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(71) Applicants: **DOW AGROSCIENCES LLC** [US/US]; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **FRAUNHOFER-GESELLSCHAFT ZUR FÖRDERUNG DER ANGEWANDTEN FORSCHUNG EV** [DE/DE]; Hansastraße 27 c, 80686 München (DE).

(72) Inventors: **NARVA, Kenneth E.**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **WORDEN, Sarah E.**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **FREY, Meghan**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **RANGASAMY, Murugesan**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **GANDRA, Premchand**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **VEERAMANI, Balaji**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **LO, Wendy**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US). **VILCINSKAS, Andreas**; Winchesterstr. 2, 35394 Giessen (DE). **KNORR, Eileen**; Heinrich-Buff-Ring 26-32, 35392 Giessen (DE). **FISHILEVICH, Elane**; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US).

(74) Agents: **CATAXINOS, Edgar R.** et al.; Magleby Cataxinos & Greenwood, 170 South Main Street, Suite 1100, Salt Lake City, Utah 84101 (US).

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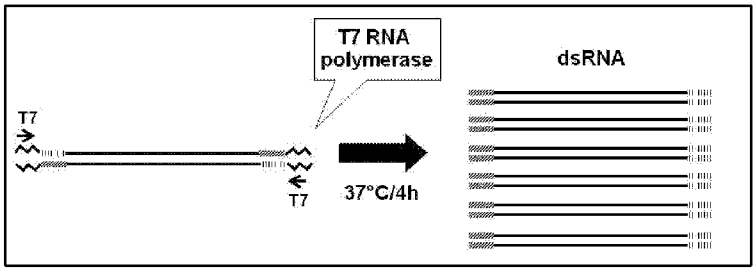
(54) **Title**: RNA POLYMERASE II33 NUCLEIC ACID MOLECULES TO CONTROL INSECT PESTS

FIG. 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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RNA POLYMERASE II33 NUCLEIC ACID MOLECULES TO CONTROL INSECT PESTS

PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial No. 62/133,210, filed March 13, 2015, for "RNA POLYMERASE II33 NUCLEIC ACID MOLECULESTO CONTROL INSECT PESTS."

TECHNICAL FIELD

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

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 has estimated that corn rootworms cause $1 billion in lost revenue each year, including $800 million in yield loss and $200 million in treatment costs.

Both WCR and NCR eggs are deposited in the soil 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 emerge from the soil as adults in July and August. Adult rootworms are about 0.25 inches in length.

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. 34:627-34. 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.

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.

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 disadvantage of placing unwanted restrictions upon the use of farmland. Moreover, oviposition of some rootworm species may occur in soybean fields, thereby mitigating the effectiveness of crop rotation practiced with corn and soybean.

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 to non-target species.

Stink bugs and other hemipteran insects (heteroptera) are 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. Hemipteran insects are present in a large number of important crops including maize, soybean, fruit, vegetables, and cereals.

Stink bugs go through multiple nymph stages before reaching the adult stage. These insects develop from eggs to adults in 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.

RNA interference (RNAi) is a process utilizing endogenous cellular pathways, whereby an interfering RNA (iRNA) molecule (*e.g.*, a dsRNA molecule) that is specific for all, or any portion of adequate size, of a target gene 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 391:806-11; Martinez *et al.* (2002) Cell 110:563-74; McManus and Sharp (2002) Nature Rev. Genetics 3:737-47.

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). Micro ribonucleic acids (miRNAs) are structurally very similar molecules that are cleaved from precursor molecules containing a polynucleotide “loop” connecting the

hybridized passenger and guide strands, and they may be similarly incorporated into RISC. Post-transcriptional gene silencing occurs when the guide strand binds specifically to a complementary mRNA molecule and induces cleavage by Argonaute, the catalytic component of the RISC complex. This process is known to spread systemically throughout the organism despite initially limited concentrations of siRNA and/or miRNA in some eukaryotes such as plants, nematodes, and some insects.

Only transcripts complementary to the siRNA and/or miRNA are cleaved and degraded, and thus the knock-down of mRNA expression is sequence-specific. In plants, several functional groups of DICER genes exist. The gene silencing effect of RNAi persists for days and, under experimental conditions, can lead to a decline in abundance of the targeted transcript of 90% or more, with consequent reduction in levels of the corresponding protein. In insects, there are at least two DICER genes, where DICER1 facilitates miRNA-directed degradation by Argonaute1. Lee *et al.* (2004) Cell 117 (1):69-81. DICER2 facilitates siRNA-directed degradation by Argonaute2.

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 beta 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.

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, 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).

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, describe 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.

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.

DISCLOSURE

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. u. undecimpunctata* Mannerheim; and *D. speciosa* Germar, 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.

In these and further examples, the native nucleic acid sequence 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 or nymph development. In some examples, post-transcriptional inhibition of the expression of a target gene by a nucleic acid molecule comprising a polynucleotide homologous thereto may be lethal to an insect pest or result in reduced growth and/or viability of an insect pest. In specific examples, *RNA polymerase II 33kD subunit* (referred to herein as, for example, *rpII33*) or a *rpII33* homolog may be selected as a target gene for post-transcriptional silencing. In particular examples, a target gene useful for post-transcriptional inhibition is a *RNA polymerase II33* gene is the gene referred to herein as *Diabrotica virgifera rpII33-1* (*e.g.*, SEQ ID NO:1), *D. virgifera rpII33-2* (*e.g.*, SEQ ID NO:3), the gene referred to herein as *Euschistus heros rpII33-1* (*e.g.*, SEQ ID NO:76), or *E. heros rpII33-2* (*e.g.*, SEQ ID NO:78). An isolated nucleic acid molecule comprising the

polynucleotide of SEQ ID NO:1; the complement of SEQ ID NO:1; SEQ ID NO:3; the complement of SEQ ID NO:3; SEQ ID NO:76; the complement of SEQ ID NO:76; SEQ ID NO:78; the complement of SEQ ID NO:78; and/or fragments of any of the foregoing (*e.g.*, SEQ ID NOs:5-8 and SEQ ID NOs:80-82) is therefore disclosed herein.

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 *rpII33* gene). For example, a nucleic acid molecule may comprise a polynucleotide encoding a polypeptide that is at least 85% identical to SEQ ID NO:2 (*D. virgifera* RPII33-1), SEQ ID NO:4 (*D. virgifera* RPII33-2), SEQ ID NO:77 (*E. heros* RPII33-1), or SEQ ID NO:79 (*E. heros* RPII33-2); and/or an amino acid sequence within a product of *D. virgifera rpII33-1*, *D. virgifera rpII33-2, E. heros rpII33-1*, or *E. heros rpII33-2*. 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.

Also disclosed are cDNA polynucleotides that may be used for the production of iRNA (*e.g.*, dsRNA, siRNA, shRNA, miRNA, and hpRNA) molecules that are complementary to all or part of an insect pest target gene, for example, an *rpII33* gene. In particular embodiments, dsRNAs, siRNAs, shRNAs, miRNAs, 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 a *rpII33* gene (*e.g.*, SEQ ID NO:1; SEQ ID NO:3; SEQ ID NO:76; and/or SEQ ID NO:78), for example, a WCR *rpII33* gene (*e.g.*, SEQ ID NO:1 and/or SEQ ID NO:3) or BSB *rpII33* gene (*e.g.*, SEQ ID NO:76 and/or SEQ ID NO:78).

Further disclosed are means for inhibiting expression of an essential gene in a coleopteran pest, and means for providing coleopteran pest protection to a plant. A means for inhibiting expression of an essential gene in a coleopteran pest is a single- or double-stranded RNA molecule consisting of a polynucleotide selected from the group consisting of SEQ ID NOs:94-97; and the complements thereof. Functional equivalents of means for inhibiting expression of an essential gene in a coleopteran pest include single- or double-stranded RNA molecules that are substantially homologous to all or part of a coleopteran *rpII33* gene comprising SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and/or SEQ ID NO:8. A means for providing coleopteran pest protection to a plant is a DNA molecule comprising a polynucleotide encoding a means for inhibiting expression of an essential gene in a coleopteran pest operably

linked to a promoter, wherein the DNA molecule is capable of being integrated into the genome of a plant.

Further disclosed are means for inhibiting expression of an essential gene in a hemipteran pest, and means for providing hemipteran pest protection to a plant. A means for inhibiting expression of an essential gene in a hemipteran pest is a single- or double-stranded RNA molecule consisting of a polynucleotide selected from the group consisting of SEQ ID NOs:100-102 and the complements thereof. Functional equivalents of means for inhibiting expression of an essential gene in a hemipteran pest include single- or double-stranded RNA molecules that are substantially homologous to all or part of a hemipteran *rpII33* gene comprising SEQ ID NO:80, SEQ ID NO:81, and/or SEQ ID NO:82. A means for providing hemipteran pest protection to a plant is a DNA molecule comprising a polynucleotide encoding a means for inhibiting expression of an essential gene in a hemipteran pest operably linked to a promoter, wherein the DNA molecule is capable of being integrated into the genome of a plant.

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.

In some embodiments, methods for controlling a population of a coleopteran pest comprises providing to the coleopteran pest an iRNA molecule that comprises all or part of a polynucleotide selected from the group consisting of: SEQ ID NO:92; the complement of SEQ ID NO:92; SEQ ID NO:93; the complement of SEQ ID NO:93; SEQ ID NO:94; the complement of SEQ ID NO:94; SEQ ID NO:95; the complement of SEQ ID NO:95; SEQ ID NO:96; the complement of SEQ ID NO:96; SEQ ID NO:97; the complement of SEQ ID NO:97; a polynucleotide that hybridizes to a native *rpII33* polynucleotide of a coleopteran pest (*e.g.*, WCR); the complement of a polynucleotide that hybridizes to a native *rpII33* polynucleotide of a coleopteran pest; a polynucleotide that hybridizes to a native coding polynucleotide of a *Diabrotica* organism (*e.g.*, WCR) comprising all or part of any of SEQ ID NOs:1, 3, and 5-8; and the complement of a polynucleotide that hybridizes to a native coding polynucleotide of a *Diabrotica* organism comprising all or part of any of SEQ ID NOs:1, 3, and 5-8.

In some embodiments, a methods for controlling a population of a hemipteran pest comprises providing to the hemipteran pest an iRNA molecule that comprises all or part of a polynucleotide selected from the group consisting of: SEQ ID NO:98; the complement of SEQ ID NO:98; SEQ ID NO:99; the complement of SEQ ID NO:99; SEQ ID NO:100; the

complement of SEQ ID NO:100; SEQ ID NO:101; the complement of SEQ ID NO:101; SEQ ID NO:102; the complement of SEQ ID NO:102; a polynucleotide that hybridizes to a native *rpII33* polynucleotide of a hemipteran pest (*e.g.*, BSB); the complement of a polynucleotide that hybridizes to a native *rpII33* polynucleotide of a hemipteran pest; a polynucleotide that hybridizes to a native coding polynucleotide of a hemipteran organism (*e.g.*, BSB) comprising all or part of any of SEQ ID NOs:76, 78, and 80-82; and the complement of a polynucleotide that hybridizes to a native coding polynucleotide of a hemipteran organism comprising all or part of any of SEQ ID NOs:76, 78, and 80-82.

In particular embodiments, an iRNA that functions upon being taken up by an insect pest to inhibit a biological function within the pest is transcribed from a DNA comprising all or part of a polynucleotide selected from the group consisting of: SEQ ID NO:1; the complement of SEQ ID NO:1; SEQ ID NO:3; the complement of SEQ ID NO:3; SEQ ID NO:76; the complement of SEQ ID NO:76; SEQ ID NO:78; the complement of SEQ ID NO:78; a native coding polynucleotide of a *Diabrotica* organism (*e.g.*, WCR) comprising all or part of any of SEQ ID NOs:1, 3, and 5-8; the complement of a native coding polynucleotide of a *Diabrotica* organism comprising all or part of any of SEQ ID NOs:1, 3, and 5-8; a native coding polynucleotide of a hemipteran organism (*e.g.*, BSB) comprising all or part of any of SEQ ID NOs:76, 78, and 80-82; and the complement of a native coding polynucleotide of a hemipteran organism comprising all or part of any of SEQ ID NOs:76, 78, and 80-82.

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

Dysdercus peruvianus, *Neomegalotomus parvus*, *Leptoglossus zonatus*, *Niesthrea sidae*, *Lygus hesperus*, or *L. lineolaris*.

The foregoing and other features will become more apparent from the following Detailed Description of several embodiments, which proceeds with reference to the accompanying **FIGs. 1-2**.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 includes a depiction of a strategy used to provide dsRNA from a single transcription template with a single pair of primers.

FIG. 2 includes a depiction of a strategy used to provide dsRNA from two transcription templates.

SEQUENCE LISTING

The nucleic acid sequences identified 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.

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 a 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)), a RNA sequence is included by any reference to the DNA sequence encoding it. In the accompanying sequence listing:

SEQ ID NO:1 shows an exemplary WCR *rpII33* DNA, referred to herein in some places as WCR *rpII33-1*:

GCCGATGCCATACATACGCTTAAAACATCGTATCTGCTCAGTTCTTTAATTAACACT
GAAGAAAATCGAATTATAAAATGCCCTACGCTAACACACCGTCAGTACAAATTTCTGAACTA
ACCGATGAAAATGTTAAGTTCGTCGTTGAGGACACAGACCTTAGCTTGGCAAACAGTCTACG
TCGTGTTTTCATCGCTGAAACTCCAACCCTAGCAATCGATTGGGTTCAATTCGAAGCCAACT
CCACTGTACTGGCAGATGAATTCCTTGCCCATCGAATTGGCTTGATTCCATTGATTTCCGAT
GAGGTAGTGGACAGAATCCAAAACACTCGTGAATGTTCATGCTTGGACTTTTGCACCGAGTG
CAGTGTGGAATTTACATTGGATGTCAAATGCAGCGACGAACATACGCGCCACGTTACCACGG
CCGATTTAAAGTCCAGTGACGCACGAGTGCTACCAGTTACGTCCAGACATCGCGATGACGAG
GACAACGAATATGGAGAGACGAACGATGAAATTCTGATCATCAAACTGCGCAAAGGTCAAGA
GCTGAAGTTGCGAGCATACGCGAAAAAGGGTTTCGGCAAGGAACATGCCAAATGGAATCCAA
CGGCTGGCGTTAGCTTTGAATACGATCCAGTCAATTCGATGAGACATACCCTGTACCCGAAG
CCGGACGAATGGCCGAAAAGTGAGCACACCGAACTTGACGATGATCAATACGAAGCTGAATA
TAACTGGGAGGCTAAGCCGAACAAGTTTTTCTTCAACGTTGAGTCGAGTGGTGCACTTCGAC
CGGAAAACATTGTGCTGATGGGAGTCAAAGTTTTGAAAAACAAATTGTCCAATCTACAGACG
CAGTTAAGTCACGAATTGACTACAAACGATGCGCTCGTGATTCAGTAAAAGCAGCGATCCCA
TTGAATTTCTTCAAAATCTTGTTTTTTTCCTCTAAG

SEQ ID NO:2 shows the amino acid sequence of a RPII33 polypeptide encoded by an exemplary WCR *rpII33* DNA, referred to herein in some places as WCR RPII33-1:

MPYANTPSVQISELTDENVKFVVEDTDLSLANSLRRVFIAETPTLAIDWVQFEANST
VLADEFLAHRIGLIPLISDEVVDRIQNTRECSCLDFCTECSVEFTLDVKCSDEHTRHVTTAD
LKSSDARVLPVTSRHRDDEDNEYGETNDEILIIKLRKGQELKLRAYAKKGFGKEHAKWNPTA
GVSFEYDPVNSMRHTLYPKPDEWPKSEHTELDDDQYEAEYNWEAKPNKFFFNVESSGALRPE
NIVLMGVKVLKNKLSNLQTQLSHELTTNDALVIQ

SEQ ID NO:3 shows a further exemplary WCR *rpII33* DNA, referred to herein in some places as WCR *rpII33-2*:

CGTTGACACTGTTGACAGTGACAGTTGAAATTGAAAACCGGATTAGAGAAGTTTTCT
TGGAAAGTTGTTTTTTTAAATAACTAACATTAAATAGAAGTTATTTGTTTAAGGGTTTAATA

TGCCATATGCAAATCAGCCATCAGTTCATATAACAGATTTAACAGATGATAATTGCAAATTT
TATATAGAAGACACTGATTTAAGTGTTGCGAATAGCATTCGCCGCGTCCTTATTGCAGAAAC
TCCTACTCTAGCTATAGACTGGGTAAAATTAGAAGCTAACTCAACTGTTCTCAGTGATGAAT
TTTTAGCACACCGAATTGGATTGATACCATTAGTTTCCGATGAAGTTGTACAAAGATTACAA
TATCCTAGGGACTGCGTATGTCTCGATTTTTGTCAAGAATGCAGTGTTGAATTTACTTTAGA
TGTAAAATGTACAGATGATCAAACTCGACATGTAACAACTGCCGATTTTAAATCTAGTGATC
CACGAGTCATACCAGCTACTTCCAAACATCGTGATGATGAATCCTCAGAGTATGGTGAAACA
GATGAAATTCTTATTATTAAACTGCGAAAGGGTCAAGAGCTTAAAGTTAAAGCGTATGCCAA
AAAAGGCTTTGGAAAAGAGCATGCCAAATGGAATCCTACATGTGGTGTTGCCTTTGAATATG
ATCCTGATAACGCTATGAGACATACATTATTTCCTAAACCAGACGAATGGCCTAAAAGTGAA
TACAGCGAATTAGAAGATGATCAGTATGAAGCTCCATATAACTGGGAATTAAAACCTAATAA
ATTCTTCTACAATGTGGAGGCTGCTGGATTGTTGAAACCAGAAAATATTGTCATCATGGGTG
TAGCTATGTTAAAAGAAAAACTGTCAAATTTGCAAACACAACTCAGCCACGAACTAACACCT
GATGTTTTGGCCATTCCAATTTAAGAAGTTAATTACAATCATAGGTAGAGTTCATTCAACCA
CAGTTATACATTTTTTTTATAATAGATAAGTAAGTTTTACACTATAGGAACAATTTTTGACA
TGTTGACTAAAGATCTTGTTCAAATAGACTAGAAATAAAATTTTGAATCCAAAAAAAAAAA

SEQ ID NO:4 shows the amino acid sequence of a WCR RPII33 polypeptide encoded by a further exemplary WCR *rpII33* DNA (*i.e.*, *rpII33-2*):

MPYANQPSVHITDLTDDNCKFYIEDTDLSVANSIRRVLIAETPTLAIDWVKLEANST
VLSDEFLAHRIGLIPLVSDEVVQRLQYPRDCVCLDFCQECSVEFTLDVKCTDDQTRHVTTAD
FKSSDPRVIPATSKHRDDESSEYGETDEILIIKLRKGQELKVKAYAKKGFGKEHAKWNPTCG
VAFEYDPDNAMRHTLFPKPDEWPKSEYSELEDDQYEAPYNWELKPNKFFYNVEAAGLLKPEN
IVIMGVAMLKEKLSNLQTQLSHELTPDVLAIPI

SEQ ID NO:5 shows an exemplary WCR *rpII33* DNA, referred to herein in some places as WCR *rpII33-1* reg1 (region 1), which is used in some examples for the production of a dsRNA:

GAATTCCTTGCCCATCGAATTGGCTTGATTCCATTGATTTCCGATGAGGTAGTGGAC
AGAATCCAAAACACTCGTGAATGTTCATGCTTGGACTTTTGCACCGAGTGCAGTGTGGAATT
TACATTGGATGTCAAATGCAGCGACGAACATACGCGCCACGTTACCACGGCCGATTTAAAGT
CCAGTGACGCACGAGTGCTACCAGTTACGTCCAGACATCGCGATGACGAGGACAACGAATAT
GGAGAGACGAACGATGAAATTCTGATCATCAAACTGCGCAAAGGTCAAGAGCTGAAGTTGCG

AGCATACGCGAAAAAGGGTTTCGGCAAGGAACATGCCAAATGGAATCCAACGGCTGGCGTTA
GCTTTGAATACGATCCAGTCAATTCGATGAGACATACCCTGTACCCGAAGCCGGACGAATGG
CCGAAAAGTGAGCACACCGAACTTGACGATGATCAATACGAAGCTGAATATAAC

SEQ ID NO:6 shows a further exemplary WCR *rplI33* DNA, referred to herein in some places as WCR *rplI33-2* reg1 (region 1), which is used in some examples for the production of a dsRNA:

GTTCTCAGTGATGAATTTTTAGCACACCGAATTGGATTGATACCATTAGTTTCCGAT
GAAGTTGTACAAAGATTACAATATCCTAGGGACTGCGTATGTCTCGATTTTTGTCAAGAATG
CAGTGTTGAATTTACTTTAGATGTAAAATGTACAGATGATCAAACTCGACATGTAACAACTG
CCGATTTTAAATCTAGTGATCCACGAGTCATACCAGCTACTTCCAAACATCGTGATGATGAA
TCCTCAGAGTATGGTGAAACAGATGAAATTCTTATTATTAAACTGCGAAAGGGTCAAGAGCT
TAAAGTTAAAGCGTATGCCAAAAAAGGCTTTGGAAAAGAGCATGCCAAATGGAATCCTACAT
GTGGTGTTGCCTTTGAATATGATCCTGATAACGCTATGAGACATACATTATTTCCTAAACCA
GACGAATGGCCTAAAAGTGAATACAGCGAATTAGAAGATGATCAGTATGAAGCTCCATATAA
CTGGG

SEQ ID NO:7 shows a further exemplary WCR *rplI33* DNA, referred to herein in some places as WCR *rplI33-2* v1 (version 1), which is used in some examples for the production of a dsRNA:

CTTTAGATGTAAAATGTACAGATGATCAAACTCGACATGTAACAACTGCCGATTTTA
AATCTAGTGATCCACGAGTCATACCAGCTACTTCCAAACATCGTGATGATGAATCCTCAGAG
TATGGTGAAACAG

SEQ ID NO:8 shows a further exemplary WCR *rplI33* DNA, referred to herein in some places as WCR *rplI33-2* v2 (version 2), which is used in some examples for the production of a dsRNA:

GCGTATGCCAAAAAAGGCTTTGGAAAAGAGCATGCCAAATGGAATCCTACATGTGGT
GTTGCCTTTGAATATGATCCTGATAACGCTATGAGACATACATTATTTCCTAAACCAGACGA
ATGGCC

SEQ ID NO:9 shows a the nucleotide sequence of T7 phage promoter.

SEQ ID NO:10 shows a fragment of an exemplary *YFP* coding sequence.

SEQ ID NOs:11-18 show primers used to amplify portions of exemplary WCR *rpII-33* sequences comprising *rpII33-1* reg1, *rpII33-2* reg1, *rpII33*-2 v1, and *rpII33*-2 v2, used in some examples for dsRNA production.

SEQ ID NO:19 shows an exemplary *YFP* gene.

SEQ ID NO:20 shows a DNA sequence of *annexin* region 1.

SEQ ID NO:21 shows a DNA sequence of *annexin* region 2.

SEQ ID NO:22 shows a DNA sequence of *beta spectrin 2* region 1.

SEQ ID NO:23 shows a DNA sequence of *beta spectrin 2* region 2.

SEQ ID NO:24 shows a DNA sequence of *mtRP-L4* region 1.

SEQ ID NO:25 shows a DNA sequence of *mtRP-L4* region 2.

SEQ ID NOs:26-53 show primers used to amplify gene regions of *annexin*, *beta spectrin 2*, *mtRP-L4*, and *YFP* for dsRNA synthesis.

SEQ ID NO:54 shows a maize DNA sequence encoding a TIP41-like protein.

*SEQ ID NO:55 s*hows the nucleotide sequence of a T20VN primer oligonucleotide.

SEQ ID NOs:56-60 show primers and probes used for dsRNA transcript expression analyses in maize.

SEQ ID NO:61 shows a nucleotide sequence of a portion of a *SpecR* coding region used for binary vector backbone detection.

SEQ ID NO:62 shows a nucleotide sequence of an *AAD1* coding region used for genomic copy number analysis.

SEQ ID NO:63 shows a DNA sequence of a maize *invertase* gene.

SEQ ID NOs:64-72 show the nucleotide sequences of DNA oligonucleotides used for gene copy number determinations and binary vector backbone detection.

SEQ ID NOs:73-75 show primers and probes used for dsRNA transcript maize expression analyses.

SEQ ID NO:76 shows an exemplary BSB *rpII33* DNA, referred to herein in some places as BSB *rpII33-1*:

GTTCGGCTCGGGTGAGTGTTTAAACCAACTACGCATCTTGTTCTCGAACCTTTGCGA
ACAGTGTTCACAAATAATGCTCGGTTGGTGTAAAGGTACCTTTAGAGCGTGACCCCAACTTC
TTTTGACTCACCTTGCAGAAACTCGATCACTAACAATTACGTGTATATAATCGATTCACTAC
ACGAACGATACATGGTTGTTTAGGTTACATTCATGTTATCTTTAGTAATGAAGTTATTGAGT
TGGCCTAATTGTTGAATGTAGTTAACAGAATGCCTTATGCCAATCAACCTTCTGTTCATGTT
TCAGATTTAACCGACGACAATGTTAAATTCCAAATAGAAGATACAGAATTAAGTGTCGCTAA

CAGCCTCAGAAGAGTCTTCATAGCTGAAACCCCAACTTTAGCTATTGATTGGGTGCAATTGT
CTGCAAATTCTACTGTTTTAAGTGATGAATTTATTGCTTCTAGAATCGGACTTATTCCTTTA
ACTTCTGATGCTGCAGTCGAAAAATTAATCTATTCTAGGGACTGTAATTGTACTGATTTCTG
CCCATCCTGTAGTGTTGAGTTTACTTTAGATGTCAAATGTGTAGATGATCAAACTAGACATG
TGACAACTGCAGATTTAAAGACTGCTGATCCATGTGTAGTTCCTGCTACATCTAAAAATAGA
GATGCTGATGCCAATGAATATGGTGAATCAGATGATATTTTGATTGTTAAATTAAGAAAAGG
ACAAGAGCTTAAATTGAGGGCCTTTGCTAAGAAAGGTTTTGGTAAGGAACATGCTAAGTGGA
ATCCTACTGCTGGGGTTTGTTTTGAGTATGACCCTGACAACTCAATGAGGCATACACTGTTT
CCAAAACCAGATGAGTGGCCAAAAAGTGAATATACTGAATTAGATGAGGATCAGTATGAAGC
TCCATTTAATTGGGAAGCCAAACCTAACAAATTTTTCTTCAATGTTGAAAGTTGTGGATCTT
TGCGCCCCGAAAACATAGTATTAAAAGGAGTAGAAGTTCTAAAATATAAACTTTCTGATTTA
TTAATTCAATTGAGTCATGAATCAGCTGGCCAAGTTGATCATATGCCTGTTTAACCAGTTTT
TGTGATAAATTATTATCTGAAATAATTCAATTATTATATTTATATTAATGTAAAATAAAAAG
AAATTTGATAACTGAAAAAAAAAAAAAAAAATCTATTGAAAGAATACATTCATTAATACCTT
TCTAAAGAAAAATTATTCAATTTAAAATTGTTGCCAAAAAGTATTCAGCATTTTTTTAAAAT
TCAATCTAGGCATATACTACTGTAAATAAATACAAACAATACTTTCATTTTTGTACTGTTCT
AAAAATTGT

SEQ ID NO:77 shows the amino acid sequence of a BSB RPII33 polypeptide encoded by an exemplary BSB *rpII33* DNA (*i.e.*, BSB *rpII33-1*):

MPYANQPSVHVSDLTDDNVKFQIEDTELSVANSLRRVFIAETPTLAIDWVQLSANST
VLSDEFIASRIGLIPLTSDAAVEKLIYSRDCNCTDFCPSCSVEFTLDVKCVDDQTRHVTTAD
LKTADPCVVPATSKNRDADANEYGESDDILIVKLRKGQELKLRAFAKKGFGKEHAKWNPTAG
VCFEYDPDNSMRHTLFPKPDEWPKSEYTELDEDQYEAPFNWEAKPNKFFFNVESCGSLRPEN
IVLKGVEVLKYKLSDLLIQLSHESAGQVDHMPV

SEQ ID NO:78 shows an exemplary BSB *rpII33* DNA, referred to herein in some places as BSB *rpII33-2*:

TGTAAAACTTGTTCTTTAAGATCTCAAGACCTTTTATTAGAACATCTACAGGCTTAA
GAGAGCCCTCTACAACTTCTACGTCCATGTGCACCGTGTCTATTTCACAAAGGAGATCTGGT
TCTTCCTCCTCAACCATCGGCCAGTCCTTCTTAAGCGTATCTTCTGTCCAGTAGTTTGTGGA
CCTAGTCTTATTGGTTCTATCATACTCGAACCCGACAACAGAGACAGGAGACCACTTGGCAT
GCATCCTCCCTATCCCCTTCCTAGCAATACACCTAATTTTCAGGCTTTGATTCTTCCCAAGT

TTTGCAATTACCGGTGTGCTTTTTATAAAAGTCTCGTCACTGTCAAATTTTATGTCTTTACA
AGTCACGTTAAGGGGGGTCTCTGAGGTGTTGCTAACATCAAGTTCCATCTCTACGGAACAAC
GAGAGCAAAGCTCATCACAGTCACACTCTTCTTTATACACAAGCTCTTTCTTTGAGTACATT
GGGATAAGCCCAAGGGACTGTGCCAATACTTCATCGGGGAGGACCGTGTTGTTTTTGATGAT
TTCGACGAGATCTATTGCGATAGTAGGTACTTCAGATAAGAGGATTCTCCTTAGAGCATTAG
CATAGGAGACTGTAATCCCAGTGAGAGTGAATTTGATGTGTTCGTCGTTTTGTTCGTGAATT
GTAATTTTCATGAGAAAGCTGGAGGGCAAAAGAAATGAAGTAAATTTAGAAGGGAACACCTG
TGAAGTATGATCGACTACG

SEQ ID NO:79 shows the amino acid sequence of a further BSB RPII33 polypeptide encoded by an exemplary BSB *rpII33* DNA (*i.e.*, BSB *rpII33-2*):

MKITIHEQNDEHIKFTLTGITVSYANALRRILLSEVPTIAIDLVEIIKNNTVLPDEV
LAQSLGLIPMYSKKELVYKEECDCDELCSRCSVEMELDVSNTSETPLNVTCKDIKFDSDETF
IKSTPVIAKLGKNQSLKIRCIARKGIGRMHAKWSPVSVVGFEYDRTNKTRSTNYWTEDTLKK
DWPMVEEEEPDLLCEIDTVHMDVEVVEGSLKPVDVLIKGLEILKNKFY

SEQ ID NO:80 shows an exemplary BSB *rpII33* DNA, referred to herein in some places as BSB_*rpII33-1* reg1 (region 1), which is used in some examples for the production of a dsRNA:

GGTGAATCAGATGATATTTTGATTGTTAAATTAAGAAAAGGACAAGAGCTTAAATTG
AGGGCCTTTGCTAAGAAAGGTTTTGGTAAGGAACATGCTAAGTGGAATCCTACTGCTGGGGT
TTGTTTTGAGTATGACCCTGACAACTCAATGAGGCATACACTGTTTCCAAAACCAGATGAGT
GGCCAAAAAGTGAATATACTGAATTAGATGAGGATCAGTATGAAGCTCCATTTAATTGGGAA
GCCAAACCTAAC

SEQ ID NO:81 shows a further exemplary BSB *rpII33* DNA, referred to herein in some places as BSB_*rpII33-1* v1 (version 1), which is used in some examples for the production of a dsRNA:

TTGTTTTGAGTATGACCCTGACAACTCAATGAGGCATACACTGTTTCCAAAACCAGA
TGAGTGGCCAAAAAGTGAATATACTGAATTAGATGAGGATCAGTATGAAGCTCC

SEQ ID NO:82 shows a further exemplary BSB *rpII33* DNA, referred to herein in some places as BSB_*rpII33-2* reg1 (region 1), which is used in some examples for the production of a dsRNA:

CGTCGAAATCATCAAAAACAACACGGTCCTCCCCGATGAAGTATTGGCACAGTCCCT
TGGGCTTATCCCAATGTACTCAAAGAAAGAGCTTGTGTATAAAGAAGAGTGTGACTGTGATG
AGCTTTGCTCTCGTTGTTCCGTAGAGATGGAACTTGATGTTAGCAACACCTCAGAGACCCCC
CTTAACGTGACTTGTAAAGACATAAAATTTGACAGTGACGAGACTTTTATAAAAAGCACACC
GGTAATTGCAAAACTTGGGAAGAATCAAAGCCTGAAAATTAGGTGTATTGCTAGGAAGGGGA
TAGGGAGGATGCATGCCAAGTGGTCTCCTGTCTCTGTTGTCGGGTTCGAGTATGATAGAACC
AATAAGACTAGGTCCACAAACTACTGGACAG

SEQ ID NOs:83-88 show primers used to amplify portions of exemplary BSB *rpII-33* sequences comprising *rpII33-1* reg1, *rpII33-2* reg1, and *rpII33-1* v1, used in some examples for dsRNA production.

SEQ ID NO:89 shows an exemplary *YFP* v2 DNA, which is used in some examples for the production of the sense strand of a dsRNA.

SEQ ID NOs:90 and 91 show primers used for PCR amplification of *YFP* sequence *YFP* v2, used in some examples for dsRNA production.

SEQ ID NOs:92-102 show exemplary RNAs transcribed from nucleic acids comprising exemplary *rpII33* polynucleotides and fragments thereof.

SEQ ID NO:103 shows an exemplary DNA encoding a *Diabrotica rpII33-2 v1* dsRNA; containing a sense polynucleotide, a loop sequence (italics), and an antisense polynucleotide (underlined font):

CTTTAGATGTAAAATGTACAGATGATCAAACTCGACATGTAACAACTGCCGATTTTA
AATCTAGTGATCCACGAGTCATACCAGCTACTTCCAAACATCGTGATGATGAATCCTCAGAG
TATGGTGAAACAG*GAAGCTAGTACCAGTCATCACGCTGGAGCGCACATATAGGCCCTCCATC*
AGAAAGTCATTGTGTATATCTCTCATAGGGAACGAGCTGCTTGCGTATTTCCCTTCCGTAGT
CAGAGTCATCAATCAGCTGCACCGTGTCGTAAAGCGGGACGTTCGCAAGCTCGTCCGCGGTA
CTGTTTCACCATACTCTGAGGATTCATCATCACGATGTTTGGAAGTAGCTGGTATGACTCGT
GGATCACTAGATTTAAAATCGGCAGTTGTTACATGTCGAGTTTGATCATCTGTACATTTTAC
ATCTAAAG

SEQ ID NO:104 shows an exemplary DNA encoding a *Diabrotica rpII33-2 v2* dsRNA; containing a sense polynucleotide, a loop sequence (italics), and an antisense polynucleotide (underlined font):

GCGTATGCCAAAAAAGGCTTTGGAAAAGAGCATGCCAAATGGAATCCTACATGTGGT
GTTGCCTTTGAATATGATCCTGATAACGCTATGAGACATACATTATTTCCTAAACCAGACGA
ATGGCC*GAAGCTAGTACCAGTCATCACGCTGGAGCGCACATATAGGCCCTCCATCAGAAAGT*
CATTGTGTATATCTCTCATAGGGAACGAGCTGCTTGCGTATTTCCCTTCCGTAGTCAGAGTC
*ATCAATCAGCTGCACCGTGTCGTAAAGCGGGACGTTCGCAAGCTCGTCCGCGGTA*GGCCATT
CGTCTGGTTTAGGAAATAATGTATGTCTCATAGCGTTATCAGGATCATATTCAAAGGCAACA
CCACATGTAGGATTCCATTTGGCATGCTCTTTTCCAAAGCCTTTTTTGGCATACGC

SEQ ID NOs:105-106 show probes used for dsRNA expression analysis.

SEQ ID NO:107 shows an exemplary DNA nucleotide sequence encoding an intervening loop in a dsRNA.

SEQ ID NOs:108-109 show exemplary dsRNAs transcribed from a nucleic acid comprising exemplary *rpII33-2* polynucleotide fragments.

SEQ ID NOs:110-111 show primers used for dsRNA transcript expression analyses in maize.

DETAILED DESCRIPTION

I. Overview of several embodiments

We developed RNA interference (RNAi) as a tool for insect pest management, using one of the most likely target pest species for transgenic plants that express dsRNA; the western corn rootworm. Thus far, most genes proposed as targets for RNAi in rootworm larvae do not actually achieve their purpose. Herein, we describe RNAi-mediated knockdown of RNA polymerase 33 (rpII33) in the exemplary insect pests, western corn rootworm and neotropical brown stink bug, which is shown to have a lethal phenotype when, for example, iRNA molecules are delivered via ingested or injected rpII33 dsRNA. In embodiments herein, the ability to deliver rpII33 dsRNA by feeding to insects confers a RNAi effect that is very useful for insect (e.g., coleopteran and hemipteran) pest management. By combining rpII33-mediated RNAi with other useful RNAi targets (e.g., ROP (U.S. Patent Application Publication No. 14/577811), RNAPII (U.S. Patent Application Publication No. 14/577854), RNA polymerase I1 RNAi targets, as described in U.S. Patent Application No. 62/133214, RNA polymerase

II215 RNAi targets, as described in U.S. Patent Application No. 62/133202, ncm (U.S. Patent Application No. 62/095487), Dre4 (U.S. Patent Application No. 14/705,807), COPI alpha (U.S. Patent Application No. 62/063,199), COPI beta (U.S. Patent Application No. 62/063,203), COPI gamma (U.S. Patent Application No. 62/063,192),and COPI delta (U.S. Patent Application No. 62/063,216)), the potential to affect multiple target sequences, for example, in larval rootworms, may increase opportunities to develop sustainable approaches to insect pest management involving RNAi technologies.

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 a 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.

Thus, some embodiments involve sequence-specific inhibition of expression of target gene products, using 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 one of SEQ ID NOs:1, 3, 76, and 78, and fragments thereof. In some embodiments, a stabilized dsRNA molecule may be expressed from these polynucleotides, fragments thereof, or a gene comprising one of these polynucleotides, 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 any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82.

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, an encoded dsRNA molecule(s) may be provided when ingested by an insect (e.g., 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 NOs:1, 3, 5-8, 76, 78, and 80-82, fragments of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82, and a polynucleotide consisting of a partial sequence of a gene comprising one of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82, and/or complements thereof.

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 SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:98, or SEQ ID NO:99 (e.g., at least one polynucleotide selected from a group comprising SEQ ID NOs:94-97 and 100-102), or the complement 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 rpII33 DNA (e.g., a DNA comprising all or part of a polynucleotide selected from the group consisting of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82) in the pest or progeny of the pest, and thereby result in cessation of growth, development, viability, and/or feeding in the pest.

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, a 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), cotton (Gossypium sp.), and plants of the family Poaceae.

Other 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 a RNA molecule capable of forming a dsRNA molecule. In particular embodiments, a polynucleotide encoding a 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 a 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.

Also disclosed is a transgenic plant comprising a vector having a polynucleotide encoding a 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 a 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 protection and/or enhanced protection to insect pest infestations. Particular transgenic plants may display protection and/or enhanced protection to one or more coleopteran and/or hemipteran pest(s) selected from the group consisting of: WCR; BSB; NCR; SCR; MCR; D. balteata LeConte; D. u. tenella; D. u. undecimpunctata Mannerheim; D. speciosa Germar; Euschistus heros (Fabr.); E. servus (Say); Nezara viridula (L.); Piezodorus guildinii (Westwood); Halyomorpha halys (Stål); Chinavia hilare (Say); C. marginatum (Palisot de Beauvois); Dichelops melacanthus (Dallas); D. furcatus (F.); Edessa meditabunda (F.); Thyanta perditor (F.); Horcias nobilellus (Berg); Taedia stigmosa (Berg); Dysdercus peruvianus (Guérin-Méneville); Neomegalotomus parvus (Westwood); Leptoglossus zonatus (Dallas); Niesthrea sidae (F.); Lygus hesperus (Knight); and L. lineolaris (Palisot de Beauvois).

Further 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.

In some embodiments, compositions (e.g., a topical composition) are provided that comprise an iRNA (e.g., dsRNA) molecule for use in 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, or an RNAi bait. 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.

The compositions and methods disclosed herein may 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 and PIP-1 polypeptides (See U.S. Patent Publication No. US 2014/0007292 A1)), and/or recombinant expression of other iRNA molecules.

II. Abbreviations

BSB	Neotropical brown stink bug (Euschistus heros)
dsRNA	double-stranded ribonucleic acid
EST	expressed sequence tag
GI	growth inhibition
NCBI	National Center for Biotechnology Information
gDNA	genomic deoxyribonucleic acid
iRNA	inhibitory ribonucleic acid
ORF	open reading frame
RNAi	ribonucleic acid interference

miRNA	micro ribonucleic acid
shRNA	small hairpin ribonucleic acid
siRNA	small inhibitory ribonucleic acid
hpRNA	hairpin ribonucleic acid
UTR	untranslated region
WCR	Western corn rootworm (Diabrotica virgifera virgifera LeConte)
NCR	Northern corn rootworm (Diabrotica barberi Smith and Lawrence)
MCR	Mexican corn rootworm (Diabrotica virgifera zeae Krysan and Smith)
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced Silencing Complex
SCR	Southern corn rootworm (Diabrotica undecimpunctata howardi Barber)
SEM	standard error of the mean
YFP	yellow fluorescent protein

III. Terms

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:

Coleopteran pest: As used herein, the term “coleopteran pest” refers to pest 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. u. undecimpunctata Mannerheim; and D. speciosa Germar.

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.

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.

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

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).

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

Hemipteran pest: As used herein, the term “hemipteran pest” refers to pest 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).

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.

Insect: As used herein with regard to pests, the term “insect pest” specifically includes coleopteran insect pests. In some examples, the term “insect pest” specifically refers to a coleopteran pest in the genus Diabrotica 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. u. undecimpunctata Mannerheim; and D. speciosa Germar. In some embodiments, the term also includes some other insect pests; e.g., hemipteran insect pests.

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.

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).

Some embodiments include nucleic acids comprising a template DNA that is transcribed into a 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:

ATGATGATG	polynucleotide
TACTACTAC	"complement" of the polynucleotide
CATCATCAT	"reverse complement" of the polynucleotide

Other embodiments of the invention may include hairpin RNA-forming RNAi molecules. In these RNAi molecules, 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 the region comprising the complementary and reverse complementary polynucleotides.

"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), shRNA (small hairpin RNA), mRNA (messenger RNA), miRNA (micro-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" and "nucleic acid," and "fragments" thereof will be understood by those in the art as a term that includes both gDNAs, ribosomal RNAs, transfer RNAs, messenger RNAs, operons, and smaller engineered polynucleotides that encode or may be adapted to encode, peptides, polypeptides, or proteins.

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 DNAs. 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.

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.

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

As used herein, "transcribed non-coding polynucleotide" refers to segments of mRNA molecules such as 5'UTR, 3'UTR, and intron segments that are not translated into a peptide, polypeptide, or protein. Further, "transcribed non-coding polynucleotide" refers to a nucleic

acid that is transcribed into a RNA that functions in the cell, for example, structural RNAs (e.g., ribosomal RNA (rRNA) as exemplified by 5S rRNA, 5.8S rRNA, 16S rRNA, 18S rRNA, 23S rRNA, and 28S rRNA, and the like); transfer RNA (tRNA); and snRNAs such as U4, U5, U6, and the like. Transcribed non-coding polynucleotides also include, for example and without limitation, small RNAs (sRNA), which term is often used to describe small bacterial non-coding RNAs; small nucleolar RNAs (snoRNA); micro RNAs (miRNA); small interfering RNAs (siRNA); Piwi-interacting RNAs (piRNA); and long non-coding RNAs. Further still, "transcribed non-coding polynucleotide" refers to a polynucleotide that may natively exist as an intragenic "spacer" in a nucleic acid and which is transcribed into a RNA molecule.

Lethal RNA interference: As used herein, the term “lethal RNA interference” refers to RNA interference that results in death or a reduction in viability of the subject individual to which, for example, a dsRNA, miRNA, siRNA, shRNA, and/or hpRNA is delivered.

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.

Sequence identity: The term "sequence identity" or "identity," as used herein in the context of two polynucleotides or polypeptides, refers to the residues in the sequences of the two molecules that are the same when aligned for maximum correspondence over a specified comparison window.

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) of a molecule 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.

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 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet et al. (1988) Nucleic Acids Res. 16:10881-90; Huang et al. (1992) Comp. Appl. Biosci. 8:155-65; Pearson et al. (1994) Methods Mol. Biol. 24:307-31; Tatiana et al. (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, e.g., Altschul et al. (1990) J. Mol. Biol. 215:403-10.

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 acids with even greater sequence similarity to the sequences of the reference polynucleotides will show increasing percentage identity when assessed by this method.

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 nucleobases 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 polynucleotide need not be 100%

complementary to its target nucleic acid to be specifically hybridizable. However, the amount of complementarity that must exist for hybridization to be specific is a function of the hybridization conditions used.

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 acids. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times 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 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.

As used herein, "stringent conditions" encompass conditions under which hybridization will only occur if there is less than 20% mismatch between the sequence of the hybridization molecule and a homologous polynucleotide 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 20% sequence mismatch will not hybridize; conditions of "high stringency" are those under which sequences with more than 10% mismatch will not hybridize; and conditions of "very high stringency" are those under which sequences with more than 5% mismatch will not hybridize.

The following are representative, non-limiting hybridization conditions.

High Stringency condition (detects polynucleotides 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.

Moderate Stringency condition (detects polynucleotides 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.

Non-stringent control condition (polynucleotides 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.

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 of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82 are those nucleic acids that hybridize under stringent conditions (e.g., the Moderate Stringency conditions set forth, supra) to the reference nucleic acid. 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.

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.

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.

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.

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.

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.

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).

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).

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.

Soybean plant: As used herein, the term “soybean plant” refers to a plant of a species from the genus Glycine; for example, G. max.

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).

Transgene: An exogenous nucleic acid. In some examples, a transgene may be a DNA that encodes one or both strand(s) of a 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 an antisense polynucleotide, wherein expression of the antisense polynucleotide inhibits expression of a target nucleic acid, thereby producing an RNAi phenotype. In still 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).

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.).

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 are targeted by the compositions and methods herein.

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

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 Sequence

A. Overview

Described herein are nucleic acid molecules useful for the control of insect pests. In some examples, the insect pest is a coleopteran (e.g., species of the genus Diabrotica) or hemipteran (e.g., species of the genus Euschistus) 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 a metabolic process or involved 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 in the pest.

In some embodiments, at least one target gene in an insect pest may be selected, wherein the target gene comprises a rpII33 polynucleotide. In some examples, a target gene in a coleopteran pest is selected, wherein the target gene comprises a polynucleotide selected from

among SEQ ID NOs:1, 3, and 5-8. In some examples, a target gene in a hemipteran pest is selected, wherein the target gene comprises a polynucleotide selected from among SEQ ID NOs:76, 78, and 80-82.

In other embodiments, a target gene may be 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 (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 a rpII33 polynucleotide. A target gene may be any rpII33 polynucleotide in an insect pest, the post-transcriptional inhibition of which has a deleterious effect on the growth, survival, and/or viability 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 SEQ ID NO:2; SEQ ID NO:4; SEQ ID NO:77; or SEQ ID NO:79.

Provided according to the invention are DNAs, the expression of which results in a 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 polynucleotide in cells of the pest may be obtained. In particular embodiments, down-regulation of the coding polynucleotide in cells of the insect pest results in a deleterious effect on the growth, development, and/or survival of the pest.

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.

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 a 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.

In particular examples, nucleic acid molecules useful for the control of a coleopteran or hemipteran pest may include: all or part of a native nucleic acid isolated from a Diabrotica organism comprising a rpII33 polynucleotide (e.g., any of SEQ ID NOs:1, 3, and 5-8); all or part of a native nucleic acid isolated from a hemipteran organism comprising a rpII33 polynucleotide (e.g., any of SEQ ID NOs:76, 78, and 80-82); DNAs that when expressed result in a RNA molecule comprising a polynucleotide that is specifically complementary to all or part of a native RNA molecule that is encoded by rpII33; 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 rpII33; 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 rpII33; 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

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.

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: SEQ ID NO:1 or 3; the complement of SEQ ID NO:1 or 3; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or 3 (e.g., any of SEQ ID NOs:5-8); the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or 3; a native coding polynucleotide of a Diabrotica organism (e.g., WCR) comprising any of SEQ ID NOs:5-8; the complement of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8.

Other 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: SEQ ID NO:76 or 78; the complement of SEQ ID NO:76 or 78; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76 or 78 (e.g., any of SEQ ID NOs:80-82); the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76 or 78; a native coding polynucleotide of a hemipteran organism (e.g., BSB) comprising any of SEQ ID NOs:80-82; the complement of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82.

In particular embodiments, contact with or uptake by an insect (e.g., coleopteran and/or hemipteran) pest of an iRNA transcribed from the isolated polynucleotide inhibits the growth, development, and/or feeding of the pest. In some embodiments, contact with or uptake by the insect occurs via feeding on plant material or bait comprising the iRNA. In some embodiments, contact with or uptake by the insect occurs via spraying of a plant comprising the insect with a composition comprising the iRNA.

In some embodiments, an isolated nucleic acid molecule of the invention may comprise at least one (e.g., one, two, three, or more) polynucleotide(s) selected from the group consisting of: SEQ ID NO:92; the complement of SEQ ID NO:92; SEQ ID NO:93; the complement of

SEQ ID NO:93; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:92 or SEQ ID NO:93 (e.g., SEQ ID NOs:94-97); the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:92 or SEQ ID NO:93; a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:94-97; the complement of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:94-97; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:94-97; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:94-97.

In other embodiments, an isolated nucleic acid molecule of the invention may comprise at least one (e.g., one, two, three, or more) polynucleotide(s) selected from the group consisting of: SEQ ID NO:98; the complement of SEQ ID NO:98; SEQ ID NO:99; the complement of SEQ ID NO:99; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:98 or SEQ ID NO:99 (e.g., SEQ ID NOs:100-102); the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:98 or SEQ ID NO:99; a native coding polynucleotide of a hemipteran (e.g., BSB) organism comprising any of SEQ ID NOs:100-102; the complement of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:100-102; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:100-102; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:100-102.

In particular embodiments, contact with or uptake by a coleopteran and/or hemipteran pest of the isolated polynucleotide inhibits the survival, growth, development, reproduction and/or feeding of the pest.

In certain embodiments, dsRNA molecules provided by the invention comprise polynucleotides complementary to a transcript from a target gene comprising any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82, and fragments thereof, 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 NOs:1, 3, 5-8, 76, 78, and 80-82; a contiguous fragment of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82; and the complement of any 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 NOs:1, 3, 5-8, 76, 78, and 80-82; a contiguous fragment of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82; and the complement of any of the foregoing.

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.

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.

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 a RNA molecule by a spacer. The spacer may constitute part of the first polynucleotide or the second polynucleotide. Expression of a 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.

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 a 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 insect pests.

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

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, viability, feeding, 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 and/or viability 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.

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 and/or survival, 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 protected against 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.

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 viability, 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.

In other 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.

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.

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.

A 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. A 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.

In particular 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

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)

In specific embodiments, a recombinant DNA molecule of the invention may comprise a polynucleotide encoding a 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.

In some embodiments, one strand of a dsRNA molecule may be formed by transcription from a polynucleotide which is substantially homologous to a polynucleotide selected from the group consisting of any of SEQ ID NOs:1, 3, 76, and 78; the complements of any of SEQ ID NOs:1, 3, 76, and 78; a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:1, 3, 76, and 78 (e.g., SEQ ID NOs:5-8 and 80-82); the complement of a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:1, 3, 76, and 78; a native coding polynucleotide

of a Diabrotica organism (e.g., WCR) comprising any of SEQ ID NOs:5-8; the complement of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; a native coding polynucleotide of a hemipteran organism (e.g., BSB) comprising any of SEQ ID NOs:80-82; the complement of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82.

In other 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 NOs:5-8 and 80-82; the complement of any of SEQ ID NOs:5-8 and 80-82; fragments of at least 15 contiguous nucleotides of any of SEQ ID NOs:5-8 and 80-82; and the complements of fragments of at least 15 contiguous nucleotides of any of SEQ ID NOs:5-8 and 80-82.

In particular embodiments, a recombinant DNA molecule encoding a 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 rpII33 gene comprising any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82) or fragment thereof. In some embodiments, however, a recombinant DNA molecule may encode a 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.

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., a rpII33 gene comprising any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82, and fragments of any of the foregoing); 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.

Certain 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.

To impart protection from an insect (e.g., coleopteran and/or hemipteran) pest 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.

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.

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).

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 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.

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).

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).

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.

In other 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 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.

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).

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 NOs:1, 3, 76, and 78), both in different populations of the same species of insect pest, or in different species of insect pests.

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.

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.

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 a coleopteran or hemipteran pest other than the one defined by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:76, and SEQ ID NO:78, 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), RPS6 (U.S. Patent Application Publication No. 2013/0097730), ROP (U.S. Patent Application Publication

No. 14/577811), RNAPII (U.S. Patent Application Publication No. 14/577854), Dre4 (U.S. Patent Application No. 14/705,807), ncm (U.S. Patent Application No. 62/095487), COPI alpha (U.S. Patent Application No. 62/063,199), COPI beta (U.S. Patent Application No. 62/063,203), COPI gamma (U.S. Patent Application No. 62/063,192), COPI delta (U.S. Patent Application No. 62/063,216), RNA polymerase I1 (U.S. Patent Application No. 62/133214), and RNA polymerase II215 (U.S. Patent Application No. 62/133202); 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 insecticidal protein, and a PIP-1 polypeptide); a 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 an Insect Pest

A. Overview

In some embodiments of the invention, at least one nucleic acid molecule useful for the control of insect (e.g., coleopteran and/or hemipteran) pests may be provided to an insect pest, wherein the nucleic acid molecule leads to RNAi-mediated gene silencing in the pest. In particular embodiments, an iRNA molecule (e.g., dsRNA, siRNA, miRNA, shRNA, and hpRNA) may be provided to a coleopteran and/or hemipteran pest. In some embodiments, a nucleic acid molecule useful for the control of insect 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 insect 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 an insect 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.

In some embodiments, a pest is contacted with the nucleic acid molecule that leads to RNAi-mediated gene silencing in the pest through contact with a topical composition (e.g., a composition applied by spraying) or an RNAi bait. RNAi baits are formed when the dsRNA is mixed with food or an attractant or both. When the pests eat the bait, they also consume the dsRNA. Baits may take the form of granules, gels, flowable powders, liquids, or solids. In particular embodiments, rpII33 may be incorporated into a bait formulation such as that described in U.S. Patent No. 8,530,440 which is hereby incorporated by reference. Generally, with baits, the baits are placed in or around the environment of the insect pest, for example, WCR can come into contact with, and/or be attracted to, the bait.

B. RNAi-mediated Target Gene Suppression

In certain 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, SCR, and 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.

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.

In particular 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).

In some 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.

In certain 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.

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; SEQ ID NO:3; the complement of SEQ ID NO:3; SEQ ID NO:5; the complement of SEQ ID NO:5; SEQ ID NO:6; the complement of SEQ ID NO:6; SEQ ID NO:7; the complement of SEQ ID NO:7; SEQ ID NO:8; the complement of SEQ ID NO:8; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3; a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; the complement of a native coding

polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a Diabrotica organism comprising any of SEQ ID NOs:5-8; SEQ ID NO:76; the complement of SEQ ID NO:76; SEQ ID NO:78; the complement of SEQ ID NO:78; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76 or SEQ ID NO:78; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76 or SEQ ID NO:78; a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; the complement of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding polynucleotide of a hemipteran organism comprising any of SEQ ID NOs:80-82. 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. In these and further embodiments, a nucleic acid molecule may be expressed that specifically hybridizes to a RNA molecule present in at least one cell of an insect (e.g., coleopteran and/or hemipteran) pest.

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.

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, a 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, a 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.

In certain embodiments, expression of a target gene in a pest (e.g., coleopteran or hemipteran) 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.

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

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.

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 a 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 a RNA molecule transcribed from a rpII33 DNA molecule, for example, comprising a polynucleotide selected from the group consisting of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82. 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.

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.

To impart insect (e.g., coleopteran and/or hemipteran) pest protection to a transgenic plant, a recombinant DNA molecule may, for example, be transcribed into an iRNA molecule, such as a dsRNA molecule, a siRNA molecule, a miRNA molecule, a shRNA molecule, or a hpRNA molecule. In some embodiments, a 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 protected against 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.

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.

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.

In other 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.

In certain 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.

iRNA molecules of the invention can be incorporated within the seeds of a plant species (e.g., corn or soybean), 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.

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.

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.

MODE(S) FOR CARRYING OUT THE INVENTION

I. Overview of several embodiments

Development of transgenic plants is becoming increasingly complex, and typically requires stacking multiple transgenes into a single locus. *See* Xie *et al.* (2001) Nat. Biotechnol. 19(7):677-9. Since each transgene usually requires a unique promoter for expression, multiple promoters are required to express different transgenes within one gene stack. In addition to increasing the size of the gene stack, this frequently leads to repeated use of the same promoter to obtain similar levels of expression patterns of different transgenes. This approach is often problematic, as the expression of multiple transgenes driven by the same promoter may lead to gene silencing or HBGS. An excess of competing transcription factor (TF)-binding sites in repeated promoters can cause depletion of endogenous TFs and lead to transcriptional downregulation. The silencing of transgenes is undesirable to the performance of a transgenic plant produced to express the transgenes. Repetitive sequences within a transgene often lead to intra-locus homologous recombination resulting in polynucleotide rearrangements and undesirable phenotypes or agronomic performance.

Plant promoters used for basic research or biotechnological application are generally unidirectional, and regulate only one gene that has been fused at its 3′ end (downstream). To produce transgenic plants with various desired traits or characteristics, it would be useful to

reduce the number of promoters that are deployed to drive expression of the transgenes that encode the desired traits and characteristics. Especially in applications where it is necessary to introduce multiple transgenes into plants for metabolic engineering and trait stacking, thereby necessitating multiple promoters to drive the expression of multiple transgenes. By developing a single Rice Ubiquitin-3 synthetic bi-directional promoter that can drive expression of two transgenes that flank the promoter, the total numbers of promoters needed for the development of transgenic crops may be reduced, thereby lessening the repeated use of the same promoter, reducing the size of transgenic constructs, and/or reducing the possibility of HBGS. Such a promoter can be generated by introducing known *cis*-elements in a novel or synthetic stretch of DNA, or alternatively by "domain swapping," wherein domains of one promoter are replaced with functionally equivalent domains from other heterologous promoters.

Embodiments herein utilize a process wherein a unidirectional promoter from a *Oryza sativa* (Rice) Ubiquitin-3 gene (*e.g.*, Rubi3) was used to design a synthetic Rice Ubiquitin-3 bi-directional promoter, such that one promoter can direct the expression of two genes, one on each end of the promoter. Synthetic Rice Ubiquitin-3 bi-directional promoters may allow those in the art to stack transgenes in plant cells and plants while lessening the repeated use of the same promoter and reducing the size of transgenic constructs. Furthermore, regulating the expression of two genes with a single synthetic Rice Ubiquitin-3 bi-directional promoter may also provide the ability to co-express the two genes under the same conditions, such as may be useful, for example, when the two genes each contribute to a single trait in the host. The use of bi-directional function of promoters in plants has been reported in some cases, including the *Zea mays* Ubiquitin 1 promoter (International Patent Publication No. WO2013101343 A1), CaMV 35 promoters (Barfield and Pua (1991) Plant Cell Rep. 10(6-7):308-14; Xie *et al.* (2001), *supra*), and the *mas* promoters (Velten *et al.* (1984) EMBO J. 3(12):2723-30; Langridge *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:3219-23).

Transcription initiation and modulation of gene expression in plant genes is directed by a variety of DNA sequence elements that are collectively arranged within the promoter. Eukaryotic promoters consist of minimal core promoter element (minP), and further upstream regulatory sequences (URSs). The core promoter element is a minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription. Core promoters in plants also comprise canonical regions associated with the initiation of transcription, such as CAAT and TATA boxes. The TATA box element is usually located approximately 20 to 35 nucleotides upstream of the initiation site of transcription.

The activation of the minP is dependent upon the URS, to which various proteins bind and subsequently interact with the transcription initiation complex. URSs comprise of DNA sequences, which determine the spatiotemporal expression pattern of a promoter comprising the URS. The polarity of a promoter is often determined by the orientation of the minP, while the URS is bipolar (*i.e.*, it functions independent of its orientation).

In specific examples of some embodiments, a minimal core promoter element (minUbi10P) of a modified *Zea mays* Ubiquitin-1 promoter (ZmUbi1) originally derived from *Zea mays*, is used to engineer a synthetic Rice Ubiquitin-3 bi-directional promoter that functions in plants to provide expression control characteristics that are unique with respect to previously described bi-directional promoters. Embodiments include a synthetic Rice Ubiquitin-3 bi-directional promoter that further includes a minimal core promoter element nucleotide sequence derived from a native *Zea mays* Ubiquitin-1 promoter (minPZmUbi1).

The ZmUbi1 promoter originally derived from *Zea mays* c.v. B73 comprises sequences located in the maize genome within about 899 bases 5′ of the transcription start site, and further within about 1,093 bases 3′ of the transcription start site. Christensen *et al.* (1992) Plant Mol. Biol. 18(4):675-89 (describing a *Zea mays* c.v. B73 ZmUbi1 gene). A modified ZmUbi1 promoter derived from B73 that is used in some examples is an approximately 2 kb promoter that contains a TATA box; two overlapping heat shock consensus elements; an 82 or 83 nucleotide (depending on the reference strand) leader sequence immediately adjacent to the transcription start site, which is referred to herein as ZmUbi1 exon; and a 1015-1016 nucleotide intron. Other maize ubiquitin promoter variants derived from *Zea* species and *Zea mays* genotypes may exhibit high sequence conservation around the minP element consisting of the TATA element and the upstream heat shock consensus elements. Thus, embodiments of the invention are exemplified by the use of this short (~200 nt) highly-conserved region (*e.g.*, SEQ ID NO:2) of a ZmUbi1 promoter as a minimal core promoter element for constructing synthetic bidirectional plant promoters.

The Rice Ubiquitin-3 promoter originally derived from *Oryza sativa* comprises sequences located in the rice genome within about 1,990 bases 5′ of the transcription start site. E Sivamani, and R Qu (2006) Expression enhancement of a rice polyubiquitin promoter. *Plant Molecular Biology* 60: 225-239. A modified Rice Ubiquitin-3 promoter derived from *Oryza sativa* that is used in some examples is an approximately 2 kb promoter that contains a TATA box, a 5′ UTR/intron sequence, and a downstream enhancing element located at the start of the Rice Ubiquitin-3 coding sequence. Other Rice Ubiquitin-3 promoter variants derived

from *Oryza* species and *Oryza sativa* genotypes may exhibit high sequence conservation around these promoter elements.

II. Abbreviations

AtUbi10	Arabidopsis thaliana Ubiquitin 10
BCA	bicinchoninic acid
CaMV	cauliflower mosaic virus
CsVMV	cassava vein mosaic virus
CTP	chloroplast transit peptide
HBGS	homology-based gene silencing
minUbi1P	ZmUbi1 minimal core promoter
OLA	oligo ligation amplification
PCR	polymerase chain reaction
RCA	rolling circle amplification
RUbi3	Rice Ubiquitin-3
RT-PCR	reverse transcriptase PCR
SNuPE	single nucleotide primer extension
URS	upstream regulatory sequence
ZmUbi1	Zea Mays Ubiquitin 1

III. Terms

Throughout the application, 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:

Introns: As used herein, the term "intron" refers to any nucleic acid sequence comprised in a gene (or expressed polynucleotide sequence of interest) that is transcribed but not translated. Introns include untranslated nucleic acid sequence within an expressed sequence of DNA, as well as the corresponding sequence in RNA molecules transcribed therefrom.

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.

Gene expression: The process by which the coded information of a nucleic acid transcriptional unit (including, *e.g.*, genomic DNA) 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).

Homology-based gene silencing: As used herein, “homology-based gene silencing” (HBGS) is a generic term that includes both transcriptional gene silencing and post-transcriptional gene silencing. Silencing of a target locus by an unlinked silencing locus can result from transcription inhibition (transcriptional gene silencing; TGS) or mRNA degradation (post-transcriptional gene silencing; PTGS), owing to the production of double-stranded RNA (dsRNA) corresponding to promoter or transcribed sequences, respectively. The involvement of distinct cellular components in each process suggests that dsRNA-induced TGS and PTGS likely result from the diversification of an ancient common mechanism. However, a strict comparison of TGS and PTGS has been difficult to achieve because it generally relies on the analysis of distinct silencing loci. We describe a single transgene locus that triggers both TGS and PTGS, owing to the production of dsRNA corresponding to promoter and transcribed sequences of different target genes. Mourrain *et al.* (2007) Planta 225:365-79. It is likely that siRNAs are the actual molecules that trigger TGS and PTGS on homologous sequences: the siRNAs would in this model trigger silencing and methylation of homologous sequences in *cis* and in *trans* through the spreading of methylation of transgene sequences into the endogenous promoter. *Id.*

Nucleic acid molecule: As used herein, the term "nucleic acid molecule" (or "nucleic acid" or "polynucleotide") may refer to a polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. A nucleotide 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. The term may refer to a molecule of RNA or DNA of indeterminate length. The term includes single- and double-stranded forms of DNA. 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.

Transcription proceeds in a 5′ to 3′ manner along a DNA strand. This means that RNA is made by the sequential addition of ribonucleotide-5′-triphosphates to the 3′ terminus of the growing chain (with a requisite elimination of the pyrophosphate). In either a linear or circular nucleic acid molecule, discrete elements (*e.g.*, particular nucleotide sequences) may be referred to as being "upstream" or "5′ " relative to a further element if they are bonded or would be bonded to the same nucleic acid in the 5′ direction from that element. Similarly, discrete elements may be "downstream" or "3′ " relative to a further element if they are or would be bonded to the same nucleic acid in the 3′ direction from that element.

A base "position," as used herein, refers to the location of a given base or nucleotide residue within a designated nucleic acid. The designated nucleic acid may be defined by alignment (see below) with a reference nucleic acid.

Hybridization: Oligonucleotides and their analogs hybridize by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid molecules consist of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will hydrogen bond to T or U, and G will bond to C. "Complementary" refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

"Specifically hybridizable" and "specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. The oligonucleotide need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions where specific binding is desired, for example, under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the chosen hybridization method 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 Mg2+ concentration) of the hybridization buffer will contribute to the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, chs. 9 and 11.

As used herein, "stringent conditions" encompass conditions under which hybridization will only occur if there is less than 50% mismatch between the hybridization molecule and the DNA target. "Stringent conditions" include further particular levels of stringency. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 50% sequence mismatch will not hybridize; conditions of "high stringency" are those under

which sequences with more than 20% mismatch will not hybridize; and conditions of "very high stringency" are those under which sequences with more than 10% mismatch will not hybridize.

In particular embodiments, stringent conditions can include hybridization at 65°C, followed by washes at 65°C with 0.1x SSC/0.1% SDS for 40 minutes.

The following are representative, non-limiting hybridization conditions:

Very High Stringency: 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.

High Stringency: 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.

Moderate Stringency: 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.

In particular embodiments, specifically hybridizable nucleic acid molecules can remain bound under very high stringency hybridization conditions. In these and further embodiments, specifically hybridizable nucleic acid molecules can remain bound under high stringency hybridization conditions. In these and further embodiments, specifically hybridizable nucleic acid molecules can remain bound under moderate stringency hybridization conditions.

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 base pairs in length. Because oligonucleotides may bind to a complementary nucleotide sequence, 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 small DNA 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.

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

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, and amino acid 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.

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 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet *et al.* (1988) Nucleic Acids Res. 16:10881-90; Huang *et al.* (1992) Comp. Appl. Biosci. 8:155-65; Pearson *et al.* (1994) Methods Mol. Biol. 24:307-31; Tatiana *et al.* (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, *e.g.*, Altschul *et al.* (1990) J. Mol. Biol. 215:403-10.

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 parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked with a coding sequence when the promoter affects the transcription or expression of the coding sequence. When recombinantly produced, operably linked nucleic acid sequences are generally contiguous and,

where necessary to join two protein-coding regions, in the same reading frame. However, elements need not be contiguous to be operably linked.

Promoter: A region of DNA that generally is located upstream (towards the 5′ region of a gene) that is needed for transcription. Promoters may permit the proper activation or repression of the gene which they control. A promoter may contain specific sequences that are recognized by transcription factors. These factors may bind to the promoter DNA sequences and result in the recruitment of RNA polymerase, an enzyme that synthesizes RNA from the coding region of the gene.

Transformed: 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; whiskers-mediated transformation; and microprojectile bombardment (Klein *et al.* (1987) Nature 327:70).

Transgene: An exogenous nucleic acid sequence. In one example, a transgene is a gene sequence (*e.g.*, an herbicide-resistance gene), a gene encoding an industrially or pharmaceutically useful compound, or a gene encoding a desirable agricultural trait. In yet another example, the transgene is an antisense nucleic acid sequence, wherein expression of the antisense nucleic acid sequence inhibits expression of a target nucleic acid sequence. A transgene may contain regulatory sequences operably linked to the transgene (*e.g.*, a promoter). In some embodiments, a nucleic acid sequence of interest is a transgene. However, in other embodiments, a polynucleotide sequence of interest is an endogenous nucleic acid sequence, wherein additional genomic copies of the endogenous nucleic acid sequence are desired, or a polynucleotide sequence that is in the antisense orientation with respect to the sequence of a target nucleic acid molecule in the host organism.

Transgenic Event: A transgenic "event" is produced by transformation of plant cells with heterologous DNA, *i.e.*, a nucleic acid construct that includes a transgene of interest, regeneration of a population of plants resulting from the insertion of the transgene into the

genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety that includes the genomic/transgene DNA. Even after repeated back-crossing to a recurrent parent, the inserted transgene DNA and flanking genomic DNA (genomic/transgene DNA) from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term "event" also refers to DNA from the original transformant and progeny thereof comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (*e.g.*, the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

Vector: A nucleic acid molecule as introduced into a cell, thereby producing a transformed cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. Examples include, but are not limited to, a plasmid, cosmid, bacteriophage, or virus that carries exogenous DNA into a cell. A vector can also include one or more genes, 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 may optionally include materials to aid in achieving entry of the nucleic acid molecule into the cell (*e.g.*, a liposome, protein coding, etc.).

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, Genes V, Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Meyers (ed.), *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

As used herein, the articles, "a," "an," and "the" include plural references unless the context clearly and unambiguously dictates otherwise.

IV. Synthetic bi-directional promoter, RUbi3, and nucleic acids comprising the same

This disclosure provides nucleic acid molecules comprising a synthetic nucleotide sequence that may function as a bi-directional promoter. In some embodiments, a synthetic bi-directional promoter may be operably linked to one or two polynucleotide sequence(s) of interest. For example, the synthetic Rice Ubiquitin 3 bi-directional promoter may be operably linked to one or two polynucleotide sequence(s) of interest that encode a gene. (*e.g.*, two genes, one on each end of the promoter), so as to regulate transcription of at least one (*e.g.*, one or both) of the nucleotide sequence(s) of interest. In some embodiments, by incorporating a URS from a Rice Ubiquitin 3 promoter in the synthetic Rice Ubiquitin 3 bi-directional promoter, particular expression and regulatory patterns (*e.g.*, such as are exhibited by genes under the control of the Rice Ubiquitin 3 promoter) may be achieved with regard to a polynucleotide sequence of interest that is operably linked to the synthetic Rice Ubiquitin 3 bi-directional promoter.

Some embodiments of the invention are exemplified herein by incorporating a minimal core promoter element from a unidirectional maize ubiquitin-1 gene (ZmUbi1) promoter into a molecular context different from that of the native promoter to engineer a synthetic bidirectional promoter. This minimal core promoter element is referred to herein as "minUbi1P," and is approximately 200 nt in length. Sequencing and analysis of minUbi1P elements from multiple *Zea* species and *Z. mays* genotypes has revealed that functional minUbi1P elements are highly conserved, such that a minUbi1P element may element may preserve its function as an initiator of transcription if it shares, for example, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, and/or at least about 100% sequence identity to the minUbi1P element of SEQ ID NO:2. Characteristics of minUbi1P elements that may be useful in some embodiments of the invention may include, for example, and without limitation, the aforementioned high conservation of nucleotide sequence, the presence of at least one TATA box, and/or the presence of at least one *(e.g.*, two) heat shock consensus element(s). In particular minUbi1P elements, more than one heat shock consensus elements may be overlapping within the minUbi1P sequence.

In embodiments, the process of incorporating a minUbi1P element into a molecular context different from that of a native promoter (*i.e.*, Rice Ubiquitin 3) to engineer a synthetic bi-directional promoter may comprise incorporating the minUbi1P element into a Rice

Ubiquitin 3 promoter nucleic acid, while reversing the orientation of the minUbi1P element with respect to the remaining sequence of the Rice Ubiquitin 3 promoter. Thus, a synthetic Rice Ubiquitin 3 bi-directional promoter may comprise a minUbi1P minimal core promoter element located 3′ of, and in reverse orientation with respect to, a Rice Ubiquitin 3 promoter nucleotide sequence, such that it may be operably linked to a nucleotide sequence of interest located 3′ of the Rice Ubiquitin 3 promoter nucleotide sequence. For example, the minUbi1P element may be incorporated at the 3′ end of a Rice Ubiquitin 3 promoter in reverse orientation.

A synthetic bi-directional Rice Ubiquitin 3 promoter may also comprise one or more additional sequence elements in addition to a minUbi1P element and elements of a native Rice Ubiquitin 3 promoter. In some embodiments, a synthetic bi-directional Rice Ubiquitin 3 promoter may comprise a promoter URS, an exon (*e.g.*, a leader or signal peptide), an intron, a spacer sequence, and/or combinations of one or more of any of the foregoing. For example and without limitation, a synthetic bi-directional Rice Ubiquitin 3 promoter may comprise a URS sequence from a Rice Ubiquitin 3 or ZmUbi1 promoter, an intron from a Rice Ubiquitin 3 or ZmUbi1 gene, an exon encoding a leader peptide from an Rice Ubiquitin 3 or ZmUbi1 gene, an intron from an Rice Ubiquitin 3 or ZmUbi1 gene, and combinations of these.

A synthetic bi-directional Rice Ubiquitin 3 promoter may also comprise one or more additional sequence elements in addition to a minUBi1P element and elements of a native promoter Rice Ubiquitin 3 including the minUbi1P. In some embodiments, a synthetic bi-directional Rice Ubiquitin 3 promoter may comprise a promoter URS, an exon (*e.g.*, a leader or signal peptide), an intron, a spacer sequence, and or combinations of one or more of any of the foregoing. For example and without limitation, a synthetic bi-directional Rice Ubiquitin 3 promoter may comprise a URS sequence from a *Zea mays* Ubiquitin 1 promoter, an intron from a ADH gene, an exon encoding a leader peptide from an *Zea mays* Ubiquitin gene, an intron from an *Zea mays* Ubiquitin gene, and combinations of these.

In some embodiments of a promoter comprising a promoter URS, the URS may be selected to confer particular regulatory properties on the synthetic promoter. Known promoters vary widely in the type of control they exert on operably linked genes (*e.g.*, environmental responses, developmental cues, and spatial information), and a URS incorporated into a heterologous promoter typically maintains the type of control the URS exhibits with regard to its native promoter and operably linked gene(s). Langridge *et al.* (1989), *supra.* Examples of eukaryotic promoters that have been characterized and may contain a URS comprised within a synthetic bi-directional Rice Ubiquitin 3 promoter according to some embodiments include, for

example and without limitation: those promoters described in U.S. Patent Nos. 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); 6,433,252 (maize L3 oleosin promoter); 6,429,357 (rice actin 2 promoter, and rice actin 2 intron); 5,837,848 (root-specific promoter); 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 (bi-directional promoters); 6,635,806 (gamma-coixin promoter); and U.S. Patent Application Serial No. 09/757,089 (maize chloroplast aldolase promoter).

Additional exemplary prokaryotic promoters include the nopaline synthase (NOS) promoter (Ebert *et al.* (1987) Proc. Natl. Acad. Sci. USA 84(16):5745-9); the octopine synthase (OCS) promoter (which is 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); CaMV35S (U.S. Patent Nos. 5,322,938, 5,352,605, 5,359,142, and 5,530,196); FMV35S (U.S. Patent Nos. 6,051,753, and 5,378,619); a PC1SV promoter (U.S. Patent No. 5,850,019); the SCP1 promoter (U.S. Patent No. 6,677,503); and *Agrobacterium tumefaciens* 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), and the like.

In some embodiments, a synthetic Rice Ubiquitin 3 bi-directional promoter may further comprise an exon. For example, it may be desirable to target or traffic a polypeptide encoded by a polynucleotide sequence of interest operably linked to the promoter to a particular subcellular location and/or compartment. In these and other embodiments, a coding sequence (exon) may be incorporated into a nucleic acid molecule between the remaining synthetic Rice Ubiquitin 3 bi-directional promoter sequence and a nucleotide sequence encoding a polypeptide. These elements may be arranged according to the discretion of the skilled practitioner such that the synthetic Rice Ubiquitin 3 bi-directional promoter promotes the expression of a polypeptide (or one or both of two polypeptide-encoding sequences that are operably linked to the promoter) comprising the peptide encoded by the incorporated coding sequence in a functional relationship with the remainder of the polypeptide. In particular

examples, an exon encoding a leader, transit, or signal peptide (*e.g.*, a *Zea mays* Ubi1 leader peptide) may be incorporated.

Peptides that may be encoded by an exon incorporated into a synthetic Rice Ubiquitin 3 bi-directional promoter include, for example and without limitation: a Ubiquitin (*e.g.*, *Zea mays* Ubi1) leader peptide, a chloroplast transit peptide (CTP) (*e.g.*, the *A. thaliana* EPSPS CTP (Klee *et al.* (1987) Mol. Gen. Genet. 210:437-42), and the *Petunia hybrida* EPSPS CTP (della-Cioppa *et al.* (1986) Proc. Natl. Acad. Sci. USA 83:6873-7)), as exemplified for the chloroplast targeting of dicamba monooxygenase (DMO) in International PCT Publication No. WO 2008/105890.

Introns may also be incorporated in a synthetic Rice Ubiquitin 3 bi-directional promoter in some embodiments of the invention, for example, between the remaining synthetic Rice Ubiquitin 3 bi-directional promoter sequence and a polynucleotide sequence of interest that is operably linked to the promoter. In some examples, an intron incorporated into a synthetic Rice Ubiquitin 3 bi-directional promoter may be, without limitation, a 5′ UTR that functions as a translation leader sequence that is present in a fully processed mRNA upstream of the translation start sequence (such a translation leader sequence may affect processing of a primary transcript to mRNA, mRNA stability, and/or translation efficiency). Examples of translation leader sequences include maize and petunia heat shock protein leaders (U.S. Patent No. 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 No. 5,659,122), PhDnaK (U.S. Patent No. 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). In particular examples, a *Zea mays* Ubiquitin 1 intron may be incorporated in a synthetic Rice Ubiquitin-3 bi-directional promoter.

Additional sequences that may optionally be incorporated into a synthetic Rice Ubiquitin-3 bi-directional promoter include, for example and without limitation: 3′ non-translated sequences, 3′ transcription termination regions, and polyadenylation regions. These are genetic elements located downstream of a polynucleotide sequence of interest (*e.g.*, a sequence of interest that is operably linked to a synthetic Rice Ubiquitin-3 bi-directional promoter), and include polynucleotides that provide polyadenylation signal, and/or other regulatory signals capable of affecting transcription, mRNA processing, or gene expression. A polyadenylation signal may function in plants to cause the addition of polyadenylate nucleotides to the 3′ end of a mRNA precursor. The polyadenylation sequence may be derived from the

natural gene, 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′ nontranslated 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 *Agrobacterium tumefaciens* Nos gene (GenBank Accession No. E01312).

In some embodiments, a synthetic Rice Ubiquitin-3 bi-directional promoter comprises one or more nucleotide sequence(s) that facilitate targeting of a nucleic acid comprising the promoter to a particular locus in the genome of a target organism. For example, one or more sequences may be included that are homologous to segments of genomic DNA sequence in the host (*e.g.*, rare or unique genomic DNA sequences). In some examples, these homologous sequences may guide recombination and integration of a nucleic acid comprising a synthetic Rice Ubiquitin-3 bi-directional promoter at the site of the homologous DNA in the host genome. In particular examples, a synthetic Rice Ubiquitin-3 bi-directional promoter comprises one or more nucleotide sequences that facilitate targeting of a nucleic acid comprising the promoter to a rare or unique location in a host genome utilizing engineered nuclease enzymes that recognize sequence at the rare or unique location and facilitate integration at that rare or unique location. Such a targeted integration system employing zinc-finger endonucleases as the nuclease enzyme is described in U.S. Patent Application No. 13/011,735, the contents of the entirety of which are incorporated herein by this reference.

In other embodiments, the disclosure further includes as an embodiment the polynucleotide sequence of interest comprising a trait. The trait can be an insecticidal resistance trait, herbicide tolerance trait, nitrogen use efficiency trait, water use efficiency trait, nutritional quality trait, DNA binding trait, selectable marker trait, and any combination thereof.

In further embodiments the traits are integrated within the transgenic plant cell as a transgenic event. In additional embodiments, the transgenic event produces a commodity product. Accordingly, a composition is derived from transgenic plant cells of the subject disclosure, wherein said composition is a commodity product selected from the group consisting of meal, flour, protein concentrate, or oil. In further embodiments, commodity products produced by transgenic plants derived from transformed plant cells are included, wherein the commodity products comprise a detectable amount of a nucleic acid sequence

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 nucleic acid sequences 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 acid sequences of the invention. The detection of one or more of the sequences 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 agronomic traits.

Nucleic acids comprising a synthetic Rice Ubiquitin-3 bi-directional promoter may be produced using any technique known in the art, including, for example and without limitation: RCA, PCR amplification, RT-PCR amplification, OLA, and SNuPE. These and other equivalent techniques are well known to those of skill in the art, and are further described in detail in, for example and without limitation: Sambrook *et al. Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory, 2001; and Ausubel *et al. Current Protocols in Molecular Biology*, John Wiley & Sons, 1998. All of the references cited above, including both of the foregoing manuals, are incorporated herein by this reference in their entirety, including any drawings, figures, and/or tables provided therein.

V. Delivery to a cell of a nucleic acid molecule comprising synthetic bi-directional promoter, RUbi3

The present disclosure also provides methods for transforming a cell with a nucleic acid molecule comprising a synthetic Rice Ubiquitin-3 bi-directional promoter. Any of the large number of techniques known in the art for introduction of nucleic acid molecules into plants may be used to transform a plant with a nucleic acid molecule comprising a synthetic Rice Ubiquitin-3 bi-directional promoter according to some embodiments, for example, to introduce one or more synthetic Rice Ubiquitin-3 bi-directional promoters into the host plant genome, and/or to further introduce one or more polynucleotides of interest operably linked to the promoter.

Suitable methods for transformation of plants include any method by which DNA can be introduced into a cell, for example and without limitation: electroporation (*see*, *e.g.*, U.S. Patent 5,384,253), microprojectile bombardment (*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), *Agrobacterium*-mediated

transformation (*see*, *e.g.*, U.S. Patents 5,635,055, 5,824,877, 5,591,616, 5,981,840, and 6,384,301), and protoplast transformation (*see*, *e.g.*, U.S. Patent 5,508,184). Through the application of techniques such as the foregoing, the cells of virtually any plant species may be stably transformed, and these cells may be developed into transgenic plants by techniques known to those of skill in the art. For example, techniques that may be particularly useful in the context of cotton transformation are described in U.S. Patents 5,846,797, 5,159,135, 5,004,863, and 6,624,344; techniques for transforming *Brassica* plants in particular are described, for example, in U.S. Patent 5,750,871; techniques for transforming soya are described, for example, in U.S. Patent 6,384,301; and techniques for transforming maize are described, for example, in U.S. Patents 7,060,876 and 5,591,616, and International PCT Publication WO 95/06722.

After effecting delivery of an exogenous nucleic acid to a recipient cell, the transformed cell is generally identified for further culturing and plant regeneration. In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene with the transformation vector used to generate the transformant. In this case, the potentially transformed cell population can be assayed by exposing the cells to a selective agent or agents, or the cells can be screened for the desired marker gene trait.

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 media 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 maturity.

To confirm the presence of the desired nucleic acid molecule comprising a synthetic Rice Ubiquitin-3 bi-directional promoter 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 and PCR; 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.

Targeted integration events may be screened, for example, by PCR amplification using, *e.g.*, oligonucleotide primers specific for nucleic acid molecules of interest. PCR genotyping is understood to include, but not be limited to, polymerase-chain reaction (PCR) amplification of genomic DNA 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 (*see*, *e.g.*, Rios *et al.* (2002) Plant J. 32:243-53), and may be applied to genomic DNA derived from any plant species or tissue type, including cell cultures. Combinations of oligonucleotide primers that bind to both target sequence and introduced sequence may be used sequentially or multiplexed in PCR amplification reactions. Oligonucleotide primers designed to anneal to the target site, introduced nucleic acid sequences, and/or combinations of the two may be produced. Thus, PCR genotyping strategies may include, for example and without limitation: amplification of specific sequences in the plant genome, amplification of multiple specific sequences in the plant genome, amplification of non-specific sequences in the plant genome, and combinations of any of the foregoing. One skilled in the art may devise additional combinations of primers and amplification reactions to interrogate the genome. For example, a set of forward and reverse oligonucleotide primers may be designed to anneal to nucleic acid sequence(s) specific for the target outside the boundaries of the introduced nucleic acid sequence.

Forward and reverse oligonucleotide primers may be designed to anneal specifically to an introduced nucleic acid molecule, for example, at a sequence corresponding to a coding region within a polynucleotide sequence of interest comprised therein, or other parts of the nucleic acid molecule. These primers may be used in conjunction with the primers described above. Oligonucleotide primers may be synthesized according to a desired sequence, and are commercially available (*e.g.*, from Integrated DNA Technologies, Inc., Coralville, IA). Amplification may be followed by cloning and sequencing, or by direct sequence analysis of amplification products. One skilled in the art might envision alternative methods for analysis of amplification products generated during PCR genotyping. In one embodiment, oligonucleotide primers specific for the gene target are employed in PCR amplifications.

VI. Cells, cell cultures, tissues, and organisms comprising synthetic bi-directional promoter, RUbi3

Some embodiments of the present invention also provide cells comprising a synthetic Rice Ubiquitin-3 bi-directional promoter, for example, as may be present in a nucleic acid construct. In particular examples, a synthetic Rice Ubiquitin-3 bi-directional promoter according to some embodiments may be utilized as a regulatory sequence to regulate the expression of transgenes in plant cells and plants. In some such examples, the use of a synthetic bi-directional RUbi3 promoter operably linked to a polynucleotide sequence of interest (*e.g.*, a transgene) may reduce the number of homologous promoters needed to regulate expression of a given number of polynucleotide sequences of interest, and/or reduce the size of the nucleic acid construct(s) required to introduce a given number of nucleotide sequences of interest. Furthermore, use of a synthetic Rice Ubiquitin-3 bi-directional promoter may allow co-expression of two operably linked nucleotide sequence of interest under the same conditions (*i.e.*, the conditions under which the RUbi3 promoter is active). Such examples may be particularly useful, *e.g.*, when the two operably linked polynucleotide sequences of interest each contribute to a single trait in a transgenic host comprising the nucleotide sequences of interest, and co-expression of the nucleotide sequences of interest advantageously impacts expression of the trait in the transgenic host.

In some embodiments, a transgenic plant comprising one or more synthetic Rice Ubiquitin-3 bi-directional promoter(s) and/or nucleotide sequence(s) of interest may have one or more desirable traits conferred (*e.g.*, introduced, enhanced, or contributed to) by expression of the nucleotide sequence(s) of interest in the plant. Such traits may include, for example and without limitation: resistance to insects, other pests, and disease-causing agents; tolerance to herbicides; enhanced stability, yield, or shelf-life; environmental tolerances; pharmaceutical production; industrial product production; and nutritional enhancements. In some examples, a desirable trait may be conferred by transformation of a plant with a nucleic acid molecule comprising a synthetic Rice Ubiquitin-3 bi-directional promoter operably linked to a polynucleotide sequence of interest. In some examples, a desirable trait may be conferred to a plant produced as a progeny plant *via* breeding, which trait may be conferred by one or more nucleotide sequences of interest operably linked to a synthetic Rice Ubiquitin-3 bi-directional promoter that is/are passed to the plant from a parent plant comprising a nucleotide sequence of interest operably linked to a synthetic Rice Ubiquitin-3 bi-directional promoter.

A transgenic plant according to some embodiments may be any plant capable of being transformed with a nucleic acid molecule of the invention, or of being bred with a plant transformed with a nucleic acid molecule of the invention. Accordingly, the plant may be a dicot or monocot. Non-limiting examples of dicotyledonous plants for use in some examples include: alfalfa, beans, broccoli, cabbage, canola, carrot, cauliflower, celery, Chinese cabbage, cotton, cucumber, eggplant, lettuce, melon, pea, pepper, peanut, potato, pumpkin, radish, rapeseed, spinach, soybean, squash, sugarbeet, sunflower, tobacco, tomato, and watermelon. Non-limiting examples of monocotyledonous plants for use in some examples include: *Brachypodium*, corn, onion, rice, sorghum, wheat, rye, millet, sugarcane, oat, triticale, switchgrass, and turfgrass.

In some embodiments, a transgenic plant may be used or cultivated in any manner, wherein presence a synthetic Rice Ubiquitin-3 bi-directional promoter and/or operably linked polynucleotide sequence of interest is desirable. Accordingly, such transgenic plants may be engineered to, *inter alia*, have one or more desired traits or transgenic events, by being transformed with nucleic acid molecules according to the invention, and may be cropped or cultivated by any method known to those of skill in the art.

The following examples are provided to illustrate certain particular features and/or embodiments. The examples should not be construed to limit the disclosure to the particular features or embodiments exemplified.

EXAMPLES

EXAMPLE 1: Materials and Methods

Sample preparation and bioassays

A number of dsRNA molecules (including those corresponding to *rpII33-1* reg1 (SEQ ID NO:5), *rpII33-2* reg1 (SEQ ID NO:6), *rpII33-2* v1 (SEQ ID NO:7), and *rpII33-2* v2 (SEQ ID NO:8) were synthesized and purified using a MEGASCRIPT® T7 RNAi kit (LIFE TECHNOLOGIES, Carlsbad, CA) or T7 Quick High Yield RNA Synthesis Kit (NEW ENGLAND BIOLABS, Whitby, Ontario). 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).

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).

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 µL/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.

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).

The statistical analysis was done using JMP™ software (SAS, Cary, NC).

The LC_{50} (Lethal Concentration) is defined as the dosage at which 50% of the test insects are killed. The 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.

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

Insects from 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 protection technology.

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):

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).

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.

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 hrs.

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).

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.

Candidate genes for RNAi targeting were hypothesized to be essential for survival and growth in pest 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.

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 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.

Several candidate target genes encoding *Diabrotica rpII33* (SEQ ID NO:1 and SEQ ID NO:3) were identified as genes that may lead to coleopteran pest mortality, inhibition of growth, inhibition of development, and/or inhibition of feeding in WCR.

The polynucleotides of SEQ ID NO:1 and SEQ ID NO:3 are novel. The sequences are not provided in public databases, and are not disclosed in PCT International Patent Publication No. 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; U.S. Patent 7,612,194; or U.S. Patent Application No. 2013192256. The *Diabrotica rpII33-1* (SEQ ID NO:1) is somewhat related to a fragment of a sequence from *Drosophila willistoni* (GENBANK Accession No. XM_002064757.1). There was no significant homologous nucleotide sequence to the *Diabrotica rpII33-2* (SEQ ID NO:3) found in GENBANK. The closest homolog of the *Diabrotica* RPII33-1 amino acid sequence (SEQ ID NO:2) is a *Aedes aegypti* protein having GENBANK Accession No. XP_001659470.1 (94% similar; 87% identical over the homology region). The closest homolog of the *Diabrotica* RPII33-2 amino acid sequence (SEQ ID NO:4) is a *Dendroctonus ponderosae* protein having GENBANK Accession No. AAE63493.1 (96% similar; 91% identical over the homology region).

RpII33 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 *rpII33* are useful for preventing root feeding damage by corn rootworm. *RpII33* dsRNA transgenes represent new modes of action for combining with *Bacillus thuringiensis* 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.

EXAMPLE 3: Amplification of Target Genes to produce dsRNA

Full-length or partial clones of sequences of *rpII33* candidate genes were used to generate PCR amplicons for dsRNA synthesis. 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:9) was incorporated into the 5' ends of the amplified sense or antisense strands. *See* Table 1. Total RNA was extracted from WCR using TRIzol® (Life Technologies, Grand Island, NY), and was then used to make first-strand cDNA with SuperScriptIII® First-Strand Synthesis System and

manufacturers Oligo dT primed instructions (Life Technologies, Grand Island, NY). 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:10; 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 *rpII-33* target gene and *YFP* negative control gene.

	Gene ID	Primer ID	Sequence
Pair 1	*rpII33-1* Reg1	Dvv-rpII33-1_For	TTAATACGACTCACTATAGGGAGAGAATTCCTT GCCCATCGAATTG (SEQ ID NO:11)
		Dvv-rpII33-1_Rev	TTAATACGACTCACTATAGGGAGAGTTATATTC AGCTTCGTATTGATC (SEQ ID NO:12)
Pair 2	*rpII33-2* Reg1	Dvv-rpII33-2_For	TTAATACGACTCACTATAGGGAGAGTTCTCAGT GATGAATTTTTAGCAC (SEQ ID NO:13)
		Dvv-rpII33-2_Rev	TTAATACGACTCACTATAGGGAGACCCAGTTAT ATGGAGCTTCATACTG (SEQ ID NO:14)
Pair 3	*rpII33-2 v1*	Dvv-rpII33-2 v1_For	TTAATACGACTCACTATAGGGAGACTTTAGATG TAAAATGTACAGATG (SEQ ID NO:15)
		Dvv-rpII33-2 v1_Rev	TTAATACGACTCACTATAGGGAGACTGTTTCAC CATACTCTGAG (SEQ ID NO:16)
Pair 4	*rpII33-2 v2*	Dvv-rpII33-2_v2_For	TTAATACGACTCACTATAGGGAGAGCGTATGCC AAAAAAGGCTTTG (SEQ ID NO:17)
		Dvv-rpII33-2_v2_Rev	TTAATACGACTCACTATAGGGAGAGGCCATTCG TCTGGTTTAGG (SEQ ID NO:18)
Pair 5	YFP	YFP-F_T7	TTAATACGACTCACTATAGGGAGACACCATGGGC TCCAGCGGCGCCC (SEQ ID NO:26)
		YFP-R_T7	TTAATACGACTCACTATAGGGAGAAGATCTTGAA GGCGCTCTTCAGG (SEQ ID NO:29)

EXAMPLE 4: RNAi Constructs

Template preparation by PCR and dsRNA synthesis.

A strategy used to provide specific templates for *rpII33* and *YFP* dsRNA production is shown in FIG. 1. Template DNAs intended for use in *rpII33* 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 eggs, first-instar larvae, or adults. For each selected *rplI33* 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 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* FIG. 1. The sequences of the dsRNA templates amplified with the particular primer pairs were: SEQ ID NO:5 (*rplI33-1* reg1), SEQ ID NO:6 (*rplI33-2* reg1), SEQ ID NO:7 (*rplI33-2* ver1), SEQ ID NO:8 (*rplI33-2* v2), and YFP (SEQ ID NO:10). Double-stranded RNA for insect bioassay was synthesized and purified using an AMBION® MEGASCRIPT® RNAi kit following the manufacturer's instructions (INVITROGEN) or HiScribe® T7 In Vitro Transcription Kit following the manufacturer's instructions (New England Biolabs, Ipswich, MA). The concentrations of dsRNAs were measured using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE).

Construction of plant transformation vectors.

Entry vectors harboring a target gene construct for hairpin formation comprising segment of *rplI33* (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:76, or SEQ ID NO:78) 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 the *rplI33* target gene segment in opposite orientation to one another, the two segments being separated by a linker polynucleotide (*e.g.*, SEQ ID NO:107, and an ST-LS1 intron (Vancanneyt *et al.* (1990) Mol. Gen. Genet. 220(2):245-50)). Thus, the primary mRNA transcript contains the two *rplI33* gene segment sequences as large inverted repeats of one another, separated by the intron sequence. A copy of a promoter (*e.g.* maize ubiquitin 1, U.S. Patent No. 5,510,474; 35S from Cauliflower Mosaic Virus (CaMV); Sugarcane bacilliform badnavirus (ScBV) promoter; promoters from rice actin genes; ubiquitin promoters; pEMU; MAS; maize H3 histone promoter; ALS promoter; phaseolin gene promoter; *cab*; *rubisco*; *LAT52*; *Zm13*; and/or *apg*) is used to drive production of the primary mRNA hairpin transcript, and a fragment comprising a 3' untranslated region (*e.g.*, a maize peroxidase 5 gene (ZmPer5 3'UTR v2; U.S. Patent No. 6,699,984), AtUbi10, AtEf1, or StPinII) is used to terminate transcription of the hairpin-RNA-expressing gene.

Entry vectors pDAB126158 and pDAB126159 comprise a *rplI33*-RNA construct (SEQ ID NOs:103 and 104, respectively) that comprises a segment of *rplI33* (SEQ ID NOs:7 and 8, respectively).

Entry vectors described above are used in standard GATEWAY® recombination reactions with a typical binary destination vector to produce *rplI33* hairpin RNA expression transformation vectors for *Agrobacterium*-mediated maize embryo transformations.

The binary destination vector comprises a herbicide tolerance gene (aryloxyalknoate dioxygenase; AAD-1 v3) (U.S. Patent 7,838,733(B2), and Wright *et al.* (2010) Proc. Natl. Acad. Sci. U.S.A. 107:20240-5) under the regulation of a plant operable promoter (*e.g.*, sugarcane bacilliform badnavirus (ScBV) promoter (Schenk *et al.* (1999) Plant Mol. Biol. 39:1221-30) and ZmUbi1 (U.S. Patent 5,510,474)). A 5'UTR and intron are positioned between the 3' end of the 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 7,179,902) is used to terminate transcription of the AAD-1 mRNA.

A negative control binary vector, comprising a gene that expresses a YFP protein, is constructed by means of standard GATEWAY® recombination reactions with a typical binary destination vector and entry vector. The binary destination vector comprises a herbicide tolerance 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).

EXAMPLE 5: Screening of Candidate Target Genes

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.

Replicated bioassays demonstrated that ingestion of dsRNA preparations derived from *rplI33-2* reg1, *rplI33-2* v1, and *rplI33-2* v2 each resulted in mortality and 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 dsRNA, 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:10).

Table 2. Results of *rplI33* 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)	N	Mean (%Mortality) ± SEM*	Mean (GI) ± SEM
***rpII33-2* Reg1**	500	2	97.06±2.94 (A)	1.00±0.01 (A)
***rpII33-2* v1**	500	10	88.83±3.09 (A)	0.97±0.01 (A)
***rpII33-2* v2**	500	10	89.41±1.18 (A)	0.94±0.02 (A)
TE**	0	13	13.62±2.30 (B)	0.06±0.06 (B)
WATER	0	13	18.32±3.19 (B)	-0.03±0.07 (B)
YFP***	500	13	14.87±2.37 (B)	-0.04±0.08 (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 (0.1 mM) buffer, pH 7.2.

***YFP = Yellow Fluorescent Protein

Table 3. Summary of oral potency of *rpII33* dsRNA on WCR larvae (ng/cm^2).

Gene Name	LC_{50}	Range	GI_{50}	Range
*rpII33-2*_v1	6.63	8.80 - 11.57	7.03	3.57 - 13.84
*rpII33-2*_v2	15.84	20.6 - 26.77	15.76	8.38 - 29.64

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 *rpII33-2* v1, *rpII33-2* v2, and *rpII33-2* reg1 each provide surprising and unexpected superior control of *Diabrotica*, compared to other genes suggested to have utility for RNAi-mediated insect control.

For example, *annexin*, *beta spectrin 2*, and *mtRP-L4* were each suggested in U.S. Patent 7,612,194 to be efficacious in RNAi-mediated insect control. SEQ ID NO:20 is the DNA sequence of *annexin* region 1 (Reg 1) and SEQ ID NO:21 is the DNA sequence of *annexin* region 2 (Reg 2). SEQ ID NO:22 is the DNA sequence of *beta spectrin* 2 region 1 (Reg 1) and

SEQ ID NO:23 is the DNA sequence of *beta spectrin* 2 region 2 (Reg2). SEQ ID NO:24 is the DNA sequence of *mtRP-L4* region 1 (Reg 1) and SEQ ID NO:25 is the DNA sequence of *mtRP-L4* region 2 (Reg 2). A YFP sequence (SEQ ID NO:10) was also used to produce dsRNA as a negative control.

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 FIG. 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* FIG. 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 *annexin* Reg1, *annexin* Reg2, *beta spectrin* 2 Reg1, *beta spectrin* 2 Reg2, *mtRP-L4* Reg1, *mtRP-L4* Reg2, and YFP dsRNA molecules. 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**	**Sequence**
Pair 6	YFP	YFP-F_T7	TTAATACGACTCACTATAGGGAGACACCATGGGCTCCAGCGGCGCCC (SEQ ID NO:26)
	YFP	YFP-R	AGATCTTGAAGGCGCTCTTCAGG (SEQ ID NO:27)

Pair 7	YFP	YFP-F	CACCATGGGCTCCAGCGGCGCCC (SEQ ID NO:28)
	YFP	YFP-R_T7	TTAATACGACTCACTATAGGGAGAAGATCT TGAAGGCGCTCTTCAGG (SEQ ID NO:29)
Pair 8	Annexin (Reg 1)	Ann-F1_T7	TTAATACGACTCACTATAGGGAGAGCTCCA ACAGTGGTTCCTTATC (SEQ ID NO:30)
	Annexin (Reg 1)	Ann-R1	CTAATAATTCTTTTTTAATGTTCCTGAGG (SEQ ID NO:31)
Pair 9	Annexin (Reg 1)	Ann-F1	GCTCCAACAGTGGTTCCTTATC (SEQ ID NO:32)
	Annexin (Reg 1)	Ann-R1_T7	TTAATACGACTCACTATAGGGAGACTAATA ATTCTTTTTTAATGTTCCTGAGG (SEQ ID NO:33)
Pair 10	Annexin (Reg 2)	Ann-F2_T7	TTAATACGACTCACTATAGGGAGATTGTTA CAAGCTGGAGAACTTCTC (SEQ ID NO:34)
	Annexin (Reg 2)	Ann-R2	CTTAACCAACAACGGCTAATAAGG (SEQ ID NO:35)
Pair 11	Annexin (Reg 2)	Ann-F2	TTGTTACAAGCTGGAGAACTTCTC (SEQ ID NO:36)
	Annexin (Reg 2)	Ann-R2T7	TTAATACGACTCACTATAGGGAGACTTAAC CAACAACGGCTAATAAGG (SEQ ID NO:37)
Pair 12	Beta-spect2 (Reg 1)	Betasp2-F1_T7	TTAATACGACTCACTATAGGGAGAAGATGT TGGCTGCATCTAGAGAA (SEQ ID NO:38)
	Beta-spect2 (Reg 1)	Betasp2-R1	GTCCATTCGTCCATCCACTGCA (SEQ ID NO:39)
Pair 13	Beta-spect2 (Reg 1)	Betasp2-F1	AGATGTTGGCTGCATCTAGAGAA (SEQ ID NO:40)

	Beta-spect2 (Reg 1)	Betasp2-R1_T7	TTAATACGACTCACTATAGGGAGAGTCCATTCGTCCATCCACTGCA (SEQ ID NO:41)
Pair 14	Beta-spect2 (Reg 2)	Betasp2-F2_T7	TTAATACGACTCACTATAGGGAGAGCAGATGAACACCAGCGAGAAA (SEQ ID NO:42)
	Beta-spect2 (Reg 2)	Betasp2-R2	CTGGGCAGCTTCTTGTTTCCTC (SEQ ID NO:43)
Pair 15	Beta-spect2 (Reg 2)	Betasp2-F2	GCAGATGAACACCAGCGAGAAA (SEQ ID NO:44)
	Beta-spect2 (Reg 2)	Betasp2-R2_T7	TTAATACGACTCACTATAGGGAGACTGGGCAGCTTCTTGTTTCCTC (SEQ ID NO:45)
Pair 16	mtRP-L4 (Reg 1)	L4-F1_T7	TTAATACGACTCACTATAGGGAGAAGTGAAATGTTAGCAAATATAACATCC (SEQ ID NO:46)
	mtRP-L4 (Reg 1)	L4-R1	ACCTCTCACTTCAAATCTTGACTTTG (SEQ ID NO:47)
Pair 17	mtRP-L4 (Reg 1)	L4-F1	AGTGAAATGTTAGCAAATATAACATCC (SEQ ID NO:48)
	mtRP-L4 (Reg 1)	L4-R1_T7	TTAATACGACTCACTATAGGGAGAACCTCTCACTTCAAATCTTGACTTTG (SEQ ID NO:49)
Pair 18	mtRP-L4 (Reg 2)	L4-F2_T7	TTAATACGACTCACTATAGGGAGACAAAGTCAAGATTTGAAGTGAGAGGT (SEQ ID NO:50)
	mtRP-L4 (Reg 2)	L4-R2	CTACAAATAAAACAAGAAGGACCCC (SEQ ID NO:51)
Pair 19	mtRP-L4 (Reg 2)	L4-F2	CAAAGTCAAGATTTGAAGTGAGAGGT (SEQ ID NO:52)
	mtRP-L4 (Reg 2)	L4-R2_T7	TTAATACGACTCACTATAGGGAGACTACAAATAAAACAAGAAGGACCCC (SEQ ID NO:53)

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, pH 8.

**YFP = Yellow Fluorescent Protein

EXAMPLE 6: Production of Transgenic Maize Tissues Comprising Insecticidal dsRNAs

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 *rpII33* (*e.g.*, SEQ ID NO:1 and SEQ ID NO:3)) through expression of a chimeric gene stably-integrated into the plant genome are 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 8,304,604, which is herein incorporated by reference in its entirety. Transformed tissues are selected by their ability to grow on Haloxyfop-containing medium and are screened for dsRNA production, as appropriate. Portions of such transformed tissue cultures are presented to neonate corn rootworm larvae for bioassay, essentially as described in EXAMPLE 1.

Agrobacterium Culture Initiation. Glycerol stocks of *Agrobacterium* strain DAt13192 cells (PCT International Publication No. WO 2012/016222A2) harboring a binary transformation vector described above (EXAMPLE 4) are streaked on AB minimal medium plates (Watson, *et al.* (1975) J. Bacteriol. 123:255-264) containing appropriate antibiotics, and are grown at 20 °C for 3 days. The cultures are then streaked onto YEP plates (gm/L: yeast extract, 10; Peptone, 10; NaCl, 5) containing the same antibiotics and are incubated at 20 °C for 1 day.

Agrobacterium culture. On the day of an experiment, a stock solution of Inoculation Medium and acetosyringone is 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) contains: 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 is 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 is thoroughly mixed.

For each construct, 1 or 2 inoculating loops-full of *Agrobacterium* from the YEP plate are suspended in 15 mL 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}) is measured in a spectrophotometer. The suspension is then diluted to OD_{550} of 0.3 to 0.4 using additional Inoculation Medium/acetosyringone mixtures. The tube of *Agrobacterium* suspension is 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 is performed.

Ear sterilization and embryo isolation. Maize immature embryos are 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 are harvested approximately 10 to 12 days post-pollination. On the experimental day, de-husked ears are 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) are 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) is added. For a given set of experiments, embryos from pooled ears are used for each transformation.

Agrobacterium co-cultivation. Following isolation, the embryos are placed on a rocker platform for 5 minutes. The contents of the tube are then poured onto a plate of Co-cultivation Medium, which contains 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 is removed with a sterile, disposable, transfer pipette. The embryos are then oriented with the scutellum facing up using sterile forceps with the aid of a microscope. The plate is 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).

Callus Selection and Regeneration of Transgenic Events. Following the Co-Cultivation period, embryos are transferred to Resting Medium, which is 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 are moved to each plate. The plates are 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 are then transferred (<18/plate) onto Selection Medium I, which is 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 are 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 are 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 are 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 allows transgenic callus to further proliferate and differentiate.

Proliferating, embryogenic calli are transferred (<9/plate) to Pre-Regeneration medium. Pre-Regeneration Medium contains 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 are 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 are 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 develop. Regeneration Medium contains 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 are then isolated and transferred to Elongation Medium without selection. Elongation Medium contains 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.

Transformed plant shoots selected by their ability to grow on medium containing Haloxyfop are 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 are 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, RT-qPCR assays are used to detect the presence of the linker sequence and/or of target sequence in putative transformants. Selected transformed plantlets are then moved into a greenhouse for further growth and testing.

Transfer and establishment of T_0 plants in the greenhouse for bioassay and seed production. When plants reach the V3-V4 stage, they are 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).

Plants to be used for insect bioassays are 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 are infested for bioassay.

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

EXAMPLE 7: Molecular Analyses of Transgenic Maize Tissues

Molecular analyses (*e.g.* RT-qPCR) of maize tissues are performed on samples from leaves that were collected from greenhouse grown plants on the day before or same day that root feeding damage is assessed.

Results of RT-qPCR assays for the target gene are used to validate expression of the transgene. Results of RT-qPCR assays for intervening sequence between repeat sequences (which is integral to the formation of dsRNA hairpin molecules) in expressed RNAs are alternatively used to validate the presence of hairpin transcripts. Transgene RNA expression levels are measured relative to the RNA levels of an endogenous maize gene.

DNA qPCR analyses to detect a portion of the *AAD1* coding region in gDNA are used to estimate transgene insertion copy number. Samples for these analyses are collected from plants grown in environmental chambers. Results are 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 *rpII33* transgenes) are advanced for further studies in the greenhouse.

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) are used to determine if the transgenic plants contain extraneous integrated plasmid backbone sequences. RNA transcript expression level: target qPCR. Callus cell events or transgenic plants are analyzed by real time quantitative PCR (qPCR) of the target sequence to determine the relative expression level of the transgene, as compared to the transcript level of an internal maize gene (SEQ ID NO:54; 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 is isolated using Norgen BioTek™ Total RNA Isolation Kit (Norgen, Thorold, ON). The total RNA is subjected to an on-column DNase1 treatment according to the kit's suggested protocol. The RNA is then quantified on a NANODROP 8000 spectrophotometer (THERMO SCIENTIFIC) and the concentration is normalized to 50 ng/µL. First strand cDNA is 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 is modified slightly to include the

addition of 10 µL of 100 µM T20VN oligonucleotide (IDT) (TTTTTTTTTTTTTTTTTTTTVN, where V is A, C, or G, and N is A, C, G, or T; SEQ ID NO:55) into the 1 mL tube of random primer stock mix, in order to prepare a working stock of combined random primers and oligo dT.

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

Separate real-time PCR assays for the target gene and TIP41-like transcript are performed on a LIGHTCYCLER™ 480 (ROCHE DIAGNOSTICS, Indianapolis, IN) in 10 µL reaction volumes. For the target gene assays, reactions are run with Primers rpII33 v1 FWD Set 2 (SEQ ID NO:56) and rpII33 v1 REV Set 2 (SEQ ID NO:57), and an IDT Custom Oligo probe rpII33 v1 PRB Set 2, labeled with FAM and double quenched with Zen and Iowa Black quenchers (SEQ ID NO:105); or Primers rpII33 v2 FWD Set 2 (SEQ ID NO:111) and rpII33 v2 REV Set 2 (SEQ ID NO:112), and an IDT Custom Oligo probe rpII33 v2 PRB Set 2, labeled with FAM and double quenched with Zen and Iowa Black quenchers (SEQ ID NO:106). For the TIP41-like reference gene assay, primers TIPmxF (SEQ ID NO:58) and TIPmxR (SEQ ID NO:59), and Probe HXTIP (SEQ ID NO:60) labeled with HEX (hexachlorofluorescein) are used.

All assays include negative controls of no-template (mix only). For the standard curves, a blank (water in source well) is 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 is excited at 465 nm and fluorescence is measured at 510 nm; the corresponding values for the HEX (hexachlorofluorescein) fluorescent moiety are 533 nm and 580 nm.

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

Target	**Oligonucleotide**	**Sequence**
rpII33-2 v1	RPII33-2 v1 FWD Set 2	GATCAAACTCGACATGTAACAACTG (SEQ ID NO:56)
rpII33-2 v1	RPII33-2 v1 REV Set 2	GGATTCATCATCACGATGTTTGG (SEQ ID NO:57)

rpII33-2 v1	RPII33-2 v1 PRB Set 2	/56-FAM/AGTGATCCA/ZEN/CGAGTCATACCAGCTACT /3IABkFQ/ (SEQ ID NO:105)
RpII33-2 v2	RpII33-2 v2 FWD Set 2	AAAGAGCATGCCAAATGGA (SEQ ID NO:110)
RpII33-2 v2	RpII33-2 v2 REV Set 2	GGCCATTCGTCTGGTTTAG (SEQ ID NO:111)
RpII33-2 v2	RpII33-2 v2 PRB Set 2	/56-FAM/TGTGGTGTT/ZEN/GCCTTTGAATATGATCCTGA /3IABkFQ/ (SEQ ID NO:106)
TIP41	TIPmxF	TGAGGGTAATGCCAACTGGTT (SEQ ID NO:58)
TIP41	TIPmxR	GCAATGTAACCGAGTGTCTCTCAA (SEQ ID NO:59)
TIP41	HXTIP (HEX-Probe)	TTTTTGGCTTAGAGTTGATGGTGTACTGATGA (SEQ ID NO:60)

*TIP41-like protein.

Table 7. PCR reaction recipes for transcript detection.

	rpII33	**TIP-like Gene**
Component	**Final Concentration**	
Roche Buffer	1 X	1X
rpII33 (F)	0.4 µM	0
rpII33 (R)	0.4 µM	0
rpII33 (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.

Target Gene 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

Data are 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 are 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.

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 *rpII33* hairpin dsRNA in transgenic plants expressing a *rpII33* hairpin dsRNA.

All materials and equipment are treated with RNaseZAP (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 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 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, and then centrifuged at 12,000 x g for 10 min at 4 °C to 25 °C. The supernatant is discarded and the RNA pellet is washed twice with 1 mL 70% ethanol, with centrifugation at 7,500 x g for 10 min at 4 °C 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.

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 hours and 15 minutes.

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 room temperature for up to 2 days.

The membrane is pre-hybridized 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 NOs:5-8, or 103-104, as appropriate) labeled with digoxigenin 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.

Transgene copy number determination. Maize leaf pieces approximately equivalent to 2 leaf punches are collected in 96-well collection plates (QIAGEN™). Tissue disruption is 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, gDNA is isolated in high throughput format using a BIOSPRINT96 PLANT KIT and a BIOSPRINT96 extraction robot. gDNA is diluted 1:3 DNA:water prior to setting up the qPCR reaction.

qPCR analysis. Transgene detection by hydrolysis probe assay is performed by real-time PCR using a LIGHTCYCLER®480 system. Oligonucleotides to be used in hydrolysis probe assays to detect the target gene (*e.g. rpII33*), the linker sequence, and/or to detect a portion of the *SpecR* gene (*i.e.*, the spectinomycin resistance gene borne on the binary vector plasmids; SEQ ID NO:61; SPC1 oligonucleotides in **Table 9**), are 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:62; GAAD1 oligonucleotides in **Table 9**) are designed using PRIMER EXPRESS software (APPLIED BIOSYSTEMS). **Table 9** shows the sequences of the primers and probes. Assays are multiplexed with reagents for an endogenous maize chromosomal gene (Invertase (SEQ ID NO:63; GENBANK Accession No: U16123; referred to herein as IVR1), which serves as an internal reference sequence to ensure gDNA is present in each assay. For amplification, LIGHTCYCLER®480 PROBES MASTER mix (ROCHE APPLIED SCIENCE) is 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 is performed as outlined in **Table 11**. Fluorophore activation and emission for the FAM- and HEX-labeled probes are 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) are 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 are 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	Sequence
GAAD1-F	TGTTCGGTTCCCTCTACCAA (SEQ ID NO:64)
GAAD1-R	CAACATCCATCACCTTGACTGA (SEQ ID NO:65)
GAAD1-P (FAM)	CACAGAACCGTCGCTTCAGCAACA (SEQ ID NO:66)
IVR1-F	TGGCGGACGACGACTTGT (SEQ ID NO:67)
IVR1-R	AAAGTTTGGAGGCTGCCGT (SEQ ID NO:68)
IVR1-P (HEX)	CGAGCAGACCGCCGTGTACTTCTACC (SEQ ID NO:69)
SPC1A	CTTAGCTGGATAACGCCAC (SEQ ID NO:70)
SPC1S	GACCGTAAGGCTTGATGAA (SEQ ID NO:71)
TQSPEC (CY5*)	CGAGATTCTCCGCGCTGTAGA (SEQ ID NO:72)
Loop- F	GGAACGAGCTGCTTGCGTAT (SEQ ID NO:73)

Loop- R	CACGGTGCAGCTGATTGATG (SEQ ID NO:74)
Loop-P (FAM)	TCCCTTCCGTAGTCAGAG (SEQ ID NO:75)

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

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. Growth and survival of target insects on the test diet is reduced compared to that of the control group.

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.

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

The soil around the maize plants growing in ROOTRANERS® is infested with 150 to 200 WCR eggs. The insects are allowed to feed for 2 weeks, after which time a "Root Rating" is given to each plant. A Node-Injury Scale is utilized for grading, essentially according to Oleson *et al.* (2005) J. Econ. Entomol. 98:1-8. Plants passing this bioassay, showing reduced

injury, are transplanted to 5-gallon pots for seed production. Transplants are treated with insecticide to prevent further rootworm damage and insect release in the greenhouses. Plants are hand pollinated for seed production. Seeds produced by these plants are saved for evaluation at the T_1 and subsequent generations of plants.

Transgenic negative control plants are generated by transformation with vectors harboring genes designed to produce a yellow fluorescent protein (YFP). Non-transformed negative control plants are grown from seeds of parental corn varieties from which the transgenic plants were produced. Bioassays are conducted with negative controls included in each set of plant materials.

EXAMPLE 9: Transgenic *Zea mays* Comprising Coleopteran Pest Sequences

10-20 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 comprise a portion of SEQ ID NO:1 or SEQ ID NO:3 (*e.g.*, the hairpin dsRNAs transcribed from SEQ ID NO:103 and SEQ ID NO:104). Additional hairpin dsRNAs are 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), RPS6 (U.S. Patent Application Publication No. 2013/0097730), ROP (U.S. Patent Application Publication No. 14/577811), RNAPII (U.S. Patent Application Publication No. 14/577854), *Dre4* (U.S. Patent Application No. 14/705,807), *ncm* (U.S. Patent Application No.62/095487), *COPI alpha* (U.S. Patent Application No. 62/063,199), *COPI beta* (U.S. Patent Application No. 62/063,203), *COPI gamma* (U.S. Patent Application No. 62/063,192), or *COPI delta* (U.S. Patent Application No. 62/063,216). 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 linker 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.

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.

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 and/or development 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*, *D. speciosa* Germar, and *D. u. undecimpunctata* Mannerheim, leads to failure to successfully infest, feed, develop, and/or leads to death of the coleopteran pest. The choice of target genes and the successful application of RNAi are then used to control coleopteran pests.

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 non-transformed 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. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are recorded. 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

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 and/or SEQ ID NO:3). 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

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 or SEQ ID NO:3) 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, Cry3, Cry6, Cry34 and Cry35 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: Screening of Candidate Target Genes in Neotropical Brown Stink Bug (*Euschistus heros*)

Neotropical Brown Stink Bug (BSB; *Euschistus heros*) colony. BSB 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, and 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 wicks. After the initial two weeks, insects were transferred into a new container once a week.

BSB artificial diet. A BSB artificial diet was prepared as follows. 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, and then cooled and stored at 4 °C. The artificial diet was used within two weeks of preparation.

BSB transcriptome assembly. 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 contained 378,457 sequences.

BSB *rpII33* ortholog identification. A tBLASTn search of the BSB pooled transcriptome was performed using as query, *Drosophila rpII-33* (protein sequence GENBANK Accession No. ABI30983). BSB *rpII33-1* (SEQ ID NO:76) and BSB *rpII33-2* (SEQ ID NO:78) were identified as *Euschistus heros* candidate target genes, the products of which have the predicted peptide sequences, SEQ ID NO:77 and SEQ ID NO:79 respectively.

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 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 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. Following manufacturer-recommended TRIzol® extraction protocol for 1 mL TRIzol®, the RNA pellet was dried at room temperature and resuspended in 200 µL 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). The RNA concentration was determined using a NANODROP™ 8000 spectrophotometer (THERMO SCIENTIFIC, Wilmington, DE).

cDNA amplification. cDNA was reverse-transcribed from 5 µg 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.

Primers as shown in **Table 12** were used to amplify BSB_*rpII33-1*, BSB_*rpII33-2*, BSB_*rpII33-3*. The DNA template was amplified by touch-down PCR (annealing temperature lowered from 60 °C to 50 °C, in a 1 °C/cycle decrease) with 1 µL cDNA (above) as the template. Fragments comprising a 255 bp segment of BSB_*rpII33*-1 (SEQ ID NO:80), a 111 bp segment of BSB_*rpII33*-1 v1 (SEQ ID NO:81), and a 398 bp segment of BSB_*rpII33*-2 (SEQ ID NO:82) were generated during 35 cycles of PCR. The above procedure was also used to amplify a 301 bp negative control template YFPv2 (SEQ ID NO:89), using YFPv2-F (SEQ ID NO:90) and YFPv2-R (SEQ ID NO:91) primers. The BSB_ *rpII33-1*, BSB_ *rpII33-1 v1*, BSB_ *rpII33-2*, and YFPv2 primers contained a T7 phage promoter sequence (SEQ ID NO:9) at their

5' ends, and thus enabled the use of YFPv2 and BSB_*rpII33* DNA fragments for dsRNA transcription.

Table 12. Primers and Primer Pairs used to amplify portions of coding regions of exemplary *rpII33* target genes and a *YFP* negative control gene.

	Gene ID	Primer ID	Sequence
Pair 20	*rpII33*-1	BSB_rpII33-1_For	TTAATACGACTCACTATAGGGAGAGGTGAA TCAGATGATATTTTGATTG (SEQ ID NO:83)
		BSB_rpII33-1_Rev	TTAATACGACTCACTATAGGGAGAGTTAGG TTTGGCTTCCCAATTAAATG (SEQ ID NO:84)
Pair 21	*rpII33*-1 v1	BSB_rpII33-1 v1_For	TTAATACGACTCACTATAGGGAGATTGTTTTGAGTATG ACCCTGACAAC (SEQ ID NO:85)
		BSB_rpII33-1 v1_Rev	TTAATACGACTCACTATAGGGAGAGGAGCTTCATACT GATCCTCATCTAATTC (SEQ ID NO:86)
Pair 22	*rpII33*-2	BSB_rpII33-2_For	TTAATACGACTCACTATAGGGAGACGTCGA AATCATCAAAAACAACACG (SEQ ID NO:87)
		BSB_rpII33-2_Rev	TTAATACGACTCACTATAGGGAGACTGTCC AGTAGTTTGTGGACCTAG (SEQ ID NO:88)
Pair 23	YFP	YFPv2-F	TTAATACGACTCACTATAGGGAGAGCATCTGG AGCACTTCTCTTTCA (SEQ ID NO:90)
		YFPv2-R	TTAATACGACTCACTATAGGGAGACCATCTCC TTCAAAGGTGATTG (SEQ ID NO:91)

dsRNA synthesis. dsRNA was synthesized using 2 µL PCR product (above) as the template with a MEGAscript™ T7 RNAi kit (AMBION) used according to the manufacturer's instructions. *See* FIG. 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, pH 7.4).

Injection of dsRNA into BSB hemocoel. BSB were reared on a green bean and seed diet, as the colony, 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 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 and then filled with 2 to 3 µL 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 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 hour light: dark photoperiod. Viability counts and weights were taken on day 7 after the injections.

BSB *rpII33* is a lethal dsRNA target. As summarized in **Table 13** and **Table 14**, in each replicate at least ten 2nd instar BSB nymphs (1 - 1.5 mg each) were injected into the hemocoel with 55.2 nL BSB_*rpII33-1*, BSB_ *rpII33-2*, and BSB_ *rpII33-1* v1 dsRNA (500 ng/µL), for an approximate final concentration of 18.4 - 27.6 µg dsRNA/g insect. The mortality determined for these dsRNAs was significantly different from that seen with the same amount of injected *YFP* v2 dsRNA (negative control), with $p < 0.05$ (Student's *t*-test).

Table 13. Results of BSB_*rpII33* dsRNA injection into the hemocoel of 2nd instar Neotropical Brown Stink Bug nymphs seven days after injection.

Treatment*	**N Trials**	**Mean % Mortality ± SEM****	**p value *t*-test**
rpII33-1	3	66.7 ± 8.82	5.78E-03***
rpII33-2	3	6.67 ± 6.67	7.25E-01
Not injected	3	0.00 ± 0.00	1.58E-01
***YFP* v2 dsRNA**	3	10.0 ± 5.77	

*Ten insects injected per trial for each dsRNA.

**Standard error of the mean.

***Significantly different from the *YFP v2* dsRNA control using a Student's *t*-test. ($p < 0.05$).

Table 14. Results of BSB_*rpII33-1* v1 dsRNA injection into the hemocoel of 2nd instar Neotropical Brown Stink Bug nymphs seven days after injection.

Treatment *	N trials	% mortality ± SEM**	p value *t*-test
BSB_*rpII33-1* v1	3	51 ± 24.7	1.98E-01
not injected	3	3 ± 3.3	5.61E-01
***YFP* v2 dsRNA**	3	10 ± 10	

*Ten insects injected per trial for each dsRNA.

**Standard error of the mean.

Example 13: Transgenic *Zea mays* Comprising Hemipteran Pest Sequences

Ten to 20 transgenic T_0 *Zea mays* plants harboring expression vectors for nucleic acids comprising any portion of SEQ ID NO:76 and/or SEQ ID NO:78 (*e.g.*, SEQ ID NOs:80-82) are generated as described in EXAMPLE 4. A further 10-20 T_1 *Zea mays* independent lines expressing hairpin dsRNA for an RNAi construct are obtained for BSB challenge. Hairpin dsRNA are derived comprising a portion of SEQ ID NO:76 and/or SEQ ID NO:78, or segments thereof (*e.g.*, SEQ ID NOs:80-82). 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 linker 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.

Moreover, RNAi molecules having mismatch sequences with more than 80% sequence identity to target genes affect hemipterans 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.

In planta delivery of dsRNA, siRNA, shRNA, hpRNA, 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/or survival of the hemipteran pest is affected, and in the case of at least one of *Euschistus heros, E. servus, Nezara viridula, Piezodorus guildinii, Halyomorpha halys, Chinavia hilare, C. marginatum, Dichelops melacanthus, D. furcatus; Edessa meditabunda, Thyanta perditor, Horcias nobilellus, Taedia stigmosa, Dysdercus peruvianus, Neomegalotomus parvus, Leptoglossus zonatus, Niesthrea sidae, Lygus hesperus,* and *L. lineolaris* leads to failure to successfully infest, feed, develop, and/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.

Phenotypic comparison of transgenic RNAi lines and non-transformed *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 non-transformed 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. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are recorded. 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

Ten to 20 transgenic T_0 *Glycine max* plants harboring expression vectors for nucleic acids comprising a portion of SEQ ID NO:76 and/or SEQ ID NO:78, or segments thereof (*e.g.*, SEQ ID NOs:80-82) 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.

Preparation of split-seed soybeans. The split soybean seed comprising a portion of an embryonic axis protocol requires 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.

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 a binary plasmid comprising SEQ ID NO:76 and/or SEQ ID NO:78, and/or segments thereof (*e.g.*, SEQ ID NOs:80-82). The *A. tumefaciens* solution is diluted to a final concentration of λ=0.6 OD_{650} before immersing the cotyledons comprising the embryo axis.

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 (*Agrobacterium* Protocols, vol. 2, 2nd Ed., Wang, K. (Ed.) Humana Press, New Jersey, 2006) in a Petri dish covered with a piece of filter paper.

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, and 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®).

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, and 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 $\mu mol/m^2sec$.

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.

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 $\mu mol/m^2sec$ 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.

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 comprising SEQ ID NO:76 and/or SEQ ID NO:78, or segments thereof (*e.g.*, SEQ ID NOs:80-82). These are confirmed through RT-PCR or other molecular analysis methods as known in the art. Total RNA preparations from selected independent T_1 lines are optionally used for RT-PCR with primers designed to bind in the linker 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.

RNAi molecules having mismatch sequences with more than 80% sequence identity to target genes affect BSB 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.

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 viability of feeding of the hemipteran pest is affected, and in the case of at least one of *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,* and *Lygus lineolaris* leads to failure to successfully infest, feed, develop, and/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.

Phenotypic comparison of transgenic RNAi lines and non-transformed *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 non-transformed 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. Plant shoot characteristics such as height, leaf numbers and sizes, time of flowering, floral size and appearance are recorded. 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.

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 (*See* 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 a *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. Significant mortality and/or growth inhibition is observed in the wells provided with *rpII33* dsRNA, compared to the control wells.

Example 16: Transgenic *Arabidopsis thaliana* Comprising Hemipteran Pest Sequences

Arabidopsis transformation vectors containing a target gene construct for hairpin formation comprising segments of *rpII33* (SEQ ID NO:76 or SEQ ID NO:78) are generated using standard molecular methods similar to EXAMPLE 4. *Arabidopsis* transformation is performed using standard *Agrobacterium*-based procedure. T_1 seeds are selected with glufosinate tolerance selectable marker. Transgenic T_1 *Arabidopsis* plants are generated and homozygous simple-copy T_2 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.

Construction of *Arabidopsis* transformation vectors. Entry clones based on an entry vector harboring a target gene construct for hairpin formation comprising a segment of *rpII33* (SEQ ID NO:76 or SEQ ID NO:78) 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 a linker sequence (SEQ ID NO:107). Thus, the primary mRNA transcript contains the two *rpII33*gene segment sequences as large inverted repeats of one another, separated by the linker sequence. A copy of a promoter (*e.g. 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 5,428,147) is used to terminate transcription of the hairpin-RNA-expressing gene.

The hairpin clones within entry vectors are used in standard GATEWAY® recombination reactions with a typical binary destination vector to produce hairpin RNA expression transformation vectors for *Agrobacterium*-mediated *Arabidopsis* transformation.

A binary destination vector comprises a herbicide tolerance gene, *DSM-2v2* (U.S. Patent Publication No. 2011/0107455), under the regulation of a Cassava vein mosaic virus promoter (CsVMV Promoter v2, U.S. Patent 7,601,885; Verdaguer *et al.* (1996) Plant Mol. Biol. 31:1129-39). 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. 172:1814-22) is used to terminate transcription of the DSM2v2 mRNA.

A negative control binary construct 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 and entry vector. The entry construct comprises a *YFP* hairpin sequence 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).

Production of transgenic *Arabidopsis* comprising insecticidal RNAs: *Agrobacterium*-mediated transformation. Binary plasmids containing hairpin dsRNA 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.

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 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*

Selection of T_1 *Arabidopsis* transformed with dsRNA constructs. Up to 200 mg of T_1 seeds from each transformation are 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 100-150 mE/m^2s.

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 reproductive 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. Significant mortality and/or growth inhibition is observed in nymphs feeding on transgenic *BSB_rpII33* dsRNA plants, compared to that of nymphs on control plants.

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.

Example 18: Transformation of Additional Crop Species

Cotton is transformed with a *rpII33* dsRNA transgene to provide control of hemipteran insects by utilizing a method known to those of skill in the art, for example, substantially the same techniques previously described in EXAMPLE 14 of U.S. Patent 7,838,733, or Example 12 of PCT International Patent Publication No. WO 2007/053482.

Example 19: *rpII33* dsRNA in Insect Management

RpII33 dsRNA transgenes are combined with other dsRNA molecules in transgenic plants to provide redundant RNAi targeting and synergistic RNAi effects. Transgenic plants including, for example and without limitation, corn, soybean, and cotton expressing dsRNA that targets *rpII33* are useful for preventing feeding damage by coleopteran and hemipteran insects. *RpII33* dsRNA transgenes are also combined in plants with *Bacillus thuringiensis* insecticidal protein technology, and or PIP-1 insecticidal polypeptides, to represent new modes of action in Insect Resistance Management gene pyramids. When combined with other dsRNA molecules that target insect pests and/or with insecticidal proteins in transgenic plants, a synergistic insecticidal effect is observed that also mitigates the development of resistant insect populations.

CLAIMS

What is 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:5; the complement of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:5; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:5; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising SEQ ID NO:5;

SEQ ID NO:3; the complement of SEQ ID NO:3; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:3; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:3; a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:6-8; the complement of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:6-8; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:6-8; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:6-8;

SEQ ID NO:76; the complement of SEQ ID NO:76; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:76; a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:80 or SEQ ID NO:81; the complement of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:80 or SEQ ID NO:81; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:80 or SEQ ID NO:81; the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:80 or SEQ ID NO:81;

SEQ ID NO:78; the complement of SEQ ID NO:78; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:78; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:78; a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:82; the complement of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:82; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:82; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Euschistus* organism comprising SEQ ID NO:82.

2. The polynucleotide of claim 1, wherein the polynucleotide is selected from the group consisting of SEQ ID NO:1; the complement of SEQ ID NO:1; SEQ ID NO:3; the complement of SEQ ID NO:3; 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 fragment of at least 15 contiguous nucleotides of SEQ ID NO:3; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:3; a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; the complement of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8.

3. 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:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and the complements of any of the foregoing.

4. The polynucleotide of claim 3, 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; and *D. u. undecimpunctata* Mannerheim.

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

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

7. A double-stranded RNA molecule produced from the expression of the polynucleotide of claim 1.

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

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

10. The double stranded RNA of claim 7, 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.

11. The RNA of claim 6, 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.

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

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

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

15. The cell of claim 13, wherein the cell is a eukaryotic cell.

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

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

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

19. A commodity product produced from the plant of claim 17, wherein the commodity product comprises a detectable amount of the polynucleotide.

20. The plant of claim 17, wherein the at least one polynucleotide is expressed in the plant as a double-stranded ribonucleic acid molecule.

21. The cell of claim 16, wherein the cell is a corn, soybean, or cotton cell.

22. The plant of claim 17, wherein the plant is corn, soybean, or cotton.

23. The plant of claim 17, 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 insect ingests a part of the plant.

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

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

26. A method for controlling a coleopteran or hemipteran pest population, the method comprising providing an agent comprising a ribonucleic acid (RNA) molecule that

functions upon contact with the pest to inhibit a biological function within the pest, wherein the RNA is specifically hybridizable with a polynucleotide selected from the group consisting of any of SEQ ID NOs:92-102; the complement of any of SEQ ID NOs:92-102; a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:92-102; the complement of a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:92-102; a transcript of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82; the complement of a transcript of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82; a fragment of at least 15 contiguous nucleotides of a transcript of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82; and the complement of a fragment of at least 15 contiguous nucleotides of a transcript of any of SEQ ID NOs:1, 3, 5-8, 76, 78, and 80-82.

27. The method according to claim 26, wherein the RNA of the agent is specifically hybridizable with a polynucleotide selected from the group consisting of SEQ ID NOs:92 and 93; the complement of SEQ ID NO:92 or 93; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:92 or 93; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:92 or 93; a transcript of SEQ ID NO:1 or 3; the complement of a transcript of SEQ ID NO:1 or 3; a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or 3; and the complement of a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or 3.

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

29. A method for controlling a coleopteran pest population, the method comprising:

providing an agent comprising a first and a second polynucleotide sequence that functions upon contact with the coleopteran pest to inhibit a biological function within the coleopteran pest, wherein the first polynucleotide sequence comprises a region that exhibits from about 90% to about 100% sequence identity to from about 15 to about 30 contiguous nucleotides of any of SEQ ID NOs:92-97, and wherein the first polynucleotide sequence is specifically hybridized to the second polynucleotide sequence.

30. A method for controlling a hemipteran pest population, the method comprising:

providing an agent comprising a first and a second polynucleotide sequence that functions upon contact with the hemipteran pest to inhibit a biological function within the hemipteran pest, wherein the first polynucleotide sequence comprises a region that exhibits from about 90% to about 100% sequence identity to from about 15 to about 30 contiguous nucleotides of any of SEQ ID NOs:98-102, and wherein the first polynucleotide sequence is specifically hybridized to the second polynucleotide sequence.

31. A method for controlling a coleopteran pest population, the method comprising:

providing in a host plant of a coleopteran pest a transformed plant cell comprising the polynucleotide of claim 2, wherein the polynucleotide is expressed to produce a ribonucleic acid molecule that functions upon contact with a coleopteran pest belonging to the population to inhibit the expression of a target sequence within the coleopteran pest and results in decreased growth and/or survival of the coleopteran pest or pest population, relative to reproduction of the same pest species on a plant of the same host plant species that does not comprise the polynucleotide.

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

33. The method according to claim 32, wherein the nucleic acid comprises SEQ ID NO:103 or SEQ ID NO:104.

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

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

SEQ ID NOs:92-97;

the complement of any of SEQ ID NOs:92-97;

a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:92-97;

the complement of a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:92-97;

a transcript of any of SEQ ID NOs:1, 3, and 5-8;

the complement of a transcript of any of SEQ ID NOs:1, 3, and 5-8;

a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or SEQ ID NO:3; and

the complement of a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:1 or SEQ ID NO:3.

36. The method according to claim 35, wherein the diet comprises a plant cell transformed to express the polynucleotide.

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

38. A method of controlling hemipteran pest infestation in a plant, the method comprising contacting a hemipteran pest with a ribonucleic acid (RNA) that is specifically hybridizable with a polynucleotide selected from the group consisting of:

SEQ ID NOs:98-102;

the complement of any of SEQ ID NOs:98-102;

a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:98-102;

the complement of a fragment of at least 15 contiguous nucleotides of any of SEQ ID NOs:98-102;

a transcript of any of SEQ ID NOs:76, 78, and 80-82;

the complement of a transcript of any of SEQ ID NOs:76, 78, and 80-82;

a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:76 or SEQ ID NO:78; and

the complement of a fragment of at least 15 contiguous nucleotides of a transcript of SEQ ID NO:76 or SEQ ID NO:78.

39. The method according to claim 38, wherein contacting the hemipteran pest with the RNA comprises spraying the plant with a composition comprising the RNA.

40. The method according to claim 38, wherein the specifically hybridizable RNA is comprised in a double-stranded RNA molecule.

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

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

cultivating the crop plant to allow the expression of the at least one polynucleotide; wherein expression of the at least one polynucleotide inhibits insect pest reproduction or growth and loss of yield due to insect pest infection,

wherein the crop plant is corn, soybean, or cotton.

42. The method according to claim 41, wherein expression of the at least one polynucleotide produces a RNA molecule that suppresses at least a first target gene in an insect pest that has contacted a portion of the crop plant.

43. The method according to claim 41, wherein the polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and the complements of any of the foregoing.

44. The method according to claim 43, wherein expression of the at least one polynucleotide produces a RNA molecule that suppresses at least a first target gene in a coleopteran insect pest that has contacted a portion of the corn plant.

45. 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.

46. The method according to claim 45, wherein the vector comprises a polynucleotide selected from the group consisting of: SEQ ID NO:1; the complement of SEQ ID NO:1; SEQ ID NO:3; the complement of SEQ ID NO:3; a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3; the complement of a fragment of at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:3; a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; the complement of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8; and the complement of a fragment of at least 15 contiguous nucleotides of a native coding sequence of a *Diabrotica* organism comprising any of SEQ ID NOs:5-8.

47. The method according to claim 45, wherein the RNA molecule is a double-stranded RNA molecule.

48. The method according to claim 47, wherein the vector comprises SEQ ID NO:103 or SEQ ID NO:104.

49. A method for producing transgenic plant protected against a coleopteran pest, the method comprising:

providing the transgenic plant cell produced by the method of claim 46; 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 pest that contacts the transformed plant.

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

transforming a plant cell with a vector comprising a means for providing coleopteran pest protection 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 coleopteran pest protection 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.

51. A method for producing a transgenic plant protected against a coleopteran pest, the method comprising:

providing the transgenic plant cell produced by the method of claim 50; 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.

52. 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 protection 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 protection 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.

53. A method for producing a transgenic plant protected against a hemipteran pest, the method comprising:

providing the transgenic plant cell produced by the method of claim 52; 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.

54. The nucleic acid of claim 1, further comprising a polynucleotide encoding a polypeptide from *Bacillus thuringiensis, Alcaligenes* spp., *Pseudomonas* spp, and/or a PIP-1 polypeptide.

55. The nucleic acid of claim 54, wherein the polynucleotide encodes a polypeptide from *B. thuringiensis* that is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry6, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and/or Cyt2C.

56. The cell of claim 16, wherein the cell comprises a polynucleotide encoding a polypeptide from *Bacillus thuringiensis, Alcaligenes* spp., *Pseudomonas* spp, and/or a PIP-1 polypeptide.

57. The cell of claim 56, wherein the polynucleotide encodes a polypeptide from *B. thuringiensis* that is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry6, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and/or Cyt2C.

58. The plant of claim 17, wherein the plant comprises a polynucleotide encoding a polypeptide from *Bacillus thuringiensis, Alcaligenes* spp., *Pseudomonas* spp, and/or a PIP-1 polypeptide.

59. The plant of claim 58, wherein the polynucleotide encodes a polypeptide from *B. thuringiensis* that is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry6, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and/or Cyt2C.

60. The method according to claim 45, wherein the transformed plant cell comprises a polynucleotide encoding a polypeptide from *Bacillus thuringiensis, Alcaligenes* spp., *Pseudomonas* spp, and/or a PIP-1 polypeptide.

61. The method according to claim 60, wherein the polynucleotide encodes a polypeptide from *B. thuringiensis* that is selected from a group comprising Cry1B, Cry1I, Cry2A, Cry3, Cry6, Cry7A, Cry8, Cry9D, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1A, and/or Cyt2C.

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

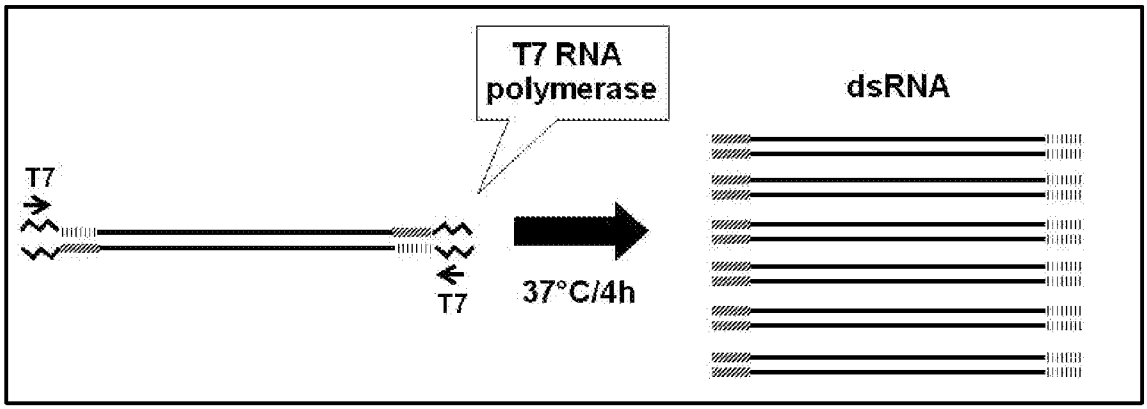
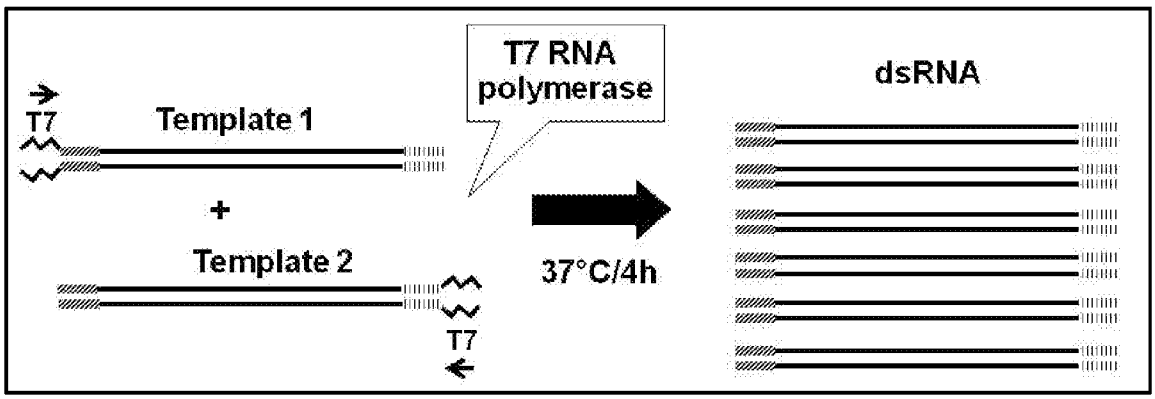


FIG. 2. Generation of dsRNA from two transcription templates.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/022304

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/82(2006.01)i, C12N 15/113(2010.01)i, C07K 14/325(2006.01)i, A01H 5/00(2006.01)i

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)
C12N 15/82; C12N 15/113; C07K 14/00; C07K 14/435; C12N 15/11; C12Q 1/68; C07K 14/325; A01H 5/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: coleopteran, diabrotica, hemipteran, nucleic acid, transform

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014-0250552 A1 (E. I. DU PONT DE NEMOURS AND COMPANY et al.) 04 September 2014 See claims 1, 3, 5; paragraphs [0006], [0016], [0057], [0075]-[0076], [0090], [0102]-[0103], [0113], [0134].	50-51
A		1-49,52-61
X	US 2013-0097735 A1 (BOWEN, DAVID J. et al.) 18 April 2013 See abstract; paragraphs [0020], [0121], [0135], [0140], [0146].	52-53
A	NCBI, NCBI Reference Sequence no. XM_002064757.1 (04 August 2008) See the whole document.	1-61
A	WO 2013-192256 A1 (SYNGENTA PARTICIPATIONS AG) 27 December 2013 See the whole document.	1-61
A	US 2014-0194306 A1 (MONSANTO TECHNOLOGY LLC) 10 July 2014 See the whole document.	1-61

☐ 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
29 June 2016 (29.06.2016)	**30 June 2016 (30.06.2016)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
Facsimile No. +82-42-481-8578

Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/022304

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

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

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

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

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

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2016/022304

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014-0250552 A1	04/09/2014	CA 2771178 A1	03/03/2011
		EP 2470662 A1	04/07/2012
		US 2011-0054007 A1	03/03/2011
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		US 2011-0154545 A1	23/06/2011
		US 7612194 B2	03/11/2009
		US 8614370 B2	24/12/2013