOH; 100 mg/L myo-inositol; 100 mg/L Casein Enzymatic Hydrolysate; 15 mg/L $AgNO_3$; 0.5 gm/L MES (2-(N-morpholino)ethanesulfonic acid monohydrate; PHYTOTECHNOLOGIES LABR.; Lenexa, KS); 250 mg/L Carbenicillin; and 2.3 gm/L GELZAN™; at pH 5.8. No more than 36 embryos were moved to each plate. The plates were placed in a clear plastic box and incubated at 27°C with continuous light at approximately 50 µmol $m^{-2}s^{-1}$ PAR for 7 to 10 days. Callused embryos were then transferred (<18/plate) onto Selection Medium I, which was comprised of Resting Medium (above) with 100 nM R-Haloxyfop acid (0.0362 mg/L; for selection of calli harboring the AAD-1 gene). The plates were returned to clear boxes and incubated at 27°C with continuous light at approximately 50 µmol $m^{-2}s^{-1}$ PAR for 7 days. Callused embryos were then transferred (<12/plate) to Selection Medium II, which is comprised of Resting Medium (above) with 500 nM R-Haloxyfop acid (0.181 mg/L). The plates were returned to clear boxes and incubated at 27°C with continuous light at approximately 50 µmol $m^{-2}s^{-1}$ PAR for 14 days. This selection step allowed transgenic callus to further proliferate and differentiate.

[00268] Proliferating, embryogenic calli were transferred (<9/plate) to Pre-Regeneration medium. Pre-Regeneration Medium contained 4.33 gm/L MS salts; 1X ISU Modified MS Vitamins; 45 gm/L sucrose; 350 mg/L L-proline; 100 mg/L myo-inositol; 50 mg/L Casein Enzymatic Hydrolysate; 1.0 mg/L $AgNO_3$; 0.25 gm/L MES; 0.5 mg/L naphthaleneacetic acid in NaOH; 2.5 mg/L abscisic acid in ethanol; 1 mg/L 6-benzylaminopurine; 250 mg/L Carbenicillin; 2.5 gm/L GELZAN™; and 0.181 mg/L Haloxyfop acid; at pH 5.8. The plates were stored in clear

boxes and incubated at 27°C with continuous light at approximately 50 μmol $m^{-2}s^{-1}$ PAR for 7 days. Regenerating calli were then transferred (<6/plate) to Regeneration Medium in PHYTATRAYS™ (SIGMA-ALDRICH) and incubated at 28°C with 16 hours light/8 hours dark per day (at approximately 160 μmol $m^{-2}s^{-1}$ PAR) for 14 days or until shoots and roots developed. Regeneration Medium contained 4.33 gm/L MS salts; 1X ISU Modified MS Vitamins; 60 gm/L sucrose; 100 mg/L myo-inositol; 125 mg/L Carbenicillin; 3 gm/L GELLAN™ gum; and 0.181 mg/L R-Haloxyfop acid; at pH 5.8. Small shoots with primary roots were then isolated and transferred to Elongation Medium without selection. Elongation Medium contained 4.33 gm/L MS salts; 1X ISU Modified MS Vitamins; 30 gm/L sucrose; and 3.5 gm/L GELRITE™: at pH 5.8.

[00269] Transformed plant shoots selected by their ability to grow on medium containing Haloxyfop were transplanted from PHYTATRAYS™ to small pots filled with growing medium (PROMIX BX; PREMIER TECH HORTICULTURE), covered with cups or HUMI-DOMES (ARCO PLASTICS), and then hardened-off in a CONVIRON growth chamber (27°C day/24°C night, 16-hour photoperiod, 50-70% RH, 200 μmol $m^{-2}s^{-1}$ PAR). In some instances, putative transgenic plantlets were analyzed for transgene relative copy number by quantitative real-time PCR assays using primers designed to detect the AAD1 herbicide tolerance gene integrated into the maize genome. Further, RNA qPCR assays were used to detect the presence of the ST-LS1 intron sequence in expressed dsRNAs of putative transformants. Selected transformed plantlets were then moved into a greenhouse for further growth and testing.

[00270] Transfer and establishment of T_0 plants in the greenhouse for bioassay and seed production When plants reached the V3-V4 stage, they were transplanted into IE CUSTOM BLEND (PROFILE/METRO MIX 160) soil mixture and grown to flowering in the greenhouse (Light Exposure Type: Photo or Assimilation; High Light Limit: 1200 PAR; 16-hour day length; 27°C day/24°C night).

[00271] Plants to be used for insect bioassays were transplanted from small pots to TINUS™ 350-4 ROOTRAINERS® (SPENCER-LEMAIRE INDUSTRIES, Acheson, Alberta, Canada); (one plant per event per ROOTRAINER®). Approximately four days after transplanting to ROOTRAINERS®, plants were infested for bioassay.

[00272] Plants of the T_1 generation were obtained by pollinating the silks of T_0 transgenic plants with pollen collected from plants of non-transgenic elite inbred line B104 or other appropriate pollen donors, and planting the resultant seeds. Reciprocal crosses were performed when possible.

EXAMPLE 7

Molecular Analyses of Transgenic Maize Tissues

[00273] Molecular analyses (*e.g.* RNA qPCR) of maize tissues were performed on samples from leaves and roots that were collected from greenhouse grown plants on the same days that root feeding damage was assessed.

[00274] Results of RNA qPCR assays for the Per5 3'UTR were used to validate expression of hairpin transgenes. (A low level of Per5 3'UTR detection is expected in nontransformed maize plants, since there is usually expression of the endogenous Per5 gene in maize tissues.) Results of RNA qPCR assays for the ST-LS1 intron sequence (which is integral to the formation of dsRNA hairpin molecules) in expressed RNAs were used to validate the presence of hairpin transcripts. Transgene RNA expression levels were measured relative to the RNA levels of an endogenous maize gene.

[00275] DNA qPCR analyses to detect a portion of the AAD1 coding region in genomic DNA were used to estimate transgene insertion copy number. Samples for these analyses were collected from plants grown in environmental chambers. Results were compared to DNA qPCR results of assays designed to detect a portion of a single-copy native gene, and simple events (having one or two copies of *COPI delta*transgenes) were advanced for further studies in the greenhouse.

[00276] Additionally, qPCR assays designed to detect a portion of the spectinomycin-resistance gene (SpecR; harbored on the binary vector plasmids outside of the T-DNA) were used to determine if the transgenic plants contained extraneous integrated plasmid backbone sequences.

[00277] Hairpin RNA transcript expression level: Per 5 3'UTR qPCR Callus cell events or transgenic plants were analyzed by real time quantitative PCR (qPCR) of the Per 5 3'UTR sequence to determine the relative expression level of the full length hairpin transcript, as compared to the transcript level of an internal maize gene (SEQ ID NO:49; GENBANK Accession No. BT069734),

which encodes a TIP41-like protein (*i.e.* a maize homolog of GENBANK Accession No. AT4G34270; having a tBLASTX score of 74% identity). RNA was isolated using an RNAEASY™ 96 kit (QIAGEN, Valencia, CA). Following elution, the total RNA was subjected to a DNAse1 treatment according to the kit's suggested protocol. The RNA was then quantified on a NANODROP 8000 spectrophotometer (THERMO SCIENTIFIC) and concentration was normalized to 25 ng/μL. First strand cDNA was prepared using a HIGH CAPACITY cDNA SYNTHESIS KIT (INVITROGEN) in a 10 μL reaction volume with 5 μL denatured RNA, substantially according to the manufacturer's recommended protocol. The protocol was modified slightly to include the addition of 10 μL of 100 μM T20VN oligonucleotide (IDT) (SEQ ID NO:50; TTTTTTTTTTTTTTTTTTTTVN, where V is A, C, or G, and N is A, C, G, or T/U) into the 1 mL tube of random primer stock mix, in order to prepare a working stock of combined random primers and oligo dT.

[00278] Following cDNA synthesis, samples were diluted 1:3 with nuclease-free water, and stored at -20°C until assayed.

[00279] Separate real-time PCR assays for the Per5 3' UTR and TIP41-like transcript were performed on a LIGHTCYCLER™ 480 (ROCHE DIAGNOSTICS, Indianapolis, IN) in 10 μL reaction volumes. For the Per5 3'UTR assay, reactions were run with Primers P5U76S (F) (SEQ ID NO:51) and P5U76A (R) (SEQ ID NO:52), and a ROCHE UNIVERSAL PROBE™ (UPL76; Catalog No. 4889960001; labeled with FAM). For the TIP41-like reference gene assay, primers TIPmxF (SEQ ID NO:53) and TIPmxR (SEQ ID NO:54), and Probe HXTIP (SEQ ID NO:55) labeled with HEX (hexachlorofluorescein) were used.

[00280] All assays included negative controls of no-template (mix only). For the standard curves, a blank (water in source well) was also included in the source plate to check for sample cross-contamination. Primer and probe sequences are set forth in **Table 6**. Reaction components recipes for detection of the various transcripts are disclosed in **Table 7**, and PCR reactions conditions are summarized in **Table 8**. The FAM (6-Carboxy Fluorescein Amidite) fluorescent moiety was excited at 465 nm and fluorescence was measured at 510 nm; the corresponding values for the HEX (hexachlorofluorescein) fluorescent moiety were 533 nm and 580 nm.

Table 6. Oligonucleotide sequences used for molecular analyses of transcript levels in transgenic maize.

Target	Oligonucleotide	SEQ ID NO.	Sequence
Per5 3'UTR	P5U76S (F)	51	TTGTGATGTTGGTGGCGTAT
Per5 3'UTR	P5U76A (R)	52	TGTTAAATAAAACCCCAAAGATCG
Per5 3'UTR	Roche UPL76 (FAM-Probe)	NAv**	Roche Diagnostics Catalog Number 488996001
TIP41	TIPmxF	53	TGAGGGTAATGCCAACTGGTT
TIP41	TIPmxR	54	GCAATGTAACCGAGTGTCTCTCAA
TIP41	HXTIP (HEX-Probe)	55	TTTTTGGCTTAGAGTTGATGGTGTACTGA TGA

*TIP41-like protein.
**NAv Sequence Not Available from the supplier.

Table 7. PCR reaction recipes for transcript detection.

	Per5 3'UTR	TIP-like Gene
Component	**Final Concentration**	
Roche Buffer	1 X	1X
P5U76S (F)	0.4 µM	0
P5U76A (R)	0.4 µM	0
Roche UPL76 (FAM)	0.2 µM	0
HEXtipZM F	0	0.4 µM
HEXtipZM R	0	0.4 µM
HEXtipZMP (HEX)	0	0.2 µM
cDNA (2.0 µL)	NA	NA
Water	To 10 µL	To 10 µL

Table 8. Thermocycler conditions for RNA qPCR.

Per5 3'UTR and TIP41-like Gene Detection			
Process	**Temp.**	**Time**	**No. Cycles**
Target Activation	95°C	10 min	1
Denature	95°C	10 sec	40

Extend	60°C	40 sec	
Acquire FAM or HEX	72°C	1 sec	
Cool	40°C	10 sec	1

[00281] Data were analyzed using LIGHTCYCLER™ Software v1.5 by relative quantification using a second derivative max algorithm for calculation of Cq values according to the supplier's recommendations. For expression analyses, expression values were calculated using the ΔΔCt method (*i.e.*, 2-(Cq TARGET – Cq REF)), which relies on the comparison of differences of Cq values between two targets, with the base value of 2 being selected under the assumption that, for optimized PCR reactions, the product doubles every cycle.

[00282] Hairpin transcript size and integrity: Northern Blot Assay In some instances, additional molecular characterization of the transgenic plants is obtained by the use of Northern Blot (RNA blot) analysis to determine the molecular size of the *COPI delta* hairpin v1 RNA in transgenic plants expressing a *COPI delta* hairpin v1 dsRNA.

[00283] All materials and equipment are treated with RNAZAP (AMBION/INVITROGEN) before use. Tissue samples (100 mg to 500 mg) are collected in 2 mL SAFELOCK EPPENDORF tubes, disrupted with a KLECKO™ tissue pulverizer (GARCIA MANUFACTURING, Visalia, CA) with three tungsten beads in 1 mL of TRIZOL (INVITROGEN) for 5 min, then incubated at room temperature (RT) for 10 min. Optionally, the samples are centrifuged for 10 min at 4°C at 11,000 rpm and the supernatant is transferred into a fresh 2 mL SAFELOCK EPPENDORF tube. After 200 µL of chloroform are added to the homogenate, the tube is mixed by inversion for 2 to 5 min, incubated at RT for 10 minutes, and centrifuged at 12,000 x g for 15 min at 4°C. The top phase is transferred into a sterile 1.5 mL EPPENDORF tube, 600 µL of 100% isopropanol are added, followed by incubation at RT for 10 min to 2 hr, then centrifuged at 12,000 x g for 10 min at 4° to 25°C. The supernatant is discarded and the RNA pellet is washed twice with 1 mL of 70% ethanol, with centrifugation at 7,500 x g for 10 min at 4° to 25°C between washes. The ethanol is discarded and the pellet is briefly air dried for 3 to 5 min before resuspending in 50 µL of nuclease-free water.

[00284] Total RNA is quantified using the NANODROP 8000® (THERMO-FISHER) and samples are normalized to 5 µg/10 µL. 10 µL of glyoxal (AMBION/INVITROGEN) are then added to each sample. Five to 14 ng of DIG RNA standard marker mix (ROCHE APPLIED SCIENCE, Indianapolis, IN) are dispensed and added to an equal volume of glyoxal. Samples and marker RNAs are denatured at 50°C for 45 min and stored on ice until loading on a 1.25% SEAKEM GOLD agarose (LONZA, Allendale, NJ) gel in NORTHERNMAX 10 X glyoxal running buffer (AMBION/INVITROGEN). RNAs are separated by electrophoresis at 65 volts/30 mA for 2 hr and 15 min.

[00285] Following electrophoresis, the gel is rinsed in 2X SSC for 5 min and imaged on a GEL DOC station (BIORAD, Hercules, CA), then the RNA is passively transferred to a nylon membrane (MILLIPORE) overnight at RT, using 10X SSC as the transfer buffer (20X SSC consists of 3 M sodium chloride and 300 mM trisodium citrate, pH 7.0). Following the transfer, the membrane is rinsed in 2X SSC for 5 minutes, the RNA is UV-crosslinked to the membrane (AGILENT/STRATAGENE), and the membrane is allowed to dry at RT for up to 2 days.

[00286] The membrane is prehybridized in ULTRAHYB buffer (AMBION/INVITROGEN) for 1 to 2 hr. The probe consists of a PCR amplified product containing the sequence of interest, (for example, the antisense sequence portion of SEQ ID NO:11 as appropriate) labeled with digoxygenin by means of a ROCHE APPLIED SCIENCE DIG procedure. Hybridization in recommended buffer is overnight at a temperature of 60°C in hybridization tubes. Following hybridization, the blot is subjected to DIG washes, wrapped, exposed to film for 1 to 30 minutes, then the film is developed, all by methods recommended by the supplier of the DIG kit.

[00287] Transgene copy number determination. Maize leaf pieces approximately equivalent to 2 leaf punches were collected in 96-well collection plates (QIAGEN). Tissue disruption was performed with a KLECKO™ tissue pulverizer (GARCIA MANUFACTURING, Visalia, CA) in BIOSPRINT96 AP1 lysis buffer (supplied with a BIOSPRINT96 PLANT KIT; QIAGEN) with one stainless steel bead. Following tissue maceration, genomic DNA (gDNA) was

isolated in high throughput format using a BIOSPRINT96 PLANT KIT and a BIOSPRINT96 extraction robot. Genomic DNA was diluted 2:3 DNA:water prior to setting up the qPCR reaction.

[00288] qPCR analysis. Transgene detection by hydrolysis probe assay was performed by real-time PCR using a LIGHTCYCLER®480 system. Oligonucleotides to be used in hydrolysis probe assays to detect the ST-LS1 intron sequence (SEQ ID NO:13), or to detect a portion of the SpecR gene (*i.e.* the spectinomycin resistance gene borne on the binary vector plasmids; SEQ ID NO:67; SPC1 oligonucleotides in **Table 9**), were designed using LIGHTCYCLER® PROBE DESIGN SOFTWARE 2.0. Further, oligonucleotides to be used in hydrolysis probe assays to detect a segment of the AAD-1 herbicide tolerance gene (SEQ ID NO:61; GAAD1 oligonucleotides in **Table 9**) were designed using PRIMER EXPRESS software (APPLIED BIOSYSTEMS). **Table 9** shows the sequences of the primers and probes. Assays were multiplexed with reagents for an endogenous maize chromosomal gene (Invertase (SEQ ID NO:58; GENBANK Accession No: U16123; referred to herein as IVR1), which served as an internal reference sequence to ensure gDNA was present in each assay. For amplification, LIGHTCYCLER®480 PROBES MASTER mix (ROCHE APPLIED SCIENCE) was prepared at 1x final concentration in a 10 µL volume multiplex reaction containing 0.4 µM of each primer and 0.2 µM of each probe (**Table 10**). A two step amplification reaction was performed as outlined in **Table 11**. Fluorophore activation and emission for the FAM- and HEX-labeled probes were as described above; CY5 conjugates are excited maximally at 650 nm and fluoresce maximally at 670 nm.

Cp scores (the point at which the fluorescence signal crosses the background threshold) were determined from the real time PCR data using the fit points algorithm (LIGHTCYCLER® SOFTWARE release 1.5) and the Relative Quant module (based on the ΔΔCt method). Data were handled as described previously (above; RNA qPCR).

Table 9. Sequences of primers and probes (with fluorescent conjugate) used for gene copy number determinations and binary vector plasmid backbone detection.

Name	SEQ ID NO:	Sequence
GAAD1-F	59	TGTTCGGTTCCCTCTACCAA
GAAD1-R	60	CAACATCCATCACCTTGACTGA
GAAD1-P (FAM)	61	CACAGAACCGTCGCTTCAGCAACA
IVR1-F	62	TGGCGGACGACGACTTGT

IVR1-R	63	AAAGTTTGGAGGCTGCCGT
IVR1-P (HEX)	64	CGAGCAGACCGCCGTGTACTTCTACC
SPC1A	65	CTTAGCTGGATAACGCCAC
SPC1S	66	GACCGTAAGGCTTGATGAA
TQSPEC (CY5*)	67	CGAGATTCTCCGCGCTGTAGA
ST-LS1- F	68	GTATGTTTCTGCTTCTACCTTTGAT
ST-LS1- R	69	CCATGTTTTGGTCATATATTAGAAAAGTT
ST-LS1-P (FAM)	70	AGTAATATAGTATTTCAAGTATTTTTTTCAAAAT

*CY5 = Cyanine-5

Table 10. Reaction components for gene copy number analyses and plasmid backbone detection.

Component	Amt. (µL)	Stock	Final Conc'n
2x Buffer	5.0	2x	1x
Appropriate Forward Primer	0.4	10 µM	0.4
Appropriate Reverse Primer	0.4	10 µM	0.4
Appropriate Probe	0.4	5 µM	0.2
IVR1-Forward Primer	0.4	10 µM	0.4
IVR1-Reverse Primer	0.4	10 µM	0.4
IVR1-Probe	0.4	5 µM	0.2
H_2O	0.6	NA*	NA
gDNA	2.0	ND**	ND
Total	10.0		

*NA = Not Applicable
**ND = Not Determined

Table 11. Thermocycler conditions for DNA qPCR

Genomic copy number analyses			
Process	**Temp.**	**Time**	**No. Cycles**
Target Activation	95°C	10 min	1
Denature	95°C	10 sec	40
Extend & Acquire FAM, HEX, or CY5	60°C	40 sec	
Cool	40°C	10 sec	1

EXAMPLE 8
Bioassay of Transgenic Maize

[00289] *In vitro* Insect Bioassays Bioactivity of dsRNA of the subject invention produced in plant cells is demonstrated by bioassay methods. *See*, *e.g.*, Baum *et al.* (2007) Nat. Biotechnol. 25(11):1322-1326. One is able to demonstrate efficacy, for example, by feeding various plant tissues or tissue pieces derived from a plant producing an insecticidal dsRNA to target insects in a controlled feeding environment. Alternatively, extracts are prepared from various plant tissues derived from a plant producing the insecticidal dsRNA and the extracted nucleic acids are dispensed on top of artificial diets for bioassays as previously described herein. The results of such feeding assays are compared to similarly conducted bioassays that employ appropriate control tissues from host plants that do not produce an insecticidal dsRNA, or to other control samples.

[00290] Insect Bioassays with Transgenic Maize Events Two western corn rootworm larvae (1 to 3 days old) hatched from washed eggs are selected and placed into each well of the bioassay tray. The wells are then covered with a "PULL N' PEEL" tab cover (BIO-CV-16, BIO-SERV) and placed in a 28°C incubator with an 18 hr/6 hr light/dark cycle. Nine days after the initial infestation, the larvae are assessed for mortality, which is calculated as the percentage of dead insects out of the total number of insects in each treatment. The insect samples are frozen at -20°C for two days, then the insect larvae from each treatment are pooled and weighed. The percent of growth inhibition is calculated as the mean weight of the experimental treatments divided by the mean of the average weight of two control well treatments. The data are expressed as a Percent Growth Inhibition (of the Negative Controls). Mean weights that exceed the control mean weight are normalized to zero.

[00291] Insect bioassays in the greenhouse Western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) eggs were received in soil from CROP CHARACTERISTICS (Farmington, MN). WCR eggs were incubated at 28°C for 10 to 11 days. Eggs were washed from the soil, placed into a 0.15% agar solution, and the concentration was adjusted to approximately 75 to 100 eggs per 0.25 mL aliquot. A hatch plate was set up in a Petri dish with an aliquot of egg suspension to monitor hatch rates.

[00292] The soil around the maize plants growing in ROOTRAINERS® was infested with 150 to 200 WCR eggs. The insects were allowed to feed for 2 weeks, after which time a "Root

Rating" was given to each plant. A Node-Injury Scale was utilized for grading essentially according to Oleson *et al.* (2005, J. Econ. Entomol. 98:1-8). Plants which passed this bioassay were transplanted to 5-gallon pots for seed production. Transplants were treated with insecticide to prevent further rootworm damage and insect release in the greenhouses. Plants were hand pollinated for seed production. Seeds produced by these plants were saved for evaluation at the T_1 and subsequent generations of plants.

[00293] Greenhouse bioassays included two kinds of negative control plants. Transgenic negative control plants were generated by transformation with vectors harboring genes designed to produce a yellow fluorescent protein (YFP) or a YFP hairpin dsRNA (*See* Example 4). Nontransformed negative control plants were grown from seeds of lines 7sh382 or B104. Bioassays were conducted on two separate dates, with negative controls included in each set of plant materials.

[00294] **Table 12** shows the combined results of molecular analyses and bioassays for *COPI delta* hairpin plants. Examination of the bioassay results summarized in **Table 12** reveals the surprising and unexpected observation that the majority of the transgenic maize plants harboring constructs that express an *COPI delta* hairpin dsRNA comprising segments of SEQ ID NO:1, for example, as exemplified in SEQ ID NO:11, are protected against root damage incurred by feeding of western corn rootworm larvae. Twenty-two of the 37 graded events had a root rating of 0.5 or lower. **Table 13** shows the combined results of molecular analyses and bioassays for negative control plants. Most of the plants had no protection against WCR larvae feeding, although five of the 34 graded plants had a root rating of 0.75 or lower. The presence of some plants having low root ratings scores amongst the negative control plant set is sometimes observed and reflects the variability and difficulty of conducting this type of bioassay in a greenhouse setting.

Table 12. Greenhouse bioassay and molecular analyses results of *COPI delta*-hairpin v1-expressing maize plants.

Sample ID		Leaf Tissue		Root Tissue		
	Batch #	ST-LS1 RTL*	PER5 UTR RTL	ST-LS1 RTL*	PER5 UTR RTL	Root Rating
***COPI delta* v1 Events**						

117220[1]-001.001	1	0.914	118.6	0.688	227.5	0.1
117220[1]-003.001	1	1.753	82.7	1.636	172.4	0.1
117220[1]-004.001	1	0.536	165.4	1.133	207.9	0.1
117220[1]-005.001	1	0.611	113.8	2.532	347.3	0.01
117220[1]-009.001	1	0.566	227.5	1.376	168.9	0.1
117220[1]-012.001	2	3.4	37.3	4.8	50.6	0.25
117220[1]-015.001	2	3.2	28.8	7.3	38.3	1
117220[1]-018.001	2	10.3	60.1	1.8	52.7	0.01
117220[1]-019.001	2	6.3	54.2	5.3	31.1	0.25
117220[1]-020.001	2	6.3	58.1	2.3	106.9	1
117220[1]-021.001	2	2.7	27.9	1.7	73.5	0.25
117220[1]-023.001	2	15.4	108.4	3.1	86.8	0.01
117220[1]-024.001	2	6.2	55.7	2.2	63.1	0.1
117220[1]-025.001	2	3.3	31.3	1.3	64.4	0.01
117220[1]-029.001	2	4.6	58.1	2.5	82.7	**
117220[1]-031.001	2	4.3	36.0	2.8	75.1	0.1
117220[1]-033.001	2	5.4	47.8	6.1	143.0	1
117220[1]-036.001	2	3.8	38.6	1.6	39.7	0.05
117220[1]-038.001	2	5.8	36.8	1.5	61.0	0.01

*RTL = Relative Transcript Level as measured against TIP4-like gene transcript levels.
**NG = Not Graded due to small plant size.
***ND = Not Done.

Table 13. Greenhouse bioassay and molecular analyses results of negative control plants comprising transgenic and nontransformed maize plants.

Sample ID		**Leaf Tissue**		**Root Tissue**		
YFP protein Events	**Batch #**	**ST-LS1 RTL***	**PER5 UTR RTL**	**ST-LS1 RTL***	**PER5 UTR RTL**	**Root Rating**
101556[691]-10720.001	1	0.000	75.1	0.000	56.1	1
101556[691]-10721.001	1	0.000	71.5	0.166	114.6	1
101556[691]-10722.001	1	0.000	259.6	0.000	0.0	**NG
101556[691]-10723.001	1	0.000	136.2	0.000	148.1	1
101556[691]-10724.001	1	0.000	82.1	0.000	16.9	1
101556[691]-	2	0.8	15.2	0.0	24.9	1

10725.001						
101556[691]-10726.001	2	0.7	16.2	0.0	55.7	0.5
101556[691]-10727.001	2	1.2	32.0	0.0	24.8	0.5
101556[691]-10728.001	2	0.0	7.9	0.0	54.9	1
101556[691]-10729.001	2	0.0	16.9	0.0	23.6	1
101556[691]-10948.001	3	0.0	21.6	***ND	***ND	0.75
101556[691]-10949.001	3	0.0	40.5	***ND	***ND	0.75
101556[691]-10950.001	3	0.0	42.2	***ND	***ND	1
101556[691]-10951.001	3	0.4	0.0	***ND	***ND	1
101556[691]-10952.001	3	0.0	58.1	***ND	***ND	1
YFP hairpin Events						
110853[9]-336.001	1	0.000	0.5	0.000	0.6	0.75
110853[9]-337.001	1	1.064	526.4	0.000	1.5	1
110853[9]-338.001	1	0.536	219.8	0.707	108.4	1
110853[9]-339.001	1	0.000	0.0	0.000	0.6	1
110853[9]-340.001	2	2.7	25.1	7.5	61.8	1
110853[9]-341.001	2	3.5	45.6	2.2	24.1	1
110853[9]-343.001	2	3.6	62.2	6.6	68.6	1
110853[9]-344.001	2	3.5	58.9	4.7	31.8	0.5
110853[9]-345.001	2	3.1	42.5	5.6	40.5	1
110853[9]-346.001	3	0.0	0.0	***ND	***ND	1
110853[9]-347.001	3	0.0	0.1	***ND	***ND	1
110853[9]-348.001	3	9.5	183.5	***ND	***ND	0.5
Nontransformed Plants						
7sh382	1	0.000	0.4	0.000	8.7	1
7sh382	1	0.000	0.3	0.000	2.3	1
7sh382	1	0.000	0.2	0.000	0.0	1
7sh382	1	0.000	0.2	0.000	4.4	0.75
7sh382	1	0.000	0.4	0.000	6.8	0.5

7sh382	2	0.0	0.1	0.0	34.8	1
7sh382	2	0.0	0.1	1.5	0.2	1
7sh382	2	0.4	0.1	***ND	***ND	1
7sh382	2	***ND	***ND	0.0	41.9	0.5
7sh382	2	1.1	0.2	0.0	2.1	1
7sh382	3	0.0	0.1	***ND	***ND	1
7sh382	3	0.0	0.1	***ND	***ND	0.5
7sh382	3	0.6	0.1	***ND	***ND	1
7sh382	3	0.0	0.1	***ND	***ND	1
7sh382	4	1.7	1.3	***ND	***ND	0.75
7sh382	4	0.6	0.1	***ND	***ND	1
7sh382	4	0.0	0.1	***ND	***ND	1
7sh382	4	0.7	0.1	***ND	***ND	1
7sh382	4	0.0	0.0	***ND	***ND	1
B104	1	0.000	0.0	0.000	1.9	1
B104	1	0.000	0.1	0.000	99.0	1
B104	1	0.000	1.1	0.000	7.1	1
B104	1	0.000	0.1	0.000	31.6	1
B104	1	0.000	0.0	0.000	2.3	1
B104	2	0.0	0.1	0.9	0.1	1
B104	2	0.3	3.6	0.0	4.3	1
B104	2	2.4	16.8	0.3	0.5	1
B104	2	0.0	0.1	0.8	0.0	1
B104	3	0.0	0.0	***ND	***ND	1
B104	3	0.0	0.0	***ND	***ND	1
B104	3	0.0	0.0	***ND	***ND	1
B104	3	0.0	0.1	***ND	***ND	1
B104	4	0.3	0.0	***ND	***ND	1
B104	4	0.4	0.0	***ND	***ND	1
B104	4	0.0	0.0	***ND	***ND	1
B104	4	0.5	0.0	***ND	***ND	1
B104	4	0.0	0.2	***ND	***ND	1

*RTL = Relative Transcript Level as measured against TIP4-like gene transcript levels.
**NG = Not Graded due to small plant size.
***ND = Not Done.

EXAMPLE 9
Transgenic *Zea mays* Comprising Coleopteran Pest Sequences

[00295] Ten to 20 transgenic T_0 *Zea mays* plants are generated as described in EXAMPLE 6. A further 10-20 T_1 *Zea mays* independent lines expressing hairpin dsRNA for an RNAi construct are obtained for corn rootworm challenge. Hairpin dsRNA may be derived as set forth in SEQ ID NO:11 or otherwise further comprising SEQ ID NO:1. Additional hairpin dsRNAs may be derived, for example, from coleopteran pest sequences such as, for example, Caf1-180 (U.S. Patent Application Publication No. 2012/0174258), VatpaseC (U.S. Patent Application Publication No. 2012/0174259), Rho1 (U.S. Patent Application Publication No. 2012/0174260), VatpaseH (U.S. Patent Application Publication No. 2012/0198586), PPI-87B (U.S. Patent Application Publication No. 2013/0091600), RPA70 (U.S. Patent Application Publication No. 2013/0091601), or RPS6 (U.S. Patent Application Publication No. 2013/0097730). These are confirmed through RT-PCR or other molecular analysis methods. Total RNA preparations from selected independent T_1 lines are optionally used for RT-PCR with primers designed to bind in the ST-LS1 intron of the hairpin expression cassette in each of the RNAi constructs. In addition, specific primers for each target gene in an RNAi construct are optionally used to amplify and confirm the production of the pre-processed mRNA required for siRNA production *in planta*. The amplification of the desired bands for each target gene confirms the expression of the hairpin RNA in each transgenic *Zea mays* plant. Processing of the dsRNA hairpin of the target genes into siRNA is subsequently optionally confirmed in independent transgenic lines using RNA blot hybridizations.

[00296] Moreover, RNAi molecules having mismatch sequences with more than 80% sequence identity to target genes affect corn rootworms in a way similar to that seen with RNAi molecules having 100% sequence identity to the target genes. The pairing of mismatch sequence with native sequences to form a hairpin dsRNA in the same RNAi construct delivers plant-processed siRNAs capable of affecting the growth, development and viability of feeding coleopteran pests.

[00297] *In planta* delivery of dsRNA, siRNA or miRNA corresponding to target genes and the subsequent uptake by coleopteran pests through feeding results in down-regulation of the target genes in the coleopteran pest through RNA-mediated gene silencing. When the function of a target gene is important at one or more stages of development, the growth, development, and reproduction

of the coleopteran pest is affected, and in the case of at least one of WCR, NCR, SCR, MCR, *D. balteata* LeConte, *D. u. tenella*, and *D. u. undecimpunctata* Mannerheim, leads to failure to successfully infest, feed, develop, and/or reproduce, or leads to death of the coleopteran pest. The choice of target genes and the successful application of RNAi is then used to control coleopteran pests.

[00298] Phenotypic comparison of transgenic RNAi lines and nontransformed *Zea mays* Target coleopteran pest genes or sequences selected for creating hairpin dsRNA have no similarity to any known plant gene sequence. Hence it is not expected that the production or the activation of (systemic) RNAi by constructs targeting these coleopteran pest genes or sequences will have any deleterious effect on transgenic plants. However, development and morphological characteristics of transgenic lines are compared with nontransformed plants, as well as those of transgenic lines transformed with an "empty" vector having no hairpin-expressing gene. Plant root, shoot, foliage and reproduction characteristics are compared. There is no observable difference in root length and growth patterns of transgenic and nontransformed plants. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are similar. In general, there are no observable morphological differences between transgenic lines and those without expression of target iRNA molecules when cultured *in vitro* and in soil in the glasshouse.

EXAMPLE 10

Transgenic *Zea mays* Comprising a Coleopteran Pest Sequence and Additional RNAi Constructs

[00299] A transgenic *Zea mays* plant comprising a heterologous coding sequence in its genome that is transcribed into an iRNA molecule that targets an organism other than a coleopteran pest is secondarily transformed *via Agrobacterium* or WHISKERS™ methodologies (*see* Petolino and Arnold (2009) Methods Mol. Biol. 526:59-67) to produce one or more insecticidal dsRNA molecules (for example, at least one dsRNA molecule including a dsRNA molecule targeting a gene comprising SEQ ID NO:1). Plant transformation plasmid vectors prepared essentially as described in EXAMPLE 4 are delivered *via Agrobacterium* or WHISKERS™-mediated transformation methods into maize suspension cells or immature maize embryos obtained from a transgenic Hi II

or B104 *Zea mays* plant comprising a heterologous coding sequence in its genome that is transcribed into an iRNA molecule that targets an organism other than a coleopteran pest.

EXAMPLE 11
Transgenic *Zea mays* Comprising an RNAi Construct and Additional Coleopteran Pest Control Sequences

[00300] A transgenic *Zea mays* plant comprising a heterologous coding sequence in its genome that is transcribed into an iRNA molecule that targets a coleopteran pest organism (for example, at least one dsRNA molecule including a dsRNA molecule targeting a gene comprising SEQ ID NO:1) is secondarily transformed *via Agrobacterium* or WHISKERS™ methodologies (*see* Petolino and Arnold (2009) Methods Mol. Biol. 526:59-67) to produce one or more insecticidal protein molecules, for example, Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C insecticidal proteins. Plant transformation plasmid vectors prepared essentially as described in EXAMPLE 4 are delivered *via Agrobacterium* or WHISKERS™-mediated transformation methods into maize suspension cells or immature maize embryos obtained from a transgenic B104 *Zea mays* plant comprising a heterologous coding sequence in its genome that is transcribed into an iRNA molecule that targets a coleopteran pest organism. Doubly-transformed plants are obtained that produce iRNA molecules and insecticidal proteins for control of coleopteran pests.

EXAMPLE 12
Mortality of Neotropical Brown Stink Bug (*Euschistus heros*) following *COPI delta* RNAi injection

[00301] Neotropical Brown Stink Bug (BSB; *Euschistus heros*) were reared in a 27°C incubator, at 65% relative humidity, with 16: 8 hour light: dark cycle. One gram of eggs collected over 2-3 days were seeded in 5L containers with filter paper discs at the bottom; the containers were covered with #18 mesh for ventilation. Each rearing container yielded approximately 300-400 adult BSB. At all stages, the insects were fed fresh green beans three times per week, a sachet of seed mixture that contained sunflower seeds, soybeans, and peanuts (3:1:1 by weight ratio) was replaced

once a week. Water was supplemented in vials with cotton plugs as a wicks. After the initial two weeks, insects were transferred onto new container once a week.

[00302] BSB artificial diet. BSB artificial diet prepared as follows (used within two weeks of preparation). Lyophilized green beans were blended to a fine powder in a MAGIC BULLET® blender while raw (organic) peanuts were blended in a separate MAGIC BULLET® blender. Blended dry ingredients were combined (weight percentages: green beans, 35%; peanuts, 35%; sucrose, 5%; Vitamin complex (e.g. Vanderzant Vitamin Mixture for insects, SIGMA-ALDRICH, Catalog No. V1007), 0.9%); in a large MAGIC BULLET® blender, which was capped and shaken well to mix the ingredients. The mixed dry ingredients were then added to a mixing bowl. In a separate container, water and benomyl anti-fungal agent (50 ppm; 25 μL of a 20,000 ppm solution/50 mL diet solution) were mixed well and then added to the dry ingredient mixture. All ingredients were mixed by hand until the solution was fully blended. The diet was shaped into desired sizes, wrapped loosely in aluminum foil, heated for 4 hours at 60°C, then cooled and stored at 4°C.

[00303] RNAi target selection Six stages of BSB development were selected for mRNA library preparation. Total RNA was extracted from insects frozen at -70°C and homogenized in 10 volumes of Lysis/Binding buffer in Lysing MATRIX A 2 mL tubes (MP BIOMEDICALS, Santa Ana, CA) on a FastPrep®-24 Instrument (MP BIOMEDICALS). Total mRNA was extracted using a mirVana™ miRNA Isolation Kit (AMBION; INVITROGEN) according to the manufacturer's protocol. RNA sequencing using an illumina® HiSeq™ system (San Diego, CA) provided candidate target gene sequences for use in RNAi insect control technology. HiSeq™ generated a total of about 378 million reads for the six samples. The reads were assembled individually for each sample using TRINITY assembler software (Grabherr *et al.* (2011) Nature Biotech. 29:644-652). The assembled transcripts were combined to generate a pooled transcriptome. This BSB pooled transcriptome contains 378,457 sequences.

[00304] BSB *COPI delta* ortholog identification A tBLASTn search of the BSB pooled transcriptome was performed using as query sequence a *Drosophila COPI delta* protein δCOP -PA

(GENBANK Accession No. NP_001162642). BSB *COPI delta* (SEQ ID NO:71) was identified as a *Euschistus heros* candidate target gene with predicted peptide sequence (SEQ ID NO:72).

[00305] The sequence SEQ ID NO:71 is novel. The sequence is not provided in public databases. The *Euschistus COPI delta* sequence (SEQ ID NO:71) is somewhat related (75% identity) to a fragment of a *coatomer subunit alpha* gene from the *Nasonia vitripennis* (GENBANK Accession No. XM_001608095.2). The closest homolog of the *Euschistus heros* COPI DELTA amino acid sequence (SEQ ID NO:72) is a *Riptortus pedestris* protein having GENBANK Accession No. BAN20389.1 (94% similar; 89% identical over the homology region).

[00306] Template preparation and dsRNA synthesis cDNA was prepared from total BSB RNA extracted from a single young adult insect (about 90 mg) using TRIzol® Reagent (LIFE TECHNOLOGIES). The insect was homogenized at room temperature in a 1.5 mL microcentrifuge tube with 200 µL of TRIzol® using a pellet pestle (FISHERBRAND Catalog No. 12-141-363) and Pestle Motor Mixer (COLE-PARMER, Vernon Hills, IL). Following homogenization, an additional 800 µL of TRIzol® was added, the homogenate was vortexed, and then incubated at room temperature for five minutes. Cell debris was removed by centrifugation and the supernatant was transferred to a new tube. 200 µL of chloroform were added and the mixture was vortexed for 15 seconds. After allowing the extraction to sit at room temperature for 2 to 3 min, the phases were separated by centrifugation at 12,000 x g at 4°C for 15 minutes. The upper aqueous phase was carefully transferred into another nuclease-free 1.5 mL microcentrifuge tube, and the RNA was precipitated with 500 µL of room temperature isopropanol. After ten-minute incubation at room temperature, the mixture was centrifuged for 10 minutes as above. The RNA pellet was rinsed with 1 mL of room-temperature 75% ethanol and centrifuged for an additional 10 minutes as above. Following manufacturer-recommended TRIzol® extraction protocol for 1 mL of TRIzol®, the RNA pellet was dried at room temperature and resuspended in 200 µL of Tris Buffer from a GFX PCR DNA AND GEL EXTRACTION KIT (illustra™; GE HEALTHCARE LIFE SCIENCES) using Elution Buffer Type 4 (*i.e.* 10 mM Tris-HCl pH8.0). RNA concentration was determined using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE).

[00307] cDNA was reverse-transcribed from 5 µg of BSB total RNA template and oligo dT primer using a SUPERSCRIPT III FIRST-STRAND SYNTHESIS SYSTEM™ for RT-PCR (INVITROGEN), following the supplier's recommended protocol. The final volume of the transcription reaction was brought to 100 µL with nuclease-free water.

[00308] Primers BSB_δCOP-1-For (SEQ ID NO:74) and BSB_δCOP-1-Rev (SEQ ID NO:75) were used to amplify BSB_*COPI delta* region 1, also referred to as BSB_*COPI delta*-1 template. The DNA templates were amplified by touch-down PCR (annealing temperature lowered from 60°C to 50°C in a 1°C/cycle decrease) with 1 µL of cDNA (above) as the template. Fragment comprising 485 bp segment of BSB_*COPI delta*-1 (SEQ ID NO:73) was generated during 35 cycles of PCR. The above procedure was also used to amplify a 301 bp negative control template YFPv2 (SEQ ID NO:76) using YFPv2-F (SEQ ID NO:77) and YFPv2-R (SEQ ID NO:78) primers. The BSB_*COPI delta* and YFPv2 primers contained a T7 phage promoter sequence (SEQ ID NO:5) at their 5' ends, and thus enabled the use of YFPv2 and BSB_*COPI delta*-1 DNA fragments for dsRNA transcription. dsRNA was synthesized using 2 µL of PCR product (above) as the template with a MEGAscript™ RNAi kit (AMBION) used according to the manufacturer's instructions. (*See* **FIGURE 1**). dsRNA was quantified on a NANODROP™ 8000 spectrophotometer and diluted to 500 ng/µL in nuclease-free 0.1X TE buffer (1 mM Tris HCL, 0.1 mM EDTA, pH7.4).

[00309] Injection of dsRNA into BSB hemoceol BSB were reared on artificial diet (above) in a 27°C incubator at 65% relative humidity and 16:8 hour light:dark photoperiod. Second instar nymphs (each weighing 1 to 1.5 mg) were gently handled with a small brush to prevent injury and were placed in a Petri dish on ice to chill and immobilize the insects. Each insect was injected with 55.2 nL of a 500 ng/µL dsRNA solution (*i.e.* 27.6 ng dsRNA; dosage of 18.4 to 27.6 µg/g body weight). Injections were performed using a NANOJECT™ II injector (DRUMMOND SCIENTIFIC, Broomhall, PA) equipped with an injection needle pulled from a Drummond 3.5 inch #3-000=203-G/X glass capillary. The needle tip was broken and the capillary was backfilled with light mineral oil, then filled with 2 to 3 µL of dsRNA. dsRNA was injected into the abdomen of the nymphs (10 insects injected per dsRNA per trial), and the trials were repeated on three different days. Injected insects (5 per well) were transferred into 32-well trays (Bio-RT-32 Rearing Tray;

BIO-SERV, Frenchtown, NJ) containing a pellet of artificial BSB diet and covered with Pull-N-Peel™ tabs (BIO-CV-4; BIO-SERV). Moisture was supplied by means of 1.25 mL of water in a 1.5 mL microcentrifuge tube with a cotton wick. The trays were incubated at 26.5°C, 60% humidity and 16:8 light:dark photoperiod. Viability counts and weights were taken on day 7 after the injections.

[00310] Injections identified BSB *COPI delta* as a lethal dsRNA dsRNA that targets segment of *YFP* coding region, YFPv2 was used as a negative control in BSB injection experiments. As summarized in **Table 14**, 27.6 ng of BSB_*COPI delta*-1 dsRNA injected into the hemoceol of 2nd instar BSB nymphs produced high mortality within seven days. The mortality caused by BSB_*COPI delta*-1 dsRNA was significantly different from that seen with the same amount of injected YFPv2 dsRNA (negative control), with p = 0.0009045 (Student's *t*-test).

Table 14 Results of BSB_*COPI delta*-1 dsRNA injection into the hemoceol of 2nd instar Brown Stink Bug nymphs seven days after injection.

Treatment*	N Trials	Mean % Mortality ± SEM	p value *t*-test
BSB *COPI delta*-1	3	96.7 ± 3.33	9.04E-04
YFP v2 dsRNA	3	13.3 ± 8.82	

*Ten insects injected per trial for each dsRNA.

EXAMPLE 13

Transgenic *Zea mays* Comprising Hemipteran Pest Sequences

[00311] Ten to 20 transgenic T_0 *Zea mays* plants harboring expression vectors for nucleic acids comprising SEQ ID NO: 71 and/or SEQ ID NO:73 are generated as described in EXAMPLE 7. A further 10-20 T_1 *Zea mays* independent lines expressing hairpin dsRNA for an RNAi construct are obtained for BSB challenge. Hairpin dsRNA may be derived as set forth in SEQ ID NO:73 or otherwise further comprising SEQ ID NO:71. These are confirmed through RT-PCR or other molecular analysis methods. Total RNA preparations from selected independent T_1 lines are optionally used for RT-PCR with primers designed to bind in the ST-LS1 intron of the hairpin expression cassette in each of the RNAi constructs. In addition, specific primers for each target gene in an RNAi construct are optionally used to amplify and confirm the production of the pre-

processed mRNA required for siRNA production *in planta*. The amplification of the desired bands for each target gene confirms the expression of the hairpin RNA in each transgenic *Zea mays* plant. Processing of the dsRNA hairpin of the target genes into siRNA is subsequently optionally confirmed in independent transgenic lines using RNA blot hybridizations.

[00312] Moreover, RNAi molecules having mismatch sequences with more than 80% sequence identity to target genes affect corn rootworms in a way similar to that seen with RNAi molecules having 100% sequence identity to the target genes. The pairing of mismatch sequence with native sequences to form a hairpin dsRNA in the same RNAi construct delivers plant-processed siRNAs capable of affecting the growth, development and viability of feeding hemipteran pests.

[00313] *In planta* delivery of dsRNA, siRNA, shRNA, or miRNA corresponding to target genes and the subsequent uptake by hemipteran pests through feeding results in down-regulation of the target genes in the hemipteran pest through RNA-mediated gene silencing. When the function of a target gene is important at one or more stages of development, the growth, development, and reproduction of the hemipteran pest is affected, and in the case of at least one of *Euschistus heros*, *Piezodorus guildinii*, *Halyomorpha halys*, *Nezara viridula*, *Acrosternum hilare*, and *Euschistus servus* leads to failure to successfully infest, feed, develop, and/or reproduce, or leads to death of the hemipteran pest. The choice of target genes and the successful application of RNAi is then used to control hemipteran pests.

[00314] Phenotypic comparison of transgenic RNAi lines and nontransformed *Zea mays*
Target hemipteran pest genes or sequences selected for creating hairpin dsRNA have no similarity to any known plant gene sequence. Hence it is not expected that the production or the activation of (systemic) RNAi by constructs targeting these hemipteran pest genes or sequences will have any deleterious effect on transgenic plants. However, development and morphological characteristics of transgenic lines are compared with nontransformed plants, as well as those of transgenic lines transformed with an "empty" vector having no hairpin-expressing gene. Plant root, shoot, foliage and reproduction characteristics are compared. There is no observable difference in root length and growth patterns of transgenic and nontransformed plants. Plant shoot characteristics such as height,

leaf numbers and sizes, time of flowering, floral size and appearance are similar. In general, there are no observable morphological differences between transgenic lines and those without expression of target iRNA molecules when cultured *in vitro* and in soil in the glasshouse.

EXAMPLE 14

Transgenic *Glycine max* Comprising Hemipteran Pest Sequences

[00315] Ten to 20 transgenic T_0 *Glycine max* plants harboring expression vectors for nucleic acids comprising SEQ ID NO: 71 and/or SEQ ID NO:73 are generated as is known in the art, including for example by *Agrobacterium*-mediated transformation, as follows. Mature soybean (*Glycine max)* seeds are sterilized overnight with chlorine gas for sixteen hours. Following sterilization with chlorine gas, the seeds are placed in an open container in a LAMINAR™ flow hood to dispel the chlorine gas. Next, the sterilized seeds are imbibed with sterile H_2O for sixteen hours in the dark using a black box at 24° C.

[00316] **Preparation of split-seed soybeans**. The split soybean seed comprising a portion of an embryonic axis protocol required preparation of soybean seed material which is cut longitudinally, using a #10 blade affixed to a scalpel, along the hilum of the seed to separate and remove the seed coat, and to split the seed into two cotyledon sections. Careful attention is made to partially remove the embryonic axis, wherein about 1/2 – 1/3 of the embryo axis remains attached to the nodal end of the cotyledon.

[00317] **Inoculation**. The split soybean seeds comprising a partial portion of the embryonic axis are then immersed for about 30 minutes in a solution of *Agrobacterium tumefaciens* (e.g., strain EHA 101 or EHA 105) containing binary plasmid comprising SEQ ID NO: 71 and/or SEQ ID NO:73. The *Agrobacterium tumefaciens* solution is diluted to a final concentration of λ=0.6 OD_{650} before immersing the cotyledons comprising the embryo axis.

[00318] **Co-cultivation**. Following inoculation, the split soybean seed is allowed to co-cultivate with the *Agrobacterium tumefaciens* strain for 5 days on co-cultivation medium (Wang, Kan. *Agrobacterium Protocols*. 2. 1. New Jersey: Humana Press, 2006. Print.) in a Petri dish covered with a piece of filter paper.

[00319] **Shoot induction**. After 5 days of co-cultivation, the split soybean seeds are washed in liquid Shoot Induction (SI) media consisting of B5 salts, B5 vitamins, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose, 0.6 g/L MES, 1.11 mg/L BAP, 100 mg/L TIMENTIN™, 200 mg/L cefotaxime, and 50 mg/L vancomycin (pH 5.7). The split soybean seeds are then cultured on Shoot Induction I (SI I) medium consisting of B5 salts, B5 vitamins, 7 g/L Noble agar, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose, 0.6 g/L MES, 1.11 mg/L BAP, 50 mg/L TIMENTIN™, 200 mg/L cefotaxime, 50 mg/L vancomycin (pH 5.7), with the flat side of the cotyledon facing up and the nodal end of the cotyledon imbedded into the medium. After 2 weeks of culture, the explants from the transformed split soybean seed are transferred to the Shoot Induction II (SI II) medium containing SI I medium supplemented with 6 mg/L glufosinate (LIBERTY®).

[00320] **Shoot elongation**. After 2 weeks of culture on SI II medium, the cotyledons are removed from the explants and a flush shoot pad containing the embryonic axis are excised by making a cut at the base of the cotyledon. The isolated shoot pad from the cotyledon is transferred to Shoot Elongation (SE) medium. The SE medium consists of MS salts, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 30 g/L sucrose and 0.6 g/L MES, 50 mg/L asparagine, 100 mg/L L-pyroglutamic acid, 0.1 mg/L IAA, 0.5 mg/L GA3, 1 mg/L zeatin riboside, 50 mg/L TIMENTIN™, 200 mg/L cefotaxime, 50 mg/L vancomycin, 6 mg/L glufosinate, 7 g/L Noble agar, (pH 5.7). The cultures are transferred to fresh SE medium every 2 weeks. The cultures are grown in a CONVIRON™ growth chamber at 24° C with an 18 h photoperiod at a light intensity of 80-90 $\mu mol/m^2sec$.

[00321] **Rooting**. Elongated shoots which developed from the cotyledon shoot pad are isolated by cutting the elongated shoot at the base of the cotyledon shoot pad, and dipping the elongated shoot in 1 mg/L IBA (Indole 3-butyric acid) for 1–3 minutes to promote rooting. Next, the elongated shoots are transferred to rooting medium (MS salts, B5 vitamins, 28 mg/L Ferrous, 38 mg/L Na_2EDTA, 20 g/L sucrose and 0.59 g/L MES, 50 mg/L asparagine, 100 mg/L L-pyroglutamic acid 7 g/L Noble agar, pH 5.6) in phyta trays.

[00322] Cultivation. Following culture in a CONVIRON™ growth chamber at 24° C, 18 h photoperiod, for 1-2 weeks, the shoots which have developed roots are transferred to a soil mix in a covered sundae cup and placed in a CONVIRON™ growth chamber (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22°C) and humidity (40-50%) for acclimatization of plantlets. The rooted plantlets are acclimated in sundae cups for several weeks before they are transferred to the greenhouse for further acclimatization and establishment of robust transgenic soybean plants.

[00323] A further 10-20 T_1 *Glycine max* independent lines expressing hairpin dsRNA for an RNAi construct are obtained for BSB challenge. Hairpin dsRNA may be derived as set forth in SEQ ID NO:73 or otherwise further comprising SEQ ID NO:71. These are confirmed through RT-PCR or other molecular analysis methods. Total RNA preparations from selected independent T_1 lines are optionally used for RT-PCR with primers designed to bind in the ST-LS1 intron of the hairpin expression cassette in each of the RNAi constructs. In addition, specific primers for each target gene in an RNAi construct are optionally used to amplify and confirm the production of the pre-processed mRNA required for siRNA production *in planta*. The amplification of the desired bands for each target gene confirms the expression of the hairpin RNA in each transgenic *Glycine max* plant. Processing of the dsRNA hairpin of the target genes into siRNA is subsequently optionally confirmed in independent transgenic lines using RNA blot hybridizations.

[00324] Moreover, RNAi molecules having mismatch sequences with more than 80% sequence identity to target genes affect corn rootworms in a way similar to that seen with RNAi molecules having 100% sequence identity to the target genes. The pairing of mismatch sequence with native sequences to form a hairpin dsRNA in the same RNAi construct delivers plant-processed siRNAs capable of affecting the growth, development and viability of feeding hemipteran pests.

[00325] *In planta* delivery of dsRNA, siRNA, shRNA, or miRNA corresponding to target genes and the subsequent uptake by hemipteran pests through feeding results in down-regulation of the target genes in the hemipteran pest through RNA-mediated gene silencing. When the function

of a target gene is important at one or more stages of development, the growth, development, and reproduction of the hemipteran pest is affected, and in the case of at least one of *Euschistus heros*, *Piezodorus guildinii*, *Halyomorpha halys*, *Nezara viridula*, *Acrosternum hilare*, and *Euschistus servus* leads to failure to successfully infest, feed, develop, and/or reproduce, or leads to death of the hemipteran pest. The choice of target genes and the successful application of RNAi is then used to control hemipteran pests.

[00326] Phenotypic comparison of transgenic RNAi lines and nontransformed Glycine max Target hemipteran pest genes or sequences selected for creating hairpin dsRNA have no similarity to any known plant gene sequence. Hence it is not expected that the production or the activation of (systemic) RNAi by constructs targeting these hemipteran pest genes or sequences will have any deleterious effect on transgenic plants. However, development and morphological characteristics of transgenic lines are compared with nontransformed plants, as well as those of transgenic lines transformed with an "empty" vector having no hairpin-expressing gene. Plant root, shoot, foliage and reproduction characteristics are compared. There is no observable difference in root length and growth patterns of transgenic and nontransformed plants. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are similar. In general, there are no observable morphological differences between transgenic lines and those without expression of target iRNA molecules when cultured in vitro and in soil in the glasshouse.

EXAMPLE 15
E. heros bioassays on Artificial diet

[00327] In dsRNA feeding assays on artificial diet, 32-well trays are set up with an ~18 mg pellet of artificial diet and water, as for injection experiments (EXAMPLE 12). dsRNA at a concentration of 200 ng/µl is added to the food pellet and water sample, 100 µl to each of two wells. Five 2nd instar E. heros nymphs are introduced into each well. Water samples and dsRNA that targets YFP transcript are used as negative controls. The experiments are repeated on three different days. Surviving insects are weighed and the mortality rates are determined after 8 days of treatment.

EXAMPLE 16
Transgenic *Arabidopsis thaliana* Comprising Hemipteran Pest Sequences

[00328] *Arabidopsis* transformation vectors containing a target gene construct for hairpin formation comprising segments of *COPI delta* (SEQ ID NO:71) are generated using standard molecular methods similar to EXAMPLE 4. Arabidopsis transformation is performed using standard Agrobacterium-based procedure. T1 seeds are selected with glufosinate tolerance selectable marker. Transgenic T1 Arabidopsis plants are generated and homozygous simple-copy T2 transgenic plants are generated for insect studies. Bioassays are performed on growing Arabidopsis plants with inflorescences. Five to ten insects are placed on each plant and monitored for survival within 14 days.

[00329] Construction of *Arabidopsis* transformation vectors. Entry clones based on entry vector pDAB3916 harboring a target gene construct for hairpin formation comprising a segment of *COPI delta* (SEQ ID NO:71) are assembled using a combination of chemically synthesized fragments (DNA2.0, Menlo Park, CA) and standard molecular cloning methods. Intramolecular hairpin formation by RNA primary transcripts is facilitated by arranging (within a single transcription unit) two copies of a target gene segment in opposite orientations, the two segments being separated by an ST-LS1 intron sequence (SEQ ID NO:13) (Vancanneyt *et al.* (1990) Mol. Gen. Genet. 220(2):245-50). Thus, the primary mRNA transcript contains the two *COPI delta* gene segment sequences as large inverted repeats of one another, separated by the intron sequence. A copy of a *Arabidopsis thaliana* ubiquitin 10 promoter (Callis *et al.* (1990) J. Biological Chem. 265:12486-12493) is used to drive production of the primary mRNA hairpin transcript, and a fragment comprising a 3' untranslated region from Open Reading Frame 23 of *Agrobacterium tumefaciens* (AtuORF23 3' UTR v1; US Patent No. 5,428,147) is used to terminate transcription of the hairpin-RNA-expressing gene.

[00330] The hairpin clone within entry vector pDAB3916 described above is used in standard GATEWAY® recombination reaction with a typical binary destination vector pDAB101836 to produce hairpin RNA expression transformation vectors for *Agrobacterium*-mediated *Arabidopsis* transformation.

[00331] Binary destination vector pDAB101836 comprises a herbicide tolerance gene, DSM-2v2 (U.S. Patent App. No. 2011/0107455), under the regulation of a Cassava vein mosaic

virus promoter (CsVMV Promoter v2, U.S. Patent No. US 7601885; Verdaguer et al, (1996) Plant Molecular Biology, 31:1129-1139). A fragment comprising a 3' untranslated region from Open Reading Frame 1 of *Agrobacterium tumefaciens* (AtuORF1 3' UTR v6; Huang et al, (1990) J. Bacteriol, 172:1814-1822) is used to terminate transcription of the DSM2v2 mRNA.

[00332] A negative control binary construct, pDAB114507, which comprises a gene that expresses a YFP hairpin RNA, is constructed by means of standard GATEWAY® recombination reactions with a typical binary destination vector (pDAB101836) and entry vector pDAB3916. Entry construct pDAB112644 comprises a YFP hairpin sequence (hpYFP v2-1, SEQ ID NO:79) under the expression control of an *Arabidopsis* Ubiquitin 10 promoter (as above) and a fragment comprising an ORF23 3' untranslated region from *Agrobacterium tumefaciens* (as above).

[00333] Production of transgenic *Arabidopsis* comprising insecticidal hairpin RNAs: *Agrobacterium*-mediated transformation. Binary plasmids containing hairpin sequences are electroporated into *Agrobacterium* strain GV3101 (pMP90RK). The recombinant *Agrobacterium* clones are confirmed by restriction analysis of plasmids preparations of the recombinant *Agrobacterium* colonies. A Qiagen Plasmid Max Kit (Qiagen, Cat# 12162) is used to extract plasmids from *Agrobacterium* cultures following the manufacture recommended protocol.

[00334] *Arabidopsis* transformation and T_1 Selection. Twelve to fifteen Arabidopsis plants (c.v. Columbia) are grown in 4" pots in the green house with light intensity of 250 µmol/m^2, 25°C, and 18:6 hours of light: dark conditions. Primary flower stems are trimmed one week before transformation. *Agrobacterium* inoculums are prepared by incubating 10 µl of recombinant *Agrobacterium* glycerol stock in 100 ml LB broth (Sigma L3022) +100 mg/L Spectinomycin + 50 mg/L Kanamycin at 28°C and shaking at 225 rpm for 72 hours. *Agrobacterium* cells are harvested and suspended into 5% sucrose + 0.04% Silwet-L77 (Lehle Seeds Cat # VIS-02) +10 µg/L benzamino purine (BA) solution to OD_{600} 0.8~1.0 before floral dipping. The above-ground parts of the plant are dipped into the *Agrobacterium* solution for 5-10 minutes, with gentle agitation. The plants are then transferred to the greenhouse for normal growth with regular watering and fertilizing until seed set.

EXAMPLE 17

Growth and bioassays of transgenic *Arabidopsis*.

[00335] Selection of T_1 *Arabidopsis* transformed with hairpin RNAi constructs. Up to 200 mg of T_1 seeds from each transformation is stratified in 0.1% agarose solution. The seeds are planted in germination trays (10.5" x 21" x 1"; T.O. Plastics Inc., Clearwater, MN.) with #5 sunshine media. Transformants are selected for tolerance to Ignite® (glufosinate) at 280 g/ha at 6 and 9 days post planting. Selected events are transplanted into 4" diameter pots. Insertion copy analysis is performed within a week of transplanting via hydrolysis quantitative Real-Time PCR (qPCR) using Roche LightCycler480. The PCR primers and hydrolysis probes are designed against DSM2v2 selectable marker using LightCycler Probe Design Software 2.0 (Roche). Plants are maintained at 24°C, with a 16:8 hour light: dark photoperiod under fluorescent and incandescent lights at intensity of 100-150mE/m2×s.

[00336] *E. heros* plant feeding bioassay. At least four low copy (1-2 insertions), four medium copy (2-3 insertions), and four high copy (≥4 insertions) events are selected for each construct. Plants are grown to a flowering stage (plants containing flowers and siliques). The surface of soil is covered with ~ 50 ml volume of white sand for easy insect identification. Five to ten 2nd instar *E. heros* nymphs are introduced onto each plant. The plants are covered with plastic tubes that are 3" in diameter, 16" tall, and with wall thickness of 0.03" (Item No. 484485, Visipack Fenton MO); the tubes are covered with nylon mesh to isolate the insects. The plants are kept under normal temperature, light, and watering conditions in a conviron. In 14 days, the insects are collected and weighed; percent mortality as well as growth inhibition (1 – weight treatment/weight control) are calculated. YFP hairpin-expressing plants are used as controls.

[00337] T_2 *Arabidopsis* seed generation and T_2 bioassays. T_2 seed is produced from selected low copy (1-2 insertions) events for each construct. Plants (homozygous and/or heterozygous) are subjected to *E. heros* feeding bioassay, as described above. T_3 seed is harvested from homozygotes and stored for future analysis.

[00338] While the present disclosure may be susceptible to various modifications and alternative forms, specific embodiments have been described by way of example in detail herein. However, it should be understood that the present disclosure is not intended to be limited to the

particular forms disclosed. Rather, the present disclosure is to cover all modifications, equivalents, and alternatives falling within the scope of the present disclosure as defined by the following appended claims and their legal equivalents.

CLAIMS

What may be claimed is:

1. An isolated nucleic acid comprising at least one polynucleotide operably linked to a heterologous promoter, wherein the polynucleotide is selected from the group consisting of:

SEQ ID NO:1; the complement of SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1; a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:1; and

SEQ ID NO:71; the complement of SEQ ID NO: 71; a fragment of at least 15 contiguous nucleotides of SEQ ID NO: 71; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO: 71; a native coding sequence of a *Euschistus* organism comprising SEQ ID NO: 71; the complement of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO: 71; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO: 71; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO: 71.

2. The polynucleotide of claim 1, wherein the polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:11, SEQ ID NO:71, SEQ ID NO:73, and the complements of any of the foregoing.

3. A plant transformation vector comprising the polynucleotide of claim 1.

4. The polynucleotide of claim 1, wherein the organism is selected from the group consisting of *D. v. virgifera* LeConte; *D. barberi* Smith and Lawrence; *D. u. howardi*; *D. v. zeae*; *D. balteata* LeConte; *D. u. tenella*; *D. speciosa* Germar; *D. u. undecimpunctata* Mannerheim;

Euschistus heros (Fabr.) (Neotropical Brown Stink Bug), *Nezara viridula* (L.) (Southern Green Stink Bug), *Piezodorus guildinii* (Westwood) (Red-banded Stink Bug), *Halyomorpha halys* (Stål) (Brown Marmorated Stink Bug), *Chinavia hilare* (Say) (Green Stink Bug), *Euschistus servus* (Say) (Brown Stink Bug), *Dichelops melacanthus* (Dallas), *Dichelops furcatus* (F.), *Edessa meditabunda* (F.), *Thyanta perditor* (F.) (Neotropical Red Shouldered Stink Bug), *Chinavia marginatum* (Palisot de Beauvois), *Horcias nobilellus* (Berg) (Cotton Bug), *Taedia stigmosa* (Berg), *Dysdercus peruvianus* (Guérin-Méneville), *Neomegalotomus parvus* (Westwood), *Leptoglossus zonatus* (Dallas), *Niesthrea sidae* (F.), *Lygus hesperus* (Knight) (Western Tarnished Plant Bug), and *Lygus lineolaris* (Palisot de Beauvois).

5. A ribonucleic acid (RNA) molecule transcribed from the polynucleotide of claim 1.

6. A double-stranded ribonucleic acid molecule produced from the expression of the polynucleotide of claim 1.

7. The double-stranded ribonucleic acid molecule of claim 6, wherein contacting the polynucleotide sequence with a coleopteran or hemipteran pest inhibits the expression of an endogenous nucleotide sequence specifically complementary to the polynucleotide.

8. The double-stranded ribonucleic acid molecule of claim 7, wherein contacting said ribonucleotide molecule with a coleopteran or hemipteran pest kills or inhibits the growth, and/or feeding of the pest.

9. The double stranded RNA of claim 6, comprising a first, a second and a third RNA segment, wherein the first RNA segment comprises the polynucleotide, wherein the third RNA segment is linked to the first RNA segment by the second polynucleotide sequence, and wherein the third RNA segment is substantially the reverse complement of the first RNA segment, such that the

first and the third RNA segments hybridize when transcribed into a ribonucleic acid to form the double-stranded RNA.

10. The RNA of claim 5, selected from the group consisting of a double-stranded ribonucleic acid molecule and a single-stranded ribonucleic acid molecule of between about 15 and about 30 nucleotides in length.

11. A plant transformation vector comprising the polynucleotide of claim 1, wherein the heterologous promoter is functional in a plant cell.

12. A cell transformed with the polynucleotide of claim 1.

13. The cell of claim 12, wherein the cell is a prokaryotic cell.

14. The cell of claim 12, wherein the cell is a eukaryotic cell.

15. The cell of claim 14, wherein the cell is a plant cell.

16. A plant transformed with the polynucleotide of claim 1.

17. A seed of the plant of claim 16, wherein the seed comprises the polynucleotide.

18. A commodity product produced from the plant of claim 16, wherein the commodity product comprises a detectable amount of the polynucleotide.

19. The plant of claim 16, wherein the at least one polynucleotide is expressed in the plant as a double-stranded ribonucleic acid molecule.

20. The cell of claim 15, wherein the cell is a maize, soybean, or cotton cell.

21. The plant of claim 16, wherein the plant is maize, soybean, or cotton.

22. The plant of claim 16, wherein the at least one polynucleotide is expressed in the plant as a ribonucleic acid molecule, and the ribonucleic acid molecule inhibits the expression of an endogenous polynucleotide that is specifically complementary to the at least one polynucleotide when a coleopteran or hemipteran pest ingests a part of the plant.

23. The polynucleotide of claim 1, further comprising at least one additional polynucleotide that encodes an RNA molecule that inhibits the expression of an endogenous pest gene.

24. A plant transformation vector comprising the polynucleotide of claim 23, wherein the additional polynucleotide(s) are each operably linked to a heterologous promoter functional in a plant cell.

25. A method for controlling an insect pest population, the method comprising providing an agent comprising a ribonucleic acid (RNA) molecule that functions upon contact with the insect pest to inhibit a biological function within the pest, wherein the RNA is specifically hybridizable with a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71; the complement of a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71; a fragment of at least 15 contiguous nucleotides of a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71; the complement of a fragment of at least 15 contiguous nucleotides of a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71; a transcript of a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71; and the complement of a transcript of a polynucleotide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:71.

26. The method according to claim 25, wherein the agent is a double-stranded RNA molecule.

27. The method according to claim 25, wherein the insect pest is a coleopteran or hemipteran pest.

28. A method for controlling a coleopteran or hemipteran pest population, the method comprising:

providing in a host plant of a coleopteran or hemipteran pest a transformed plant cell comprising the polynucleotide of claim 1, wherein the polynucleotide is expressed to produce a ribonucleic acid molecule that functions upon contact with a coleopteran or hemipteran pest belonging to the population to inhibit the expression of a target sequence within the coleopteran or hemipteran pest and results in decreased growth and/or survival of the coleopteran or hemipteran pest or pest population, relative to the same pest species on a plant of the same host plant species that does not comprise the polynucleotide.

29. The method according to claim 28, wherein the ribonucleic acid molecule is a double-stranded ribonucleic acid molecule.

30. The method according to claim 28, wherein the coleopteran or hemipteran pest population is reduced relative to a population of the same pest species infesting a host plant of the same host plant species lacking the transformed plant cell.

31. The method according to claim 28, wherein the ribonucleic acid molecule is a double-stranded ribonucleic acid molecule.

32. The method according to claim 29, wherein the coleopteran or hemipteran pest population is reduced relative to a coleopteran or hemipteran pest population infesting a host plant of the same species lacking the transformed plant cell.

33. A method of controlling an insect pest infestation in a plant, the method comprising providing in the diet of the insect pest a ribonucleic acid (RNA) that is specifically hybridizable with a polynucleotide selected from the group consisting of:

SEQ ID NO:1 or SEQ ID NO:71;

the complement of SEQ ID NO:1 or SEQ ID NO:71;

a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71;

the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:71;

a transcript of SEQ ID NO:1 or SEQ ID NO:71;

the complement of a transcript of SEQ ID NO:1 or SEQ ID NO:71;

a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or SEQ ID NO:71; and

the complement of a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or SEQ ID NO:71.

34. The method according to claim 33, wherein the diet comprises a plant cell transformed to express the polynucleotide.

35. The method according to claim 33, wherein the specifically hybridizable RNA is comprised in a double-stranded RNA molecule.

36. A method for improving the yield of a corn crop, the method comprising:

introducing the nucleic acid of claim 1 into a corn plant to produce a transgenic corn plant; and

cultivating the corn plant to allow the expression of the at least one polynucleotide; wherein expression of the at least one polynucleotide inhibits the development or growth of a coleopteran and/or hemipteran pest and loss of yield due to infection by the coleopteran and/or hemipteran pest.

37. The method according to claim 36, wherein expression of the at least one polynucleotide produces an RNA molecule that suppresses at least a first target gene in a coleopteran and/or hemipteran pest that has contacted a portion of the corn plant.

38. A method for producing a transgenic plant cell, the method comprising:

transforming a plant cell with a vector comprising the nucleic acid of claim 1;

culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture comprising a plurality of transformed plant cells;

selecting for transformed plant cells that have integrated the at least one polynucleotide into their genomes;

screening the transformed plant cells for expression of a ribonucleic acid (RNA) molecule encoded by the at least one polynucleotide; and

selecting a plant cell that expresses the RNA.

39. The method according to claim 38, wherein the RNA molecule is a double-stranded RNA molecule.

40. A method for producing a coleopteran and/or hemipteran pest-resistant transgenic plant, the method comprising:

providing the transgenic plant cell produced by the method of claim 38; and

regenerating a transgenic plant from the transgenic plant cell, wherein expression of the ribonucleic acid molecule encoded by the at least one polynucleotide is sufficient to modulate the expression of a target gene in a coleopteran and/or hemipteran pest that contacts the transformed plant.

41. A method for producing a transgenic plant cell, the method comprising:

transforming a plant cell with a vector comprising a means for protecting a plant from coleopteran pests;

culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture comprising a plurality of transformed plant cells;

selecting for transformed plant cells that have integrated the means for providing coleopteran pest resistance to a plant into their genomes;

screening the transformed plant cells for expression of a means for inhibiting expression of an essential gene in a coleopteran pest; and

selecting a plant cell that expresses the means for inhibiting expression of an essential gene in a coleopteran pest.

42. A method for producing a coleopteran pest-resistant transgenic plant, the method comprising:

providing the transgenic plant cell produced by the method of claim 41; and

regenerating a transgenic plant from the transgenic plant cell, wherein expression of the means for inhibiting expression of an essential gene in a coleopteran pest is sufficient to modulate the expression of a target gene in a coleopteran pest that contacts the transformed plant.

43. A method for producing a transgenic plant cell, the method comprising:

transforming a plant cell with a vector comprising a means for providing hemipteran pest resistance to a plant;

culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture comprising a plurality of transformed plant cells;

selecting for transformed plant cells that have integrated the means for providing hemipteran pest resistance to a plant into their genomes;

screening the transformed plant cells for expression of a means for inhibiting expression of an essential gene in a hemipteran pest; and

selecting a plant cell that expresses the means for inhibiting expression of an essential gene in a hemipteran pest.

44. A method for producing a hemipteran pest-resistant transgenic plant, the method comprising:

providing the transgenic plant cell produced by the method of claim 43; and

regenerating a transgenic plant from the transgenic plant cell, wherein expression of the means for inhibiting expression of an essential gene in a hemipteran pest is sufficient to modulate the expression of a target gene in a hemipteran pest that contacts the transformed plant.

45. The nucleic acid of claim 1, further comprising a polynucleotide encoding a polypeptide from *Bacillus thuringiensis*, *Alcaligenes* spp., or *Pseudomonas* spp.

46. The nucleic acid of claim 45, wherein the polypeptide from *B. thuringiensis* is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C.

47. The cell of claim 15, wherein the cell comprises a polynucleotide encoding a polypeptide from *Bacillus thuringiensis*, *Alcaligenes* spp., or *Pseudomonas* spp.

48. The cell of claim 47, wherein the polypeptide from *B. thuringiensis* is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C.

49. The plant of claim 16, wherein the plant comprises a polynucleotide encoding a polypeptide from *Bacillus thuringiensis*, *Alcaligenes* spp., or *Pseudomonas* spp.

50. The plant of claim 49, wherein the polypeptide from *B. thuringiensis* is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C.

51. The method according to claim 38, wherein the transformed plant cell comprises a nucleotide sequence encoding a polypeptide from *Bacillus thuringiensis*, *Alcaligenes* spp., or *Pseudomonas* spp.

52. The method according to claim 51, wherein the polypeptide from *B. thuringiensis* is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and Cyt2C.

Figure 1. Generation of dsRNA from a single transcription template with a single pair of primers

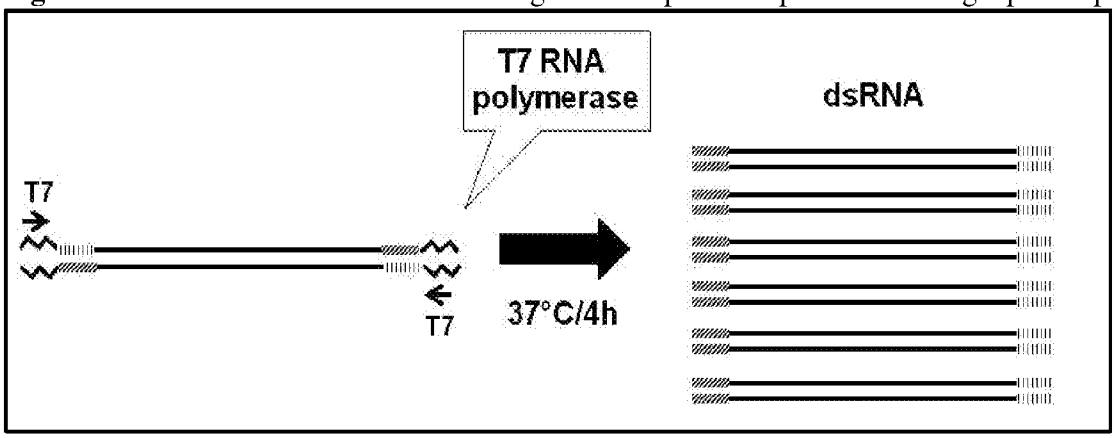
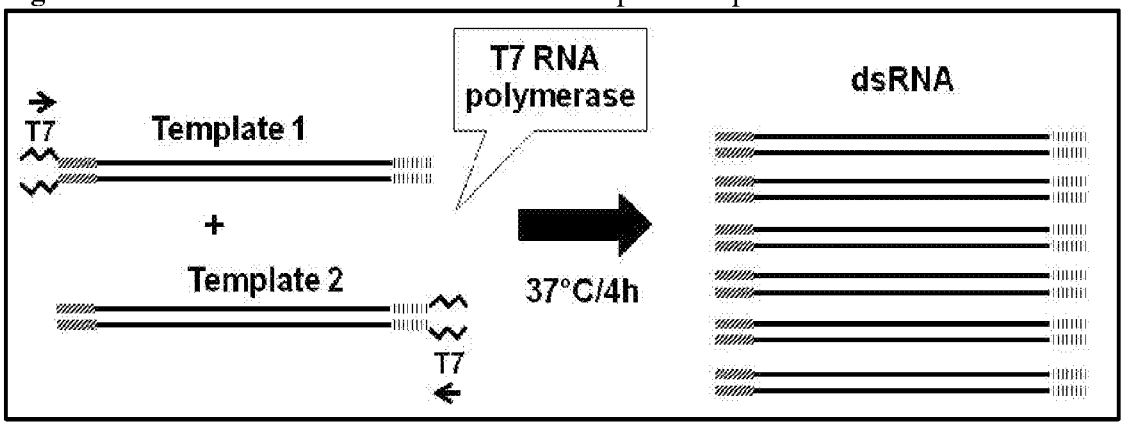


Figure 2. Generation of dsRNA from two transcription templates.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/54481

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 57/16; C12N 15/113; C07K 14/115 (2016.01)
CPC - A01N 37/46; C12N 15/113; C07K 14/225
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classification(s): A01N 57/16; C12N 15/113; C07K 14/115 (2016.01);
CPC Classification(s): A01N 37/46; C12N 15/113; C07K 14/225; USPC Classification(s): 514/44A

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO); Google; Google Scholar; EBSCO; Entrez Pubmed; Science Direct; Search terms -- transgenic, pesticidal, polynucleotide, corn, dsRNA, cultivation, screening, 'crop yield', Cry1B, B. thuringiensis, expression inhibition, Coleoptera, Hemiptera, resistance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2014/0275208 A1 (HU, X et al.) September 18, 2014; abstract; paragraphs [0005], [0006], [0015]-[0017], [0028], [0033], [0035]-[0040], [0042], [0045], [0068], [0071], [0091], [0096], [0104], [0115], [0116], [0120], [0121], [0123], [0130], [0133], [0144], [0147], [0148], [0150]; claims 1, 2, 23; SEQ ID NO: 504	1-35, 38-40, 45, 47, 49, 51 ---------- 36, 37, 46, 48, 50, 52
Y	US 2014/0033361 A1 (PIONEER HI BRED INTERNATIONAL INC., et al.) January 30, 2014; abstract; paragraphs [0010], [0287], [0288], [0301], [0444]	36, 37, 46, 48, 50, 52
A	US 2012/0137387 A1 (BAUM, JA et al.) May 31, 2012; abstract	1-40, 45-52
A	WO 2014/159829 A1 (E. I. DUPONT DE NEMOURS AND COMPANY) October 2, 2014; abstract	1-40, 45-52

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2016 (22.02.2016)	04 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/54481

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Continued Within the Next Supplemental Box-***-

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-40, 45-52; SEQ ID NO: 1

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US15/54481

-***-Continued from Box III: Lack of Unity of Invention-***-

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

Groups I+: Claims 1-40 and 45-52 are directed toward an isolated nucleic acid; and methods, additional nucleic acids and constructs related thereto.

The isolated nucleic acid, methods, additional nucleic acids and constructs will be searched to the extent that the nucleic acid encompasses SEQ ID NO: 1 (first exemplary isolated nucleic acid sequence). It is believed that Claims 1 (in-part), 2 (in-part), 3 (in-part), 4 (in-part), 5 (in-part), 6 (in-part), 7 (in-part), 8 (in-part), 9 (in-part), 10 (in-part), 11 (in-part), 12 (in-part), 13 (in-part), 14 (in-part), 15 (in-part), 16 (in-part), 17 (in-part), 18 (in-part), 19 (in-part), 20 (in-part), 21 (in-part), 22 (in-part), 23 (in-part), 24 (in-part), 25 (in-part), 26 (in-part), 27 (in-part), 28 (in-part), 29 (in-part), 30 (in-part), 31 (in-part), 32 (in-part), 33 (in-part), 34 (in-part), 35 (in-part), 36 (in-part), 37 (in-part), 38 (in-part), 39 (in-part), 40 (in-part), 45 (in-part), 46 (in-part), 47 (in-part), 48 (in-part), 49 (in-part), 50 (in-part), 51 (in-part) and 52 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (diabrotica virgifera DNA sequence). Applicant is invited to elect additional nucleic acid(s) with specified SEQ ID NO: for each, to be searched. Additional nucleic acid sequence(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected nucleic acid sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NO: 71 (Euschistus heros DNA sequence).

Group II: Claims 41-44 are directed toward methods for producing transgenic plant cells.

The inventions listed as Groups I+-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I + include SEQ ID NO: 1, which is not present in Group II, the special technical features of Group II include an essential gene in a hemipteran pest, which is not present in any of Groups I+.

However, these shared technical features are previously disclosed by WO 2014/159829 A1 (E. I. Du Pont De Nemours and Company) (hereinafter 'Du Pont') in view of US 2014/0275208 A1 to Hu, et al. (hereinafter 'Hu').

Du Pont discloses a method for producing a transgenic plant cell (a method for producing a transgenically transformed plant cell; paragraphs [000108], [000109]), the method comprising: transforming a plant cell (paragraph [000108]) with a vector (with a vector comprising a transfer cassette; paragraphs [00063], [000108]) comprising a means for protecting a plant from insect pests (comprising a silencing element that reduces the expression of a target polynucleotide in a desired pest, for protecting a plant from the pest; paragraphs [00061], [00082]); culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture (transforming the cells and culturing the cells in selective media to produce a plant cell culture comprising transformed cells; paragraphs [000136], [000137]) comprising a plurality of transformed plant cells (paragraphs [000136], [000137]); selecting for transformed plant cells (culturing the cells in selective media to select transformed plant cells; paragraphs [00136], [000137]) that have integrated (paragraphs [000130], [000137]) the means for providing insect pest resistance to a plant into their genomes (the silencing element that confers insect resistance into their genomes; paragraphs [00061], [00082], [000130], [000137]); screening the transformed plant cells for a means for inhibiting expression of a gene in an insect pest (screening transformed plants grown from the plant cells for zygosity for the silencing element, which inhibits expression of a gene in an insect pest; paragraphs [00061], [00082], [000139]); and selecting a plant cell that comprises the means for inhibiting expression of a gene in an insect pest (and selecting homozygous plant seeds for growth and examination of insect protection; paragraphs [00061], [00082], [000169]); an isolated nucleic acid (Claim 65) comprising at least one polynucleotide (comprising a nucleotide sequence; Claim 65) operably linked to a heterologous promoter (operably-linked to a heterologous promoter; paragraphs [00050], [00066]), wherein the polynucleotide is a fragment of at least 15 contiguous nucleotides (paragraphs [0008], [00052]) of a coatomer polypeptide amino acid sequence (paragraphs [0008], [0050], Table 2); a plant transformation vector comprising the polynucleotide (paragraph [00063]); a ribonucleic acid (RNA) molecule transcribed from the polynucleotide (paragraphs [00029], [00032]); a double-stranded ribonucleic acid molecule produced from the expression of the polynucleotide (paragraphs [00029], [00032]); a plant transformation vector comprising the polynucleotide (paragraph [00063]) wherein the heterologous promoter is functional in a plant cell (wherein the promoter allows for expression of the polynucleotide; paragraphs [00050], [00064], [00066]); a cell transformed with the polynucleotide (paragraphs [00136], [000137]); a plant transformed with the polynucleotide (paragraphs [000136], [000137], [000139]); a seed of the plant (paragraph [000109]), wherein the seed comprises the polynucleotide (wherein the seed is from a stably transformed plant line; paragraph [000109]); a commodity product produced from the plant (seed; paragraph [000109]); a plant transformation vector comprising the polynucleotide (paragraph [00063]) wherein additional polynucleotide(s) are operably linked to a heterologous promoter functional in a plant cell (wherein a single polynucleotide comprises more than one silencing element operably-linked to a heterologous promoter for expression thereof; paragraphs [00022], [0050], [00066]); a method for controlling a hemipteran pest population (paragraph [0009]), the method comprising: providing in a host plant of a hemipteran pest (paragraph [0009]) a transformed plant cell comprising the polynucleotide (paragraphs [00063], [000136], [000137]), wherein the polynucleotide is expressed (wherein the silencing element (polynucleotide) is expressed; paragraph [0009]) to produce a ribonucleic acid molecule (to produce a RNA molecule; paragraph [00024]) that functions upon contact with a hemipteran pest belonging to the population (that, when fed to a hemipteran pest; paragraphs [0009], [00022]) to inhibit the expression of a target sequence within the hemipteran pest (paragraph [00022]) and results in decreased growth and/or survival of the hemipteran pest or pest population, relative to the same pest species on a plant of the same host plant species that does not comprise the polynucleotide (and controls the hemipteran pest; paragraph [0009]); a method comprising: introducing the nucleic acid (paragraphs [00063], [000136], [000137]) into a corn plant (paragraph [000111]) to produce a transgenic (paragraphs [00063], [000111], [000137], [000139]) corn plant (paragraph [000111]); and cultivating the corn plant (growing (cultivating) the corn plant; paragraphs [000109], [000111]) to allow the expression of the at least one polynucleotide (to ensure (allow) the expression of the at least one polynucleotide; paragraph [000109]); wherein expression of the at least one polynucleotide (paragraphs [0009], [00022]) inhibits the development or growth of a hemipteran pest (controls a hemipteran pest; paragraphs [0009], [00022]); a method for producing a transgenic plant cell (a method for producing a transgenically transformed plant cell; paragraphs [000108], [000109]), the method comprising: ... Continued on Next Supplemental Page ...

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Box III-***-

... Continued from Previous Supplemental Page ... transforming a plant cell (paragraph [000108]) with a vector (with a vector comprising a transfer cassette; paragraphs [00063], [000108]) comprising the nucleic acid (paragraphs [00022], [000108], [000109]); culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture transforming the cells and culturing the cells in selective media to produce a plant cell culture comprising transformed cells (culturing the transformed plant cell under conditions sufficient to allow for development of a plant cell culture); paragraphs [000136], [000137]); comprising a plurality of transformed plant cells (paragraphs [000136], [000137]); selecting for transformed plant cells (culturing the cells in selective media to select transformed plant cells; paragraphs [00136], [000137]) that have integrated (paragraphs [000130], [000137]) the at least one polynucleotide into their genomes (paragraphs [000136], [000137], [000139]); a method for producing a hemipteran pest-resistant transgenic plant (a method for producing a plant expressing a hemipteran pest controlling silencing element; paragraph [0009]), the method comprising: providing the transgenic plant cell produced by the method (paragraphs [0009], [000136], [000137]); and regenerating a transgenic plant from the transgenic plant cell (paragraphs [000137]-[000139]), wherein expression of the ribonucleic acid molecule encoded by the at least one polynucleotide (paragraphs [00022], [00024]) is sufficient to modulate the expression of a target gene in a hemipteran pest that contacts the transformed plant (paragraphs [0009], [000139]); a method for controlling an insect pest population (paragraph [0009]), the method comprising providing an agent comprising a ribonucleic acid (RNA) molecule (the method comprising providing an RNA silencing element; paragraphs [0009], [00022], [00024]) that functions upon contact with the insect pest to inhibit a biological function within the pest (that, when ingested by the insect, inhibits expression of a target nucleic acid within the pest; paragraphs [0009], [00022], [00024]) wherein the RNA is specifically hybridizable with a fragment of at least 15 contiguous nucleotides (wherein the RNA comprises a sequence complementary to a fragment of at least 15 contiguous nucleotides; paragraphs [0008], [00024], [00028], [00029], [00052]) of a coatomer polypeptide amino acid sequence (of a coatomer polypeptide amino acid sequence; paragraphs [0008], [0050], Table 2);

and a method of controlling an insect pest infestation in a plant (a method of controlling an insect pest infestation in a plant; paragraph [0009]), the method comprising providing in the diet of the insect pest a ribonucleic acid (RNA) (the method comprising providing to the insect for ingestion a RNA (the method comprising providing in the diet of the insect pest a ribonucleic acid (RNA); paragraphs [0009], [00022], [00024]) that is specifically hybridizable with a fragment of at least 15 contiguous nucleotides (wherein the RNA comprises a sequence complementary to a fragment of at least 15 contiguous nucleotides; paragraphs [0008], [00024], [00028], [00029], [00052]) of a coatomer polypeptide amino acid sequence (of a coatomer polypeptide amino acid sequence; paragraphs [0008], [0050], Table 2); wherein the transformed plants stably maintain the desired phenotypic protection, and harvesting seeds from stably transformed plant lines (paragraph [000109]).

Du Pont does not disclose screening the transformed plant cells for expression of a means for inhibiting expression of a gene in an insect pest, such as a ribonucleic acid molecule encoded by at least one polynucleotide, and selecting a plant cell that expresses the means for inhibiting expression of a gene in an insect pest, such as the RNA molecule; wherein the commodity product comprises a detectable amount of the polynucleotide; a method for improving the yield of a corn crop, and loss of yield due to infection by the coleopteran and/or hemipteran pest; and SEQ ID NO: 1.

Hu discloses methods of employing a silencing element that, when ingested by a Coleopteran pest, is capable of decreasing the expression of a target nucleic acid in the pest (paragraph [0005]), and thereby providing protection to the plant from said pest (paragraph [0005]), wherein the silencing elements include fragments of sequences including coatomer proteins (figure 1b, paragraph [0025]) selected from the set consisting of SEQ ID NO: 1 (SEQ ID NO: 504 of the instant PCT application (SEQ ID NO: 1); figure 1B, paragraphs [0005], [0025], wherein SEQ ID NO: 504 of the instant PCT application comprises a sequence, starting from ORF initiating atg at nucleotide 142, which is 100% identical to SEQ ID NO: 1 of the instant PCT application), SEQ ID NO: 321, SEQ ID NO: 501 and SEQ ID NO: 324 (SEQ ID NO: 321, SEQ ID NO: 501 and SEQ ID NO: 324; figure 1b, paragraph [0025], wherein the 4 sequences listed are the only cotomer proteins disclosed by Hu).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Du Pont to have assessed expression of a RNA silencing element in the homozygous cells selected as disclosed by Du Pont in order to better enable the correlation of silencing element expression with control of pest infestations. Further, although, as above, Du Pont does not disclose wherein the commodity product comprises a detectable amount of the polynucleotide, It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Du Pont to have indicated wherein seeds produced by stably transformed plant lines comprised detectable amounts of the polynucleotide to verify the stable transformation of the plant lines. Furthermore, although, as above, Du Pont does not disclose a method for improving the yield of a corn crop, and loss of yield due to infection by the coleopteran and/or hemipteran pest, It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Du Pont to have provided said method, as Du Pont, as above, discloses the protection of plants, including corn, from insect infestations by controlling the insects. It would have been obvious to a person of ordinary skill in the art at the time of the invention was made that controlling populations of insects infesting crop plants, such as corn, as disclosed by Du Pont, would have resulted in enhanced yields of crops, such as corn, based on reduced losses of plants to destruction by the infesting insects. finally, it would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Du Pont to have also used coatomer proteins from other pest species useful in the protection of plants against pests, such as SEQ ID NO: 1, as disclosed by Hu, in order to enable protection against a wider range of pest species, dependent upon prevalent pests of a particular crop or geographical region to be protected.

Since none of the special technical features of the Groups I+-II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Du Pont and Hu references, unity of invention is lacking.