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(54) **Titre : SELECTION ET STABILISATION DE CONSTRUCTIONS D'ARN DOUBLE BRIN**
(54) **Title: METHOD FOR MANAGING CROP PEST RESISTANCE**

(57) **Abrégé/Abstract:**
A method for managing crop pest resistance to an agent for controlling the crop pest. The crop pest is contacted with a nucleic acid segment produced by: (a) obtaining a starting nucleic acid molecule substantially complementary to a target gene; (b) preparing a plurality of segments from the starting nucleic acid molecule; (c) assaying the nucleic acid segments for the ability to suppress expression of the target gene when expressed as a dsRNA in a cell comprising the target gene; and (d) identifying at least a first nucleic acid segment from the plurality of segments that provides a desired level of suppression of the target gene when expressed as a dsRNA, and one additional agent selected from a patatin, a Bacillus thuringiensis insecticidal protein, a Xenorhabdus insecticidal protein, a Photorhabdus insecticidal protein, a Bacillus laterosporus insecticidal protein, a Bacillus sphaericus insecticidal protein, a biocontrol agent, and an insecticide.

ABSTRACT

A method for managing crop pest resistance to an agent for controlling the crop pest. The crop pest is contacted with a nucleic acid segment produced by: (a) obtaining a starting nucleic acid molecule substantially complementary to a target gene; (b) preparing a plurality of segments from the starting nucleic acid molecule; (c) assaying the nucleic acid segments for the ability to suppress expression of the target gene when expressed as a dsRNA in a cell comprising the target gene; and (d) identifying at least a first nucleic acid segment from the plurality of segments that provides a desired level of suppression of the target gene when expressed as a dsRNA, and one additional agent selected from a patatin, a *Bacillus thuringiensis* insecticidal protein, a *Xenorhabdus* insecticidal protein, a *Photorhabdus* insecticidal protein, a *Bacillus laterosporus* insecticidal protein, a *Bacillus sphaericus* insecticidal protein, a biocontrol agent, and an insecticide.

DESCRIPTION

METHOD FOR MANAGING CROP PEST RESISTANCE

BACKGROUND OF THE INVENTION

This is a division of Canadian Patent Application No. 2,637,363 filed February 12, 2007.

5 **1. Field of the Invention**

The present invention relates to stable expression of RNAi constructs in plants to enable genetic control of plant pathogens and pests. The invention provides methods and compositions for improving the efficacy of dsRNAs derived from such constructs.

2. Description of Related Art

10 Short strands of complementary double stranded RNA (dsRNA) when present in, or introduced into, living cells may specifically affect the expression of a "target" gene when regions of nucleotide sequence similarity are shared between the dsRNA and the target gene transcript. Such RNA molecules may comprise complementary sequences separated by a "spacer" region such that double stranded regions of RNA are formed. The dsRNA may be cleaved by enzymes known as dimeric RNase III ribonucleases (also called "dicer" enzymes) into segments
15 approximately 21-25 base pairs in length; called siRNAs ("short interfering RNAs" or "small interfering RNAs"). The siRNA causes specific RNAse activity in a RNA-induced silencing complex ("RISC") to hydrolyze the target gene mRNA, thereby post-transcriptionally suppressing expression of the target gene. Only transcripts complementary to the siRNA are, cleaved and degraded, and thus the effect, sometimes called RNA interference (RNAi), is gene specific. RNAi has been used to specifically disrupt gene expression in a number of organisms including *Caenorhabditis*
20 *elegans* (Fire *et al.*, 1998), *Drosophila melanogaster*, insects including *Coleoptera* (Bucher *et al.*, 2002) and *Lepidoptera* (Uhlirova *et al.*, 2003; Bettencourt *et al.*, 2002), fungi (Cogoni *et al.*, 2000), and plants such as *Arabidopsis thaliana*, among others. dsRNA present in plants may also guide DNA methylation of targeted chromatin regions, resulting in gene silencing (*e.g.* Wassenegger *et al.*, 1994; Carthew, 2001; Zilberman *et al.*, 2004).

Effective use of RNAi leads to suppression of expression of a specific target gene, and thus stable expression
25 of RNAi constructs in transgenic crops can allow for novel genetic approaches to pest control. However dsRNA produced from a transgene *in planta*, although targeted to another organism, may evoke *in planta* responses such as cleavage ("dicing") of a transgene transcript, as well as silencing of the cognate transgene in the transgenic host plant. These responses could reduce or eliminate dsRNA production and hence efficacy against a target organism.

There have been reports concerning design of constructs for evoking dsRNA-mediated suppression of gene expression (Wesley *et al.*, 2001; Yuan *et al.*, 2004; Reynolds *et al.*, 2004; Arziman *et al.*, 2005). Mechanisms for systemic transport of sRNA ("small RNA") molecules (including dsRNA) are known in some organisms (*e.g.* Voinnet 2005), and the sequence of the
5 ribonucleotide being transported is known to have an effect on the efficiency of its uptake (Winston *et al.*, 2002). For instance, *C. elegans* requires a dsRNA of roughly 100 base pairs (bp) in length to be productively taken up into gut cells *e.g.* via SID1 protein (Feinberg and Hunter, 2003), and WO9953050 describes dsRNA constructs comprising intron sequences in spacer regions. However the parameters leading to optimized production, stabilization, and uptake of
10 dsRNA active against a target pest, while ensuring stable expression of a transgene encoding such dsRNA, and avoiding transgene silencing in a host cell, are not well understood. Thus there exists a need to ensure stable transcription of specific effective dsRNA-encoding transgenes within plants, and subsequent transport and uptake of the resulting dsRNA, to yield effective and specific gene suppression in target plant pathogen and pest species.

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BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

20 **FIG. 1A-1B**: Alignment of a 100 bp segment of the Dv49 target with related sequences from other organisms representing multiple genera, orders and phyla. Sequences differing from *Diabrotica virgifera virgifera* (Dv49) are highlighted. Amino acid alignment (a.a.) for the Dv49 conceptual translation is shown below the nucleotide sequence. Reynolds scores were calculated for the Dv49 sequence and are shown below the amino acid alignment – the score position
25 corresponds to nucleotide 19 of the antisense strand 21mer. Data from the embedded 26mer efficacy scan are presented below the Reynolds score. The potential 21mers that could be produced from each scan segment are underlined and the WCR mortality resulting from each embedded segment fed at 0.2 ppm in artificial diet bio-assay is shown below each scan segment. * significantly different from untreated control, P value <0.05, Planned Contrasts.

30 **FIG. 2**: Segments of coding sequence from a Na/K-exchanging ATPase (putative Drosophila gene, CG9261, ortholog) aligned from multiple *Diabrotica* spp. Sequence conforming to the group consensus is boxed and shaded. Sequencing has shown presence of alleles in some instances (e.g. "R" at position 49 of NCR sequence).

FIG. 3: Phylogenetic tree determined using a 559 bp segment of Dv26 and the ClustalW algorithm in the DNASTAR software package (Madison, WI).

FIG. 4: Design for transgene that reduces direct contiguous sequence identity between transcript of gene and resulting dsRNA transcript. Transcription unit could be terminated by a synthetic sequence derived from siRNAs that are not productively incorporated into RISC.

FIG. 5: Small efficacious dsRNA segments for insertion into expression cassette at indicated sites.

FIG. 6: 300 bp segments of *Diabrotica virgifera* V-ATPase subunit A for assay as dsRNA in WCR diet bio-assay. UTC = untreated control. EST = a short V-ATPase subunit A cDNA clone that lacked sections 1 and 2.

FIG. 7: Dv49 embedded approx. 26mer efficacy scan fed at 1 ppm.

FIG. 8: Dv49 embedded approx. 26mer efficacy scan fed at 0.2 ppm.

FIG. 9: Dv49 scan 14 27mer segment scanned as 21mers and tested for efficacy at 0.2 ppm.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of obtaining a nucleic acid segment providing a desired level of suppression of a target gene, comprising: a) obtaining a starting nucleic acid molecule substantially complementary to a target gene; b) preparing a plurality of nucleic acid segments from the starting nucleic acid molecule; c) assaying the nucleic acid segments for the ability to suppress expression of the target gene when expressed as a dsRNA in a cell comprising the target gene; and d) identifying at least a first nucleic acid segment from the plurality of nucleic acid segments that provides a desired level of suppression of the target gene when expressed as a dsRNA. In the method, the nucleic acid segments may comprise from about 21 to about 26 contiguous nucleotide portions of said starting nucleic acid molecule, including about 22, 23, 24, and 25 nucleotide portions. In certain embodiments, the segments comprise overlapping portions of said starting nucleic acid molecule and in specific embodiments may be adjoining segments. In further embodiments, the nucleic acid segments may be defined as comprising from about 0.1% to about 98% of said target gene, for example, including about 0.2%, .4%, .75%, 2%, 5%, 10%, 15%, 25%, 40%, 60%, 75% and 90%.

In one embodiment of the invention, nucleic acid segments may be ranked according to the level of suppression of the target gene obtained when the nucleic acid segments are expressed as dsRNA. The desired level of suppression of the target gene may be from about 1% to about 100% suppression of the expression of said target gene. In certain embodiments, the desired level of suppression may be complete suppression or incomplete suppression of the target gene. In

specific embodiments, the target gene may be a plant, insect, fungal, bacterial or vertebrate organism, including a crop pest or pathogen gene. Assaying the nucleic acid segments for the ability to suppress the target gene may comprise expressing the segments as a dsRNA in a cell comprising the target gene and determining the level of suppression of the target gene. In one
5 embodiment, this may comprise calculating a Reynolds score for the nucleic acid segments. In another embodiment, assaying the nucleic acid segments for the ability to suppress the target gene comprises providing said segments as dsRNA molecules in the diet of an organism comprising the target gene and determining the level of suppression of the target gene. Determining the level of suppression of the target gene may comprise observing morbidity, mortality, or stunting of said
10 organism.

In another aspect, the invention provides a method of suppressing the expression of a target gene in a cell comprising a) obtaining a nucleic acid segment according to a method provided herein; and b) providing a dsRNA expressed from the nucleic acid to a host cell comprising the target gene to suppress the expression of the target gene. In the method, providing
15 the dsRNA expressed from the nucleic acid segment to the host cell may comprise expressing the nucleic acid segment in the host cell in sense and antisense orientation. Providing the dsRNA expressed from the nucleic acid segment to the host cell may comprise providing a diet comprising the dsRNA to the cell or an organism comprising the cell and allowing the cell to take up the dsRNA. In one embodiment, the host cell is a pest cell and providing the dsRNA
20 expressed from the nucleic acid to the pest cell comprises expressing the dsRNA in a plant cell and allowing a pest comprising the cell to feed on the plant cell. In specific embodiments, suppressing the expression of the target gene in the pest cell is manifested by a phenotypic effect on said cell or the pest comprising the cell. The phenotypic effect may be programmed cell death.

In yet another aspect, the invention provides a method for modulating the expression of at
25 least a first gene in an organism comprising (a) providing as a dsRNA at least a first nucleic acid segment obtained by a method of the invention to said organism, wherein said dsRNA segment is specific for said gene in said organism; and (b) observing a phenotypic effect in said organism. In the method, the phenotypic effect may be selected from the group consisting of cessation of vegetative growth, cessation of reproductive growth, cessation of feeding, mortality, morbidity,
30 stunting, paralysis, inhibition of sexual reproduction, molt inhibition, flightless, and failure to emerge from pupal stage.

In yet another aspect, the invention provides a method for modulating the level of expression of a gene in a plant pest comprising providing in the diet of said pest at least a first dsRNA molecule, and observing a phenotypic effect of suppression of one or more genes in said
35 pest, wherein said dsRNA molecule is produced from a nucleotide sequence that exhibits substantial homology with a corresponding DNA sequence of one or more essential genes in said

pest, and wherein said nucleotide sequence is a nucleic acid segment identified according to a method provided herein.

In still yet another aspect, the invention provides a method for inhibiting plant pest infestation comprising expressing a dsRNA molecules obtained according to a method of the invention in a transgenic plant and providing the plant or a part or tissue thereof to one or more pests comprising said nucleotide sequence, and observing a phenotypic effect in said organism, wherein the phenotypic effect is sufficient to inhibit infestation of said transgenic plant by said pest.

In still yet another aspect, the invention provides a method for protecting a plant from pest infestation comprising expressing a dsRNA molecules obtained according to the invention in a transgenic plant, providing said plant or a part or tissue thereof to one or more pests comprising said nucleotide sequence, and observing a phenotypic effect in the organism, wherein the phenotypic effect is sufficient to inhibit infestation of the transgenic plant by the pest. The invention also provides a plant protected from pest infestation according to any of the methods described herein, as well as a plant regenerated from such a cell, and also a seed or progeny produced from such a plant, wherein said seed or progeny comprises a nucleotide sequence obtained according to the invention.

In still yet another aspect, the invention provides a method of producing an expression construct for expressing a dsRNA with reduced transgene silencing in a plant cell, comprising: (a) preparing an expression construct comprising a first sequence, a second sequence, and a third polynucleotide sequence, wherein the third polynucleotide sequence is linked to the first polynucleotide sequence by the second polynucleotide sequence and the third polynucleotide sequence is substantially the reverse complement of the first polynucleotide sequence; and (b) introducing an intron into at least one of the first and third polynucleotide sequences or introducing said expression construct into the intron, wherein the first and third polynucleotide sequences hybridize when transcribed into RNA and form a dsRNA molecule stabilized by the second polynucleotide sequence after intron splicing, and wherein the expression construct exhibits reduced transgene silencing in a plant cell transformed with the expression construct relative to an expression construct that lacks the intron. In one embodiment, the intron is introduced into at least one of the first and third polynucleotide sequences. In another embodiment, the intron is introduced into the first and third polynucleotide sequences. In further embodiments, the expression construct is introduced into the intron.

In still yet another aspect, the invention provides a method of controlling feeding by a target crop pest or pathogen or progeny thereof on a plant comprising introducing into the plant an expression construct prepared by any of the methods disclosed herein. The construct may be

introduced, for example, by direct genetic transformation or by transformation of a parent plant and/or progenitor cell. The invention further provides an expression construct prepared according to any of the methods disclosed herein. Still further provided are transgenic plants and plant cell transformed with an expression construct disclosed herein.

5 In still yet another aspect, the invention provides a method of increasing the pest or pathogen-inhibitory activity of a dsRNA, comprising: (a) obtaining a first nucleic acid segment that when expressed as a dsRNA and taken up by a target crop pest or pathogen inhibits feeding by the target crop pest or pathogen or progeny thereof; and (b) linking the first nucleic acid segment to a second nucleic acid segment to create a longer nucleic acid segment, wherein the 10 second nucleic acid segment is a nucleic acid that does not inhibit feeding by the target crop pest or pathogen or progeny thereof when expressed as a dsRNA, and wherein a dsRNA expressed from the longer nucleic acid exhibits increased potency of inhibition of feeding by the target crop pest or pathogen or progeny thereof relative to the dsRNA expressed from the first nucleic acid segment alone. In one embodiment, the first nucleic acid segment is obtained by a method 15 comprising the steps of: I) obtaining a starting nucleic acid molecule that when expressed as a dsRNA and taken up by a target crop pest or pathogen inhibits feeding by the target crop pest or pathogen or progeny thereof; II) selecting at least a first portion of the starting nucleic acid molecule that inhibits feeding by a target crop pest or pathogen or a progeny thereof following uptake of a dsRNA expressed from said portion; and III) employing the portion as said the first 20 nucleic acid segment in step a). The starting nucleic acid molecule may be a cDNA. In one embodiment, step II) comprises preparing a series of overlapping or consecutive portions from the starting nucleic acid molecule and identifying from said portions at least a first portion that inhibits feeding by a target crop pest or pathogen or a progeny thereof when expressed as a dsRNA and taken up by the target crop pest or pathogen.

25 The method of increasing the pest or pathogen-inhibitory activity of a dsRNA may further comprise in particular embodiments producing a recombinant vector comprising a first, a second and a third polynucleotide sequence, wherein the first polynucleotide sequence comprises the longer nucleotide segment and wherein the third polynucleotide sequence is linked to the first polynucleotide sequence by the second polynucleotide sequence, and wherein the third 30 polynucleotide sequence is substantially the reverse complement of the first polynucleotide sequence such that the first and the third polynucleotide sequences hybridize when transcribed into a ribonucleic acid to form the double stranded ribonucleotide molecule stabilized by the linked second ribonucleotide sequence. In specific embodiments the second nucleotide segment is not substantially complementary to a nucleotide sequence of the target crop pest or pathogen. 35 In further embodiments, one or both of the first nucleic acid segment and the third nucleic acid segment comprises an intron. The method may also comprise introducing an intron into said first

nucleic acid segment. In further embodiments, the first nucleic acid segment may comprise about 19 to about 80, about 19 to about 50 and about 21 to about 30 contiguous bases substantially complementary to a coding sequence of the target crop pest or pathogen. The longer nucleic acid segment may comprise at least about 80 bases, including at least about 100 bases and from about
5 80 bp to about 250 bases. In one embodiment, the target crop pest or pathogen is an insect and may be a Coleopteran, Lepidopteran, Homopteran, or Hemipteran, *e.g.* a *Diabrotica spp*. In other embodiments the target crop pest or pathogen is a nematode.

In another aspect, the invention further provides a method for producing an expression construct for expressing a dsRNA with increased specificity of pest or pathogen-inhibitory
10 activity comprising: (a) obtaining a starting nucleic acid molecule substantially complementary to at least a first coding sequence of a target crop pest or pathogen; (b) selecting a region within the starting molecule that when expressed as a dsRNA inhibits feeding by the target crop pest or pathogen or progeny thereof following uptake of the dsRNA expressed from the region by the target crop pest or pathogen; (c) linking the region to a second nucleic acid molecule to produce
15 an expression construct, wherein the second nucleic acid molecule when expressed as a dsRNA does not inhibit feeding by a target crop pest or pathogen or progeny thereof following uptake of the dsRNA. The starting nucleic acid molecule utilized by the method may be a cDNA from the target crop pest or pathogen, such as an insect or nematode. In particular embodiments, the insect may be a Coleopteran, Lepidopteran, Homopteran, or Hemipteran insect, including an insect
20 selected from the group consisting of: *D. virgifera virgifera; D. virgifera zeae; D. undecimpunctata; D. balteata; D. barberi;* and *D. speciosa*. In further embodiments, the first nucleic acid segment may comprise about 19 to about 80, about 19 to about 50 and about 21 to about 30 contiguous bases substantially complementary to a coding sequence of the target crop pest or pathogen. The longer nucleic acid segment may comprise at least about 80 bases,
25 including at least about 100 bases and from about 80 bp to about 250 bases.

A further aspect of the invention provides a method comprising identifying at least a second region within the starting molecule that when expressed as a dsRNA inhibits feeding by the target crop pest or pathogen or progeny thereof, and linking the second region to the second nucleic acid molecule or a third nucleic acid molecule that when expressed as a dsRNA does not
30 inhibit feeding by a target crop pest or pathogen or progeny thereof following uptake of the dsRNA expressed from the third nucleic acid molecule by the target plant pest or pathogen. In some embodiments, the region is not substantially complementary to a nucleic acid of a non-target crop pest or pathogen. In other embodiments, the region is complementary to a nucleic acid unique to the species in which the target crop pest or pathogen is classified. In yet other
35 embodiments, the region is complementary to a nucleic acid unique to the genus in which the target crop pest or pathogen is classified. In still further embodiments, the region is unique to

Diabrotica spp., including those selected from the group consisting of *Diabrotica undecimpunctata howardii* (Southern Corn Rootworm (SCR)), *Diabrotica virgifera virgifera* (Western Corn Rootworm (WCR)), *Diabrotica barberi* (Northern Corn Rootworm (NCR)), *Diabrotica virgifera zeae* (Mexican Corn Rootworm (MCR)), *Diabrotica balteata*, *Diabrotica*
5 *viridula*, and *Diabrotica speciosa* (Brazilian Corn Rootworm (BZR)).

In another aspect, the invention provides a method of controlling feeding by a target crop plant pest or pathogen or progeny thereof on a plant comprising introducing into the plant an expression construct or dsRNA prepared by the foregoing method. The invention also provides a plant cell transformed with an expression construct prepared by the foregoing method.

10 In yet another aspect, the invention provides a method of enhancing the control of a target crop pest or pathogen in a plant comprising expressing in the cells of the plant at least two dsRNA sequences that function upon uptake by the pest or pathogen to inhibit the expression of at least a first target coding sequence within the target crop pest or pathogen, wherein the two dsRNA sequences are substantially complementary to two non-contiguous portions of the first target
15 coding sequence or to two different coding sequences of the target crop pest or pathogen. In further embodiments, the invention provides a method wherein the two dsRNA sequences comprises about 19 bp to about 80 bp, or about 19 bp to about 50 bp, or about 21 bp to about 30 bp in length. In another embodiment, the two dsRNA sequences are substantially complementary to at least two target coding sequences of the target crop pest or pathogen. The method may
20 further comprise expressing in the cells of the plant at least a third dsRNA sequence that functions upon uptake by the pest or pathogen to inhibit the expression of a third target coding sequence within the target crop pest or pathogen, wherein the third dsRNA sequence is substantially complementary to a portion of the third target coding sequence. In yet another embodiment, a method is provided wherein the two dsRNA sequences are expressed from regions selected from a
25 starting nucleic acid molecule that when expressed as a dsRNA inhibits feeding by a target crop pest or pathogen or progeny thereof following uptake of the dsRNA by the target crop pest or pathogen. The starting nucleic acid molecule may further be a cDNA from the target crop pest or pathogen.

In another embodiment, the provided method further comprises expressing a
30 polynucleotide sequence in the cell selected from the group consisting of a patatin, a *Bacillus thuringiensis* insecticidal protein, a *Xenorhabdus* insecticidal protein, a *Photorhabdus* insecticidal protein, a *Bacillus laterosporus* insecticidal protein, and a *Bacillus sphaericus* insecticidal protein. In further embodiments, exemplary polynucleotides may encode a *Bacillus thuringiensis* insecticidal protein selected from the group consisting of a Cry1, a Cry2, a Cry3, or a coleopteran
35 toxic protein selected from the group consisting of a TIC851, a CryET70, ET29, a binary insecticidal protein CryET33 and CryET34, a binary insecticidal protein CryET80 and CryET76,

a binary insecticidal protein ET29 and TIC810, a binary insecticidal protein TIC100 and TIC101, and a binary insecticidal protein PS149B1, or other coleopteran toxic protein (*e.g.* deMaagd *et al.,* 2003). Other insecticidal compositions directed to controlling additional plant pests are possible. Thus, in certain embodiments, combinations of control agent(s) include one or more polynucleotides of the present invention that express a dsRNA and at least one other agent toxic to a plant pest such as an insect or a nematode.

The invention further provides a method wherein the target coding sequence encodes a protein, the predicted function of which is selected from the group consisting of muscle formation, juvenile hormone formation, juvenile hormone regulation, ion regulation and transport, digestive enzyme synthesis, maintenance of cell membrane potential, feeding site formation, feeding site development, feeding site maintenance, infection, molting, amino acid biosynthesis, amino acid degradation, sperm formation, pheromone synthesis, pheromone sensing, antennae formation, wing formation, leg formation, development and differentiation, egg formation, larval maturation, digestive enzyme formation, haemolymph synthesis, haemolymph maintenance, neurotransmission, cell division, energy metabolism, respiration, and apoptosis. In another embodiment, the invention provides a method wherein two coding sequences are targeted. The two target coding sequences may perform at least two functions essential for target crop pest or pathogen survival that are suppressed by the dsRNA sequences, the functions being selected from the group consisting of feeding by the pest or pathogen, cell apoptosis, cell differentiation and development, capacity or desire for sexual reproduction, muscle formation, muscle twitching, muscle contraction, juvenile hormone formation, juvenile hormone regulation, ion regulation and transport, maintenance of cell membrane potential, amino acid biosynthesis, amino acid degradation, sperm formation, pheromone synthesis, pheromone sensing, antennae formation, wing formation, leg formation, egg formation, larval maturation, digestive enzyme formation, haemolymph synthesis, haemolymph maintenance, neurotransmission, larval stage transition, pupation, emergence from pupation, cell division, energy metabolism, respiration, and formation of cytoskeletal structure.

The invention further provides a method of resistance management, comprising contacting a target organism with at least a first nucleic acid segment of the present invention, and one or more agent(s) selected from the group consisting of: a patatin, a *Bacillus thuringiensis* insecticidal protein, a *Xenorhabdus* insecticidal protein, a *Photorhabdus* insecticidal protein, a *Bacillus laterosporus* insecticidal protein, a *Bacillus sphaericus* insecticidal protein, or other insecticidal Bt toxin, a biocontrol agent, and an insecticide.

According to one embodiment, there is provided a method for managing crop pest resistance, comprising: (a) preparing a nucleic acid by: (a1) obtaining a starting nucleic acid molecule substantially complementary to a target pest gene wherein the target pest gene is a Western Corn Rootworm gene; (a2) preparing a plurality of nucleic acid segments from the starting nucleic acid molecule; (a3) assaying the nucleic acid segments for the ability to suppress expression of the target pest gene when expressed as a first dsRNA in a cell comprising the target pest gene; (a4) identifying at least a first nucleic acid segment from the plurality of nucleic acid segments that suppresses the target pest gene when expressed as a dsRNA wherein the first nucleic acid segment comprises 19-50 contiguous nucleotides that are substantially complementary to the target pest gene; and (a5) linking the first nucleic acid segment to a second nucleic acid segment to create a third nucleic acid segment that has 80 to 250 bases and is expressed as a third dsRNA, wherein the second nucleic acid segment is a neutral carrier sequence and does not inhibit feeding by the crop pest or progeny thereof when expressed as a dsRNA, and wherein the third dsRNA exhibits increased potency of inhibition of feeding by the crop pest or progeny thereof relative to the first dsRNA alone, (b) contacting the crop pest with the nucleic acid and an additional agent selected from the group consisting of a patatin, a *Bacillus thuringiensis* insecticidal protein, a *Xenorhabdus* insecticidal protein, a *Photorhabdus* insecticidal protein, a *Bacillus laterosporus* insecticidal protein, a *Bacillus sphaericus* insecticidal protein, a biocontrol agent, and an insecticide; wherein the crop pest is Western Corn Rootworm; and wherein the target pest gene comprises SEQ ID NO: 1 or SEQ ID NO: 2.

DETAILED DESCRIPTION OF THE INVENTION

Described herein are methods and compositions for improving efficacy and expression of dsRNA molecules that modulate gene expression in plant pests and pathogens. The methods enhance the specificity of small interfering RNA (siRNA) or related segments produced from 5 plant transgenes that encode dsRNA, and that provide dsRNA-mediated suppression of target gene expression in plant pests and plant pathogens. The transgene construct and target sequence size is optimized for production and delivery of one or more ribonucleotides effective in the cells of specific target species, while avoiding production of non-specific siRNAs that might otherwise modulate gene expression in an unintended manner. At the same time, by optimizing 10 arrangement of target sequences, the invention reduces the potential for silencing of the transgene in the plant by disrupting continuous target sequence with introns, thereby preventing feedback that would recognize the gene and lead to silencing in the plant.

Sequences that specifically target pest or pathogen species may be engineered into plant expression constructs, such as those with inverted repeats or by use of other methods for 15 eliciting the formation of dsRNA. By cloning siRNAs or by empirical determination via presentation of dsRNA segments to cells or whole pests that scan across a target sequence, 21-24mers that effectively lead to target message degradation can be determined. Using this information novel sequence structure for expression *in planta* can be created. This sequence structure can be further designed to yield dsRNA molecules, encoding one or more siRNA 20 molecules that are effectively taken up by the target species, while at the same time resulting in formation of siRNAs specific for modulating expression of a specific ortholog, homolog, or allele of a target gene in a target species. Expression of a specific member of a gene family may be suppressed by designing a dsRNA construct that targets that member based on sequence polymorphism between the members of a gene family. Thus, specific target sequences (*e.g.* 25 siRNA-sized, approximately 20-25 base pairs in length) may be included in a dsRNA construct based upon their empirically determined or predicted efficacy toward specific target species, populations, or sub-populations, and less specific or non-specific sequences may be excluded, while still achieving transport of effective transgene-encoded dsRNA into a cell of a target organism. The efficacy of specific siRNA-sized ribonucleotide sequences can be determined by 30 practical evaluations in bio-assays or through the use of predictive tools (*e.g.* Reynolds scores; Reynolds *et al.*, 2004) that consider biophysical parameters that are common to effective or ineffective siRNAs.

Understanding specific requirements needed to target pest species with exogenous (*e.g.* transgenic plant-produced) RNA enhances the ability to produce highly effective and specific 35 transgenic constructs. In western corn rootworm (WCR), it was determined that a 50 bp segment of the WCR V-ATPase subunit A is sufficient to elicit mortality when tandemly duplicated 5

times (250 bp total), but is ineffective as a 50 bp monomer. The 50 bp segment embedded in a neutral carrier sequence to yield a total dsRNA of 100 bp was also effective. Thus there is a size optimum for efficient uptake into organisms susceptible to RNAi. This observation indicates the need to "stabilize" the production of appropriately sized dsRNA for pest control.

5 Using the carrier concept, one or more siRNA sequence can be embedded for transcription within longer sequences. Such sequences may be used to demonstrate the effectiveness of any candidate siRNA, independent of adjacent naturally occurring sequences, allowing for enhanced flexibility in designing transgene constructs that encode dsRNA. Naturally occurring adjacent sequences that demonstrate less efficacy or specificity may be left out of a 10 dsRNA construct, while the construct nevertheless encodes the necessary sequence, and sequence length, to yield efficacious siRNA upon expression within a plant host cell and uptake and processing in a cell of a target organism. This knowledge enables the creation of novel chimeric sequences that incorporate chosen sequences encoding siRNAs into highly effective primary suppression transcripts.

15 A transgene designed by the present methods may also have dsRNA segment(s) encoding siRNA sequences interrupted through intron placement. Inclusion of one or more intron sequences in the target sequence may enhance production and stability of a primary transcript that ultimately yields an effective siRNA, while displaying a reduced propensity to be silenced in the plant cell. Additional sequence such as 5' and 3' untranslated regions (UTRs) and other 20 sequence, for instance to make exons of at least a minimal required size for plant processing, may be produced by combining sequences (e.g. direct tandem sense sequence) that do not elicit effective siRNAs. Additional exon sequences may be created from sequence that does not give rise to productive siRNAs. This arrangement may result in a reduced potential to silence the transgene (*e.g.* via methylation and eventual transcriptional silencing in a plant host cell) because 25 the gene is distinct in sequence from the processed transcript that generates siRNAs, which might otherwise cause transgene silencing via changes in chromatin structure. The presence of introns in the siRNA regions of the primary transcript may also slow overall processing and improve the longevity or stability of the dsRNA that results (FIG. 4).

Additional target sequences may be added by extending the primary transcriptional unit 30 with more introns and exons designed as above. Overlapping potent siRNAs and placing the intron within the overlap could expand the number of target sequences while minimizing the number of required introns within the construct (FIG. 5). One or more distinct sequences, each encoding siRNAs targeting expression of one or more target genes and that modulate gene expression in a target organism, may be deployed.

35 Suppression of expression of two or more target genes allows for provision of multiple modes of action via dsRNA-mediated gene suppression against a target organism. Multiple modes of action may also be achieved in transgenic plants by combining one or more dsRNA-

mediated approaches with other means, such as *Bacillus*-derived insecticidal peptides (*e.g.* crystal proteins), to interfere with the growth and development of target organisms. Combining several or multiple sequences encoding potent siRNAs, possibly in conjunction with other means, also allows development of durable pest resistance management schemes.

5 **A. Nucleic Acid Compositions and Constructs**

The invention provides recombinant DNA constructs for use in achieving stable transformation of a host plant cell. Transformed host cells may express effective levels of preferred dsRNA molecules and hence siRNA from the recombinant DNA constructs, to modulate gene expression in target cells. Isolated and purified nucleotide segments may be 10 provided from cDNA and/or genomic libraries. Deduced nucleotide sequence information allows identification of pairs of nucleotide sequences which may be derived from any preferred invertebrate pest, such as an insect, for use as thermal amplification primers to generate the dsRNA and siRNA molecules of the present invention.

As used herein, the term "nucleic acid" refers to a single or double-stranded polymer of 15 deoxyribonucleotide or ribonucleotide bases. The "nucleic acid" may also optionally contain non-naturally occurring or altered nucleotide bases that permit correct read through by a polymerase and do not reduce expression of a polypeptide encoded by that nucleic acid. 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 20 inclusive of RNAi (inhibitory RNA), dsRNA (double stranded RNA), siRNA (small interfering RNA), mRNA (messenger RNA), miRNA (micro-RNA), sRNA (small RNA), tRNA (transfer RNA, whether charged or discharged with a corresponding acylated amino acid), and cRNA (complementary RNA) and the term "deoxyribonucleic acid" (DNA) is inclusive of cDNA and genomic DNA and DNA-RNA hybrids. The words "nucleic acid segment", "nucleotide sequence 25 segment", or more generally "segment" will be understood by those in the art as a functional term that includes both genomic sequences, ribosomal RNA sequences, transfer RNA sequences, messenger RNA sequences, operon sequences and smaller engineered nucleotide sequences that express, or may be adapted to express, polynucleotides, proteins, polypeptides or peptides.

Provided according to the invention are nucleotide sequences, the expression of which 30 results in an RNA sequence which is substantially homologous to an RNA molecule of a targeted gene in a target organism, such as a plant pest or pathogen. Thus, after taking up the stabilized RNA sequence, down-regulation of the expression of the nucleotide sequence of the target gene in the cells of the target organism may be obtained, resulting in a deleterious effect on the maintenance, feeding, viability, proliferation, or reproduction of the target organism.

35 As used herein, the term "substantially homologous" or "substantial homology", with reference to a nucleic acid sequence, includes a nucleotide sequence that hybridizes under

stringent conditions to a coding sequence as set forth in the sequence listing, or the complements thereof. Sequences that hybridize under stringent conditions are those that allow an antiparallel alignment to take place between the two sequences, and the two sequences are then able, under stringent conditions, to form hydrogen bonds with corresponding bases on the opposite strand to
5 form a duplex molecule that is sufficiently stable under the stringent conditions to be detectable using methods well known in the art. Substantially homologous sequences have preferably from about 70% to about 80% sequence identity, or more preferably from about 80% to about 85% sequence identity, or most preferable from about 90% to about 95% sequence identity, to about 99% sequence identity, to a nucleotide sequence as set forth in the sequence listing, or the
10 complements thereof.

As used herein, the term "sequence identity", "sequence similarity" or "homology" is used to describe sequence relationships between two or more nucleotide sequences. The percentage of "sequence identity" between two sequences is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in
15 the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in
20 the window of comparison, 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 identical to the reference sequence and vice-versa. A first nucleotide sequence when observed in the 5' to 3' direction is said to be a "complement" of, or complementary to, a second or reference nucleotide sequence observed in the 3' to 5' direction if the first nucleotide sequence
25 exhibits complete complementarity with the second or reference sequence. As used herein, nucleic acid sequence molecules are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the
30 reference nucleotide sequence. These terms and descriptions are well defined in the art and are easily understood by those of ordinary skill in the art.

As used herein, a "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150, in which a sequence is compared to a reference sequence of the same number of contiguous
35 positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (*i.e.* gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences

Those skilled in the art should refer, for example, to the detailed methods used for sequence alignment in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, Wis., USA).

The present invention provides DNA sequences capable of being expressed as an RNA in
5 a cell or microorganism to inhibit target gene expression in a cell, tissue or organ of a target organism. The sequences may comprise a DNA molecule coding for one or more different nucleotide sequences, wherein each of the different nucleotide sequences comprises a sense nucleotide sequence and an antisense nucleotide sequence. The sequences may be connected by a spacer sequence. The spacer sequence can constitute part of the sense nucleotide sequence or the
10 antisense nucleotide sequence and is found within the dsRNA molecule between the sense and antisense sequences. The sense nucleotide sequence or the antisense nucleotide sequence is substantially identical to the nucleotide sequence of the target gene or a derivative thereof or a complementary sequence thereto. The dsDNA molecule may be placed operably under the control of a promoter sequence that functions in the cell, tissue or organ of the host expressing the
15 dsDNA to produce dsRNA molecules. As used herein, the term "plant expression construct" refers to a recombinant DNA molecule comprising a promoter functional in a plant cell operably linked to a DNA sequence that encodes dsRNA, and a 3' transcription termination polynucleotide molecule.

The invention also provides a DNA sequence for expression in a cell of a plant that, upon
20 expression of the DNA to RNA and being taken up by a target organism, such as a plant pathogen or plant pest, achieves suppression of a target gene in a cell, tissue or organ of a target organism. The dsRNA may comprise one or multiple structural gene sequences, wherein each of the structural gene sequences comprises a sense nucleotide sequence and an antisense nucleotide sequence that may be connected by a spacer sequence that forms a loop within the complementary
25 sense and antisense sequences. An intron sequence with appropriate splice sites may be placed in at least one of the sense and antisense nucleotide sequences. The sense nucleotide sequence or the antisense nucleotide sequence, apart from any intron present, is substantially identical to the nucleotide sequence of the target gene, derivative thereof, or sequence complementary thereto. The one or more structural gene sequences may be placed operably under the control of one or
30 more promoter sequences, at least one of which is operable in the cell, tissue or organ of a host organism for expression of the transcript.

A gene sequence or fragment for control of gene expression in a target organism according to the invention may be cloned between two tissue specific promoters, which are operable in a transgenic plant cell, and therein expressed to produce mRNA in the transgenic plant
35 cell that form dsRNA molecules thereto. The dsRNA molecules contained in plant tissues may be taken up by a target organism so that the intended suppression of the target gene expression is achieved.

A nucleotide sequence provided by the present invention may comprise an inverted repeat separated by a "spacer sequence." The spacer sequence may be a region comprising any sequence of nucleotides that facilitates secondary structure formation between each repeat, where this is required. In one embodiment of the present invention, the spacer sequence is part of the sense or antisense coding sequence for mRNA. The spacer sequence may alternatively comprise any combination of nucleotides or homologues thereof that are capable of being linked covalently to a nucleic acid molecule. The spacer sequence may comprise, for example, a sequence of nucleotides of at least about 10-100 nucleotides in length, or alternatively at least about 100-200 nucleotides in length, at least 200-400 about nucleotides in length, or at least about 400-500 nucleotides in length.

The nucleic acid molecules or fragments of the nucleic acid molecules or other nucleic acid molecules in the sequence listing are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the complement of another nucleic acid molecule if they exhibit complete complementarity. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be complementary if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook, *et al.,* (1989), and by Haymes *et al.,* (1985).

Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. Thus, in order for a nucleic acid molecule or a fragment of the nucleic acid molecule to serve as a primer or probe it needs only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

Appropriate stringency conditions which promote DNA hybridization are, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology (1989). For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. Preferably, a nucleic acid for use in the present invention will

exhibit at least from about 80%, or at least from about 90%, or at least from about 95%, or at least from about 98% or even about 100% sequence identity with one or more nucleic acid molecules as set forth in the sequence listing.

Nucleic acids of the present invention may also be synthesized, either completely or in
5 part, especially where it is desirable to provide plant-preferred sequences, by methods known in the art. Thus, all or a portion of the nucleic acids of the present invention may be synthesized using codons preferred by a selected host. Species-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular host species. Other modifications of the nucleotide sequences may result in mutants having slightly
10 altered activity.

dsRNA or siRNA nucleotide sequences comprise double strands of polymerized ribonucleotide and may include modifications to the phosphate-sugar backbone or the nucleoside. Modifications in RNA structure may be tailored to allow specific genetic inhibition. In one embodiment, the dsRNA molecules may be modified through an enzymatic process so that siRNA
15 molecules may be generated. The siRNA can efficiently mediate the down-regulation effect for some target genes in some target organisms. This enzymatic process may be accomplished by utilizing an RNAse III enzyme or a DICER enzyme, present in the cells of an insect, a vertebrate animal, a fungus or a plant in the eukaryotic RNAi pathway (Elbashir *et al.*, 2002; Hamilton and Baulcombe, 1999). This process may also utilize a recombinant DICER or RNAse III introduced
20 into the cells of an organism through recombinant DNA techniques that are readily known to those skilled in the art. Both the DICER enzyme and RNAse III, being naturally occurring in an organism, or being made through recombinant DNA techniques, cleave larger dsRNA strands into smaller oligonucleotides. The DICER enzymes specifically cut the dsRNA molecules into siRNA pieces each of which is about 19-25 nucleotides in length while the RNAse III enzymes normally
25 cleave the dsRNA molecules into 12-15 base-pair siRNA. The siRNA molecules produced by either of the enzymes have 2 to 3 nucleotide 3' overhangs, and 5' phosphate and 3' hydroxyl termini. The siRNA molecules generated by RNAse III enzyme are the same as those produced by Dicer enzymes in the eukaryotic RNAi pathway and are hence then targeted and degraded by an inherent cellular RNA-degrading mechanism after they are subsequently unwound, separated
30 into single-stranded RNA and hybridize with the RNA sequences transcribed by the target gene. This process results in the effective degradation or removal of the RNA sequence encoded by the nucleotide sequence of the target gene in the target organism. The outcome is the silencing of a particularly targeted nucleotide sequence within the target organism. Detailed descriptions of enzymatic processes can be found in Hannon (2002).

35 A nucleotide sequence of the present invention can be recorded on computer readable media. As used herein, "computer readable media" refers to any tangible medium of expression that can be read and accessed directly by a computer. Such media include, but are not limited to:

magnetic storage media, such as floppy discs, hard disc, storage medium, and magnetic tape: optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; optical character recognition formatted computer files, and hybrids of these categories such as magnetic/optical storage media. A skilled artisan can readily appreciate that any of the presently
5 known computer readable mediums can be used to create a manufacture comprising a computer readable medium having recorded thereon a nucleotide sequence of the present invention.

As used herein, "recorded" refers to a process for storing information on computer readable medium. A skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate media comprising the nucleotide
10 sequence information of the present invention. A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present
15 invention on computer readable medium. The sequence information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an ASCII text file, stored in a database application, such as DB2, Sybase, Oracle, or the like. The skilled artisan can readily adapt any number of data processor structuring formats (*e.g.* text file or database) in order to obtain computer readable
20 medium having recorded thereon the nucleotide sequence information of the present invention.

Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium. Software that implements the BLAST (Altschul *et al.*, 1990) and BLAZE (Brutlag, *et al.*, 1993) search algorithms on a Sybase system can be used to identify open reading frames (ORFs) within sequences such as the Unigenes and
25 EST's that are provided herein and that contain homology to ORFs or proteins from other organisms. Such ORFs are protein-encoding fragments within the sequences of the present invention and are useful in producing commercially important proteins such as enzymes used in amino acid biosynthesis, metabolism, transcription, translation, RNA processing, nucleic acid and a protein degradation, protein modification, and DNA replication, restriction, modification,
30 recombination, and repair.

The present invention further provides systems, particularly computer-based systems, which contain the sequence information described herein. Such systems are designed to identify commercially important fragments of the nucleic acid molecule of the present invention. As used herein, "a computer-based system" refers to the hardware means, software means, and data
35 storage means used to analyze the nucleotide sequence information of the present invention. The minimum hardware means of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled

artisan can readily appreciate that any one of the currently available computer-based system are suitable for use in the present invention.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequences or sequence(s) are chosen based on a three-dimensional configuration that is formed upon the folding of the target motif. There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzymatic active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, cis elements, hairpin structures, siRNAs, and inducible expression elements (protein binding sequences).

B. Recombinant Vectors and Host Cell Transformation

A recombinant DNA vector may, for example, be 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 the bacterial host. In addition, a bacterial vector may be an expression vector. Nucleic acid molecules as set forth in the sequence listing, or fragments thereof, can, for example, be suitably inserted into a vector under the control of a suitable promoter that functions in one or more microbial hosts to drive expression of a linked coding sequence or other DNA sequence. 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 (amplification of DNA or expression of DNA) and the particular host cell with which it is compatible. The vector components for bacterial transformation generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selectable marker genes, and an inducible promoter allowing the expression of exogenous DNA.

Expression and cloning vectors may contain a selection gene, also referred to as a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics, herbicides, or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, glyphosate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli. Those cells that are successfully transformed with a heterologous protein or fragment thereof produce a protein conferring drug resistance and thus survive the selection regimen.

An expression vector for producing a mRNA can also contain an inducible promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding, the nucleic acid molecule, or fragment thereof, of interest. Inducible promoters suitable for use with bacterial

hosts include β-lactamase promoter, *E. coli* λ phage PL and PR promoters, *E. coli* galactose promoter, arabinose promoter, alkaline phosphatase promoter, tryptophan (trp) promoter, and the lactose operon promoter and variations thereof and hybrid promoters such as the tac promoter. However, other known bacterial inducible promoters are suitable. Plant promoters are discussed below.

The term "operably linked", as used in reference to a regulatory sequence and a structural nucleotide sequence, means that the regulatory sequence causes regulated expression of the linked structural nucleotide sequence. "Regulatory sequences" or "control elements" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-translated sequences) of a structural nucleotide sequence, and which influence the timing and level or amount of transcription, RNA processing or stability, or translation of the associated structural nucleotide sequence. Regulatory sequences may include promoters, translation leader sequences, introns, enhancers, stem-loop structures, repressor binding sequences, and polyadenylation recognition sequences and the like.

Alternatively, the expression constructs can be integrated into the host cell genome with an integrating vector. Integrating vectors typically contain at least one sequence homologous to the chromosome that allows the vector to integrate. Integrations appear to result from recombination between homologous DNA in the vector and the chromosome in the case of bacteria. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EP 0 127,328). Integrating vectors may also be comprised of bacteriophage or transposon sequences. Suicide vectors are also known in the art.

Construction of suitable vectors containing one or more of the above-listed components employs standard recombinant DNA techniques. Isolated plasmids or DNA fragments can be cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. Examples of available bacterial expression vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as BluescriptTM (Stratagene, La Jolla, CA); pIN vectors (Van Heeke and Schuster, 1989); and the like.

A yeast recombinant construct can typically include one or more of the following: a promoter sequence, fusion partner sequence, leader sequence, transcription termination sequence, a selectable marker. These elements can be combined into an expression cassette, which may be maintained in a replicon, such as an extrachromosomal element (*e.g.*, plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 (Botstein *et al.*, 1979), pCl/1 (Brake *et al.*, 1984), and YRp17 (Stinchcomb *et al.*, 1982). In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and typically

about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20 copies.

Useful yeast promoter sequences can be derived from genes encoding enzymes in the metabolic pathway. Examples of such genes include alcohol dehydrogenase (ADH) (EP 0 5 284044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EP 0 3215447). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences (Myanohara *et al.*, 1983). In addition, synthetic promoters that do not occur in nature also function as yeast promoters. Examples of such hybrid 10 promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent No. 4,876,197 and 4,880,734). Examples of transcription terminator sequences and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes, are known to those of skill in the art.

Alternatively, the expression constructs can be integrated into the yeast genome with an 15 integrating vector. Integrating vectors typically contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombination between homologous DNA in the vector and the yeast chromosome (Orr-Weaver *et al.*, 1983). An integrating vector may be directed to a specific locus in yeast by selecting the appropriate 20 homologous sequence for inclusion in the vector. See Orr-Weaver *et al.*, supra. One or more expression constructs may integrate, possibly affecting levels of recombinant protein produced (Rine *et al.*, 1983).

The present invention also contemplates transformation of a nucleotide sequence of the present invention into a plant to achieve inhibitory levels of expression of one or more dsRNA 25 molecules. A transformation vector can be readily prepared using methods available in the art. The transformation vector typically comprises one or more nucleotide sequences capable of being transcribed to an RNA molecule substantially homologous and/or complementary to one or more nucleotide sequences encoded by the genome of the target organism, and may comprise an intron sequence within the otherwise homologous or complementary sequence such that uptake by the 30 organism of the RNA transcribed and processed from the one or more nucleotide sequences results in down-regulation of expression of at least one of the respective nucleotide sequences of the genome of the target organism.

The transformation vector may be termed a dsDNA construct and may also be defined as a recombinant molecule, a pest or disease control agent, a genetic molecule or a chimeric genetic 35 construct. A chimeric genetic construct of the present invention may comprise, for example, nucleotide sequences encoding one or more antisense transcripts, one or more sense transcripts, one or more of each of the aforementioned, wherein all or part of a transcript therefrom is

homologous to all or part of an RNA molecule comprising an RNA sequence encoded by a nucleotide sequence within the genome of a target organism.

In one embodiment, a plant transformation vector comprises an isolated and purified DNA molecule comprising a heterologous promoter operatively linked to one or more nucleotide sequences of the present invention. The nucleotide sequence may be selected from among those as set forth in the sequence listing, or a fragment thereof. The nucleotide sequence can include a segment coding for all or part of an RNA present within a targeted organism. The RNA transcript may comprise inverted repeats of all or a part of a targeted RNA. The DNA molecule comprising the expression vector may also contain a functional intron sequence positioned either upstream of the coding sequence or even within the coding sequence, and may also contain a five prime (5') untranslated leader sequence (*i.e.*, a UTR or 5'-UTR) positioned between the promoter and the point of translation initiation.

A plant transformation vector may contain sequences from one or more genes, thus allowing production of more than one dsRNA for inhibiting expression of a gene or genes in cells of a target organism. One skilled in the art will readily appreciate that segments of DNA whose sequence corresponds to that present in different genes can be combined into a single composite DNA segment for expression in a transgenic plant. Alternatively, a plasmid of the present invention already containing at least one DNA segment can be modified by the sequential insertion of additional DNA segments between the enhancer and promoter and terminator sequences. In the disease or pest control agent of the present invention designed for the inhibition of multiple genes, the genes to be inhibited can be obtained from the same target species in order to enhance the effectiveness of the control agent. In certain embodiments, the genes can be derived from different pathogen or pest organisms in order to broaden the range of pathogens 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 fabricated as illustrated and disclosed in Application Publication No. US 2004-0029283.

Promoters that function in different plant species are also well known in the art. Promoters useful for expression of polypeptides in plants include those that are inducible, viral, synthetic, or constitutive as described in Odell *et al.* (1985), and/or promoters that are temporally regulated, spatially regulated, and spatio-temporally regulated. Preferred promoters include the enhanced CaMV35S promoters, and the FMV35S promoter. A fragment of the CaMV35S promoter exhibiting root-specificity may also be preferred. A number of tissue-specific promoters have been identified and are known in the art (*e.g.* US Patents 5,110,732; 5,837,848; Hirel *et al.* 1992; Stahl *et al.* 2004; Busk *et al.*, 1997).

A recombinant DNA vector or construct of the present invention typically comprises a selectable marker that confers a selectable phenotype on plant cells. Selectable markers may also be used to select for plants or plant cells that contain the exogenous nucleic acids encoding

polypeptides or proteins of the present invention. The marker may encode biocide resistance, antibiotic resistance (*e.g.*, kanamycin, G418 bleomycin, hygromycin, *etc.*), or herbicide resistance (*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 resistance; a nitrilase gene which confers resistance to bromoxynil; a mutant acetolactate synthase gene (ALS) which confers imidazolinone or sulfonylurea resistance; and a methotrexate resistant DHFR gene. Examples of such selectable markers are illustrated in U.S. Patents 5,550,318; 5,633,435; 5,780,708 and 6,118,047.

A recombinant vector or construct 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, 1987; Jefferson *et al.*, 1987); an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a β-lactamase gene (Sutcliffe *et al.*, 1978), 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) a *xylE* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikatu *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to melanin; an α -galactosidase, which catalyzes a chromogenic α-galactose substrate.

Preferred plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens* (*e.g.* U.S. Patent Nos. 4,536,475, 4,693,977, 4,886,937, 5,501,967 and EP 0 122 791). *Agrobacterium rhizogenes* plasmids (or "Ri") are also useful and known in the art. Other preferred plant transformation vectors include those disclosed, *e.g.*, by Herrera-Estrella (1983); Bevan (1983), Klee (1985) and EPO 0 120 516.

In general it may be preferred to introduce a functional recombinant DNA at a non-specific location in a plant genome. In special cases it may be useful to insert a recombinant DNA construct by site-specific integration. Several site-specific recombination systems exist which are known to function implants include cre-lox as disclosed in U.S. Patent 4,959,317 and FLP-FRT as disclosed in U.S. Patent 5,527,695.

Suitable methods for transformation of host cells for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell (see, for example, Miki *et al.*, 1993), such as by transformation of protoplasts (U.S. Patent No. 5,508,184; Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Patent No. 5,384,253), by agitation with silicon carbide fibers (Kaeppler

et al., 1990; U.S. Patent No. 5,302,523; and U.S. Patent No. 5,464,765), by *Agrobacterium*-mediated transformation (U.S. Patent Nos. 5,563,055; 5,591,616; 5,693,512; 5,824,877; 5,981,840; 6,384,301) and by acceleration of DNA coated particles (U.S. Patent Nos. 5,015,580; 5,550,318; 5,538,880; 6,160,208; 6,399,861; 6,403,865; Padgette *et al.* 1995), etc. Through the
5 application of techniques such as these, the cells of virtually any species may be stably transformed. In the case of multicellular species, the transgenic cells may be regenerated into transgenic organisms.

The most widely utilized method for introducing an expression vector into plants is based on the natural transformation system of *Agrobacterium* (for example, Horsch *et al.*, 1985). *A.*
10 *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. Descriptions of *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are provided by numerous references, including Gruber *et al.,* 1993; Miki *et al.*, 1993, Moloney *et al.*, 1989, and U.S. Patent
15 Nos: 4,940,838 and 5,464,763. 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 (Broothaerts *et al.,* 2005).

20 Plant transformation vectors can be prepared, for instance, by inserting the dsRNA producing nucleic acids disclosed herein into plant transformation vectors and introducing these into plants. One known vector system has been derived by modifying the natural gene transfer system of *Agrobacterium tumefaciens.* The natural system comprises large Ti (tumor-inducing) plasmids containing a large segment, known as the T-DNA, which is transferred to transformed
25 plant cells. 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 the 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 sequences. The T-region may also contain a selectable marker for efficient recovery of transgenic cells and plants, and a multiple cloning site
30 for inserting sequences for transfer, such as a dsRNA encoding nucleic acid.

Transgenic plants may be regenerated from a transformed plant cell by methods well known in the field of plant cell culture. A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single simple recombinant DNA sequence inserted into one chromosome and is referred to as a transgenic event. Such transgenic plants can be
35 referred to as being heterozygous for the inserted exogenous sequence. A transgenic plant homozygous with respect to a transgene can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single exogenous gene sequence to itself,

for example an F0 plant, to produce F1 seed. One fourth of the F1 seed produced will be homozygous with respect to the transgene. Germinating F1 seed results in plants that can be tested for heterozygosity, typically using a SNP assay or a thermal amplification assay that allows for the distinction between heterozygotes and homozygotes (*i.e.*, a zygosity assay). Crossing a
5 heterozygous plant with itself or another heterozygous plant results in only heterozygous progeny.

C. Nucleic Acid Expression and Target Gene Suppression

The present invention provides, as an example, a transformed host plant for a pathogenic target organism, transformed plant cells and transformed plants and their progeny. The transformed plant cells and transformed plants may be engineered to express one or more of the
10 dsRNA sequences including siRNA, under the control of a heterologous promoter to provide a pest or pathogen-protective effect. These sequences may be used for gene suppression in a pest or pathogen, thereby reducing the level or incidence of disease caused by the pathogen on a protected transformed host organism. As used herein the words "gene suppression" are intended to refer to any of the well-known methods for reducing the levels of protein produced as a result
15 of gene transcription to mRNA and subsequent translation of the mRNA.

Gene suppression is also intended to mean the reduction of protein expression from a gene or a coding sequence including posttranscriptional gene suppression and transcriptional suppression. Posttranscriptional gene suppression is mediated by the homology between of all or a part of a mRNA transcribed from a gene or coding sequence targeted for suppression and the
20 corresponding double stranded RNA used for suppression, and refers to the substantial and measurable reduction of the amount of available mRNA available in the cell for binding by ribosomes. The transcribed RNA can be in the sense orientation to effect what is called co-suppression, in the anti-sense orientation to effect what is called anti-sense suppression, or in both orientations producing a dsRNA to effect RNA interference (RNAi).

25 Transcriptional suppression is mediated by the presence in the cell of a dsRNA gene suppression agent exhibiting substantial sequence identity to a target DNA sequence or the complement thereof. Gene suppression can be effective against target genes in plant pests or pathogens that may take up or contact plant material containing gene suppression agents, specifically designed to inhibit or suppress the expression of one or more homologous or
30 complementary sequences in the cells of the target organism. Post-transcriptional gene suppression by anti-sense or sense oriented RNA to regulate gene expression in plant cells is disclosed in U.S. Pat. Nos. 5,107,065, 5,759,829, 5,283,184, and 5,231,020. The use of dsRNA to suppress genes in plants is disclosed in WO 99/53050, WO 99/49029, U.S. Patent Application Publication No. 2003/0175965, and 2003/0061626, U.S. Patent Application Ser. No. 10/465,800,
35 and U.S. Patent Nos. 6,506,559, and 6,326,193.

A beneficial method of gene suppression employs both sense-oriented and anti-sense-oriented, transcribed RNA which is stabilized, *e.g.*, as a hairpin and stem and loop structure. A preferred DNA construct for effecting gene suppression in a target organism is one in which a first segment encodes an RNA exhibiting an anti-sense orientation exhibiting substantial identity
5 to a segment of a gene targeted for suppression, which is linked to a second "spacer" segment, and to a third segment encoding an RNA exhibiting substantial complementarity to the first segment. Such a construct forms a stem and loop structure by hybridization of the first segment with the third segment, and a loop structure from the second segment nucleotide sequences linking the first and third segments (see WO94/01550, WO98/05770, US 2002/0048814, and US 2003/0018993).
10 According to one embodiment of the present invention, there is provided a nucleotide sequence, for which *in vitro* expression results in transcription of a stabilized RNA sequence that is substantially homologous to an RNA molecule that comprises an RNA sequence encoded by a nucleotide sequence within the genome of the target organism. Thus, after the target organism takes up the stabilized RNA sequence, a down-regulation of the nucleotide sequence
15 corresponding to the target gene in the cells of a target organism is effected.

Inhibition of a target gene using the stabilized dsRNA technology of the present invention is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single
20 point mutations relative to the target sequence may also be found to be effective for inhibition. In performance of the present invention, it is preferred that the inhibitory dsRNA and the portion of the target gene share at least from about 80% sequence identity, or from about 90% sequence identity, or from about 95% sequence identity, or from about 99% sequence identity, or even about 100% sequence identity. Alternatively, the duplex region of the RNA may be defined
25 functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript. A less than full length sequence exhibiting a greater homology compensates for a longer less homologous sequence. The length of the identical nucleotide sequences may be at least about 20, 50, 100, 200, 300, 400, 500 or at least about 1000 bases. Normally, a sequence of greater than about 20 nucleotides is to be used. The introduced nucleic acid molecule may not
30 need to possess absolute homology, and may not need to be full length, relative to either the primary transcription product or fully processed mRNA of the target gene. Therefore, those skilled in the art need to realize that, as disclosed herein, 100% sequence identity between the RNA and the target gene may not be required to practice specific embodiments of the present invention. Those skilled in the art will also recognize that a greater degree of sequence similarity
35 between the introduced nucleic acid and the target sequence may result in a higher level of gene suppression.

Inhibition of target gene expression may be quantified by measuring either the endogenous target RNA or the protein produced by translation of the target RNA and the consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism. Techniques for quantifying RNA and proteins are well known to one of ordinary 5 skill in the art.

In certain embodiments gene expression is inhibited by at least 10%, preferably by at least 33%, more preferably by at least 50%, and yet more preferably by at least 80%. In particularly preferred embodiments of the invention gene expression is inhibited by at least 80%, more preferably by at least 90%, more preferably by at least 95%, or by at least 99% within cells in the 10 target organism so that a significant inhibition takes place. Significant inhibition is intended to refer to sufficient inhibition that results in a detectable phenotype (*e.g.*, cessation of vegetative or reproductive growth, feeding, mortality, *etc.*) or a detectable decrease in RNA and/or protein corresponding to the target gene being inhibited. Although in certain embodiments of the invention inhibition occurs in substantially all cells of the target organism, in other preferred 15 embodiments inhibition occurs in only a subset of cells expressing the gene.

dsRNA molecules may be synthesized either *in vivo* or *in vitro*. The dsRNA may be formed by a single self-complementary RNA strand or from two complementary RNA strands. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or *in vitro*. Inhibition may be targeted by specific 20 transcription in an organ, tissue, or cell type; stimulation of an environmental condition (*e.g.*, infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus.

25 A RNA, dsRNA, siRNA, or miRNA 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 another organism. RNA may also be produced by partial or total organic synthesis; any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (*e.g.*, T3, T7, 30 SP6). The use and production of an expression construct are known in the art (see, for example, WO 97/32016; U.S. Pat. No's. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. 35 Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA 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 the duplex strands.

For transcription from a transgene *in vivo* or an expression construct, a regulatory region (*e.g.*, promoter, enhancer, silencer, and polyadenylation) may be used to transcribe the RNA 5 strand (or strands). Therefore, in one embodiment, the nucleotide sequences for use in producing RNA molecules may be operably linked to one or more promoter sequences functional in a microorganism, a fungus or a plant host cell. Ideally, the nucleotide sequences are placed under the control of an endogenous promoter, normally resident in the host genome. The endogenous promoter is thus typically a heterologous promoter with respect to the transgene. The nucleotide 10 sequence of the present invention, under the control of an operably linked promoter sequence, may further be flanked by additional sequences that advantageously affect its transcription and/or the stability of a resulting transcript. Such sequences are generally located upstream of the operably linked promoter and/or 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, 15 although such an upstream sequence only is also contemplated.

As used herein, the term "gene suppression agent" refers to a particular RNA molecule consisting of a first RNA segment, a second RNA segment, and a third RNA segment. The first and the third RNA segments lie within the length of the RNA molecule, are substantially inverted repeats of each other, and are linked together by the second RNA segment. At least one of the 20 nucleotide sequences encoding the first and third RNA segments may comprise an intron sequence. The complementarity between the first and the third RNA segments upon removal of the intron results in the ability of the two segments to hybridize *in vivo* and *in vitro* to form a double stranded molecule, *i.e.*, a stem, linked together at one end of each of the first and third segments by the second segment which forms a loop, so that the entire structure forms into a stem 25 and loop structure, or an even more tightly hybridizing structures may form into a stem-loop knotted structure. The first and the third segments correspond invariably and not respectively to a sense and an antisense sequence with respect to the target RNA transcribed from the target gene in the target organism that is suppressed by the ingestion or uptake of the dsRNA molecule. The control agent can also be a substantially purified (or isolated) nucleic acid molecule and more 30 specifically nucleic acid molecules or nucleic acid fragment molecules thereof from a genomic DNA (gDNA) or cDNA library. Alternatively, the fragments may comprise smaller oligonucleotides having from about 15 to about 250 nucleotide residues, and more preferably, about 15 to about 30 nucleotide residues.

As used herein, the term "genome" as it applies to cells of a target organism or a host 35 plant encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components of the cell. The DNA's of the present invention introduced into plant cells can therefore be either chromosomally integrated or organelle-localized. The term

"genome" as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. The DNA's of the present invention introduced into bacterial host cells can therefore be either chromosomally integrated or plasmid-localized.

As used herein, the term "target organism" or "target crop pest" refers to Ascomycetes,
5 Basidiomycetes, Deuteromycetes, Oomycetes, viruses, nematodes, insects, and the like that are present in the environment and that may infect, cause disease, or infest host plant material transformed to express or coated with a double stranded gene suppression agent containing the gene suppression agent. As used herein, "phytopathogenic microorganism" refers to microorganisms that can cause plant disease, including viruses, bacteria, fungi, oomycetes,
10 chytrids, algae, and nematodes. As used herein, the term "plant pest" refers to insects such as beetles, grasshoppers, weevils, aphids, mites, leafhoppers, thrips, whiteflies, rootworms, borers, grubs, and the like.

As used herein, a "pathogen resistance" or "pest resistance" trait is a characteristic of a host plant that causes the plant host to be resistant to attack from a pest or pathogen that typically
15 is capable of inflicting damage or loss to the plant. Such resistance can arise from a natural mutation or more typically from incorporation of recombinant DNA that confers resistance. To impart resistance to a transgenic plant a recombinant DNA can, for example, be transcribed into a RNA molecule that forms a dsRNA molecule within the tissues or fluids of the recombinant plant. Formation of the RNA molecule may also include processing, such as intron splicing. The
20 dsRNA molecule is comprised in part of a segment of RNA that is identical to a corresponding RNA segment encoded from a DNA sequence within a pest or pathogen that prefers to cause disease on the recombinant plant. Expression of the corresponding gene within the target organism is suppressed by the dsRNA, and the suppression of expression of the gene in the target organism results in the plant being resistant to the pest or pathogen. Fire *et al.,* (U.S. Patent No.
25 6,506,599) generically described inhibition of pest infestation, providing specifics only about several nucleotide sequences that were effective for inhibition of gene function in the nematode species *Caenorhabditis elegans*. Similarly, US 2003/0061626 describes the use of dsRNA for inhibiting gene function in a variety of nematode pests. US 2003/0150017 describes using dsDNA sequences to transform host cells to express corresponding dsRNA sequences that are
30 substantially identical to target sequences in specific pests, and particularly describe constructing recombinant plants expressing such dsRNA sequences for ingestion by various plant pests, facilitating down-regulation of a gene in the genome of the pest organism and improving the resistance of the plant to the pest infestation.

The modulatory effect of dsRNA is applicable to a variety of genes expressed in a pest or
35 pathogen, including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including house keeping genes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

As used herein, the phrase "inhibition of gene expression" or "inhibiting expression of a target gene in the cell of a target organism" refers to the absence (or observable decrease) within the target organism in the level of protein and/or mRNA product from the target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell 5 and without any effects on any gene within the cell that is producing the dsRNA molecule. The inhibition of gene expression of the target gene in the target organism may result in novel phenotypic traits in the target organism. To create a durable transgenic trait, production of dsRNA and/or its processing into siRNA would need to occur over both the developmental lifetime time of the individual transgenic crop plant and over generational time of a target 10 organism.

The present invention provides in part a delivery system for the delivery of the target organism control agents by ingestion of host cells or the contents of the cells. In accordance with another embodiment, the present invention involves generating a transgenic plant cell or a plant that contains a recombinant DNA construct transcribing the stabilized dsRNA molecules of the 15 present invention. As used herein, the phrase "taking up" refers to the process of an agent coming in contact with, or entering, a cell of a target organism. This may occur, for instance, by diffusion, active uptake, ingestion, feeding, injection, or soaking. As used herein, the phrase "generating a transgenic plant cell or a plant" refers to the methods of employing the recombinant DNA technologies readily available in the art (*e.g.*, by Sambrook, *et al.*, 1989) to construct a plant 20 transformation vector transcribing the stabilized dsRNA molecules of the present invention, to transform the plant cell or the plant and to generate the transgenic plant cell or the transgenic plant that contain the transcribed, stabilized dsRNA molecules.

The invention also provides methods comprising exposure of a target organism to one or more control agent(s) of the present invention incorporated in a spray mixer and applied to the 25 surface of a host, such as a host plant, including as a seed treatment (*e.g.* US Patent 6,551,962). Such control agent(s) may thus provide for exposure of a target organism by means of a dsRNA of the invention that targets suppression of one or more essential or pathogenicity related gene(s) in the target organism in combination with one or more of the following: a Bt toxin as set forth in the website (lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html), a biocontrol agent, an 30 insecticide, and a seed treatment. Methods for formulating and applying such seed treatments are well known in the art.

Such applications, including a seed treatment, may include an insecticide known in the art. Examples are set forth in US Patent 6,551,962, including a carbaryl insecticide, fenvalerate, esfenvalerate, malathion, a carbofuran insecticide, chloropyrifos, fonophos, phorate, terbufos, 35 permethrin, a neonicotinoid, and tefluthrin among others. Thus, a combination of lethality may be provided to a target organism, yielding a means for resistance management to prevent development of resistance by a target organism to a particular pesticidal composition. Biocontrol

agents are known in the art, and may include, for instance, naturally-occurring or recombinant bacteria or fungi from the genera *Rhizobium, Bacillus, Pseudomonas, Serratia, Clavibacter, Trichoderma, Glomus, Gliocladium* and mycorrhizal fungi, among others. A method for such resistance management is also provided by the invention.

5 Combinations of control agent(s) that may be employed with the invention include one or more polynucleotides that comprise or express a dsRNA of the present invention and at least one other agent toxic to an insect such as a coleopteran. Such combinations may be used to provide a "synergistic" effect. When it is said that some effects are "synergistic", it is meant to include the synergistic effects of the combination on the pesticidal activity (or efficacy) of the combination of 10 the dsRNA and the pesticide. However, it is not intended that such synergistic effects be limited to the pesticidal activity, as such effects include unexpected advantages of increased scope of activity, advantageous activity profile as related to type and amount of damage reduction, decreased cost of pesticide and application, decreased pesticide distribution in the environment, decreased pesticide exposure of personnel who produce, handle and plant crop seed, and other 15 advantages known to those skilled in the art.

In an exemplary embodiment, ingestion of the control agent(s) by a pest or pathogen organism delivers the control agents to the cells of the organism. In yet another embodiment, the RNA molecules themselves are encapsulated in a synthetic matrix such as a polymer and applied to the surface of a host such as a plant. Ingestion of the host cells by a target organism permits 20 delivery of the control agents to the organism and results in down-regulation of a target gene in the organism.

It is envisioned that the compositions of the present invention can be incorporated within the seeds of a plant species either as a product of expression from a recombinant gene incorporated into a genome of the plant cells, or incorporated into a coating or seed treatment that 25 is applied to the seed before planting. The plant cell containing a recombinant gene is considered herein to be a transgenic event.

The present invention provides in part a delivery system for the delivery of disease control agents to target organisms. The stabilized dsRNA or siRNA molecules of the present invention may be directly introduced into the cells of a target organism, or introduced into an 30 extracellular space (e.g. the plant apoplast). Methods for introduction may include direct mixing of RNA with media for the organism, as well as engineered approaches in which a species that is a host is engineered to express the dsRNA or siRNA. In one *in vitro* embodiment, for example, the dsRNA or siRNA molecules may be incorporated into, or overlaid on the top of, growth media. In another embodiment, the RNA may be sprayed onto a plant surface. In still another 35 embodiment, the dsRNA or siRNA may be expressed by microorganisms and the microorganisms may be applied onto a plant surface or introduced into a root or stem by a physical means such as an injection. In still another embodiment, a plant may be genetically engineered to express the

dsRNA or siRNA in an amount sufficient to affect target gene expression in the target organism known to infect or infest a plant host.

It is also anticipated that dsRNA's produced by chemical or enzymatic synthesis may be formulated in a manner consistent with common agricultural practices and used as spray-on
5 products for controlling plant disease. The formulations may include the appropriate stickers and wetters required for efficient foliar coverage as well as UV protectants to protect dsRNAs from UV damage. Such additives are commonly used in the bioinsecticide industry and are well known to those skilled in the art. Such applications could be combined with other spray-on insecticide applications, biologically based or not, to enhance plant protection from infection or
10 insect feeding damage. For instance, the RNA molecules may also be combined with another control agent, for instance an insecticidal agent such as a Cry protein, or insecticidal fragment thereof.

The present invention also relates to recombinant DNA constructs for expression in a microorganism. Exogenous nucleic acids from which an RNA of interest is transcribed can be
15 introduced into a microbial host cell, such as a bacterial cell or a fungal cell, using methods known in the art.

The nucleotide sequences of the present invention may be introduced into a wide variety of prokaryotic and eukaryotic microorganism hosts to produce the stabilized dsRNA or siRNA molecules. The term "organism" includes prokaryotic and eukaryotic species such as bacteria,
20 and fungi. Fungi include yeasts and filamentous fungi, among others. Illustrative prokaryotes, both Gram-negative and Gram-positive, include Enterobacteriaceae, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, and *Proteus*; Bacillaceae; Rhizobiaceae, such as *Rhizobium*; Spirillaceae, such as photobacterium; *Zymomonas*, *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum*; Lactobacillaceae; Pseudomonadaceae, such as *Pseudomonas* and *Acetobacter*;
25 Azotobacteraceae, *Actinomycetales*, and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes which includes filamentous fungi such as *Sclerotinia, Erysiphe,* and the like, and yeast, such as *Saccharomyces* and *Schizosaccharomyces*; Basidiomycetes, such as *Rhodotorula*, *Aureobasidium*, *Sporobolomyces*, and the like; and Oomycetes, such as *Phytophthora.*

30 **D. Transgenic Plants**

The present invention provides a transgenic plant including, without limitation, alfalfa, corn, canola, rice, soybean, tobacco, turfgrass, and wheat, among others. The present invention provides seeds and plants having one or more transgenic event(s). Combinations of events are referred to as "stacked" transgenic events. These stacked transgenic events can be events that are
35 directed at the same target organism, or they can be directed at different target pathogens or pests. In one embodiment, a seed having the ability to express a nucleic acid provided herein also has

the ability to express at least one other agent, including, but not limited to, an RNA molecule the sequence of which is derived from the sequence of an RNA expressed in a target pathogen and that forms a double stranded RNA structure upon expressing in the seed or cells of a plant grown from the seed, wherein the ingestion of one or more cells of the plant by the target pathogen
5 results in the suppression of expression of the RNA in the cells of the target pathogen.

In certain embodiments, a seed having the ability to express a dsRNA the sequence of which is derived from a target organism also has a transgenic event that provides herbicide tolerance. One beneficial example of a herbicide tolerance gene provides resistance to glyphosate, N- (phosphonomethyl) glycine, including the isopropylamine salt form of such
10 herbicide.

Benefits provided by the present invention may include, but are not limited to: the ease of introducing dsRNA into the target organism's cells, the low concentration of dsRNA which can be used, the stability of dsRNA, and the effectiveness of the inhibition. The ability to use a low concentration of a stabilized dsRNA avoids several disadvantages of anti-sense interference. The
15 present invention is not limited to *in vitro* use or to specific sequence compositions, to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or to a particular delivery method, as opposed to the some of the available techniques known in the art, such as antisense and co-suppression. Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models.

20 In order to achieve inhibition of a target gene selectively within a target organism species that it is desired to control, the target gene should preferably exhibit a low degree of sequence identity with corresponding genes in a plant or a vertebrate animal. Preferably the degree of the sequence identity is less than approximately 80%. More preferably the degree of the sequence identity is less than approximately 70%. Most preferably the degree of the sequence identity is
25 less than approximately 60%.

In addition to direct transformation of a plant with a recombinant DNA construct, transgenic plants can be prepared by crossing a first plant having a recombinant DNA construct with a second plant lacking the construct. For example, recombinant DNA for gene suppression can be introduced into first plant line that is amenable to transformation to produce a transgenic
30 plant that can be crossed with a second plant line to introgress the recombinant DNA for gene suppression into the second plant line.

The present invention can be, in practice, combined with other disease control traits in a plant to achieve desired traits for enhanced control of plant disease. Combining disease control traits that employ distinct modes-of-action can provide protected transgenic plants with superior
35 consistency and durability over plants harboring a single control trait because of the reduced probability that resistance will develop in the field.

The invention also relates to commodity products containing one or more of the sequences of the present invention, and produced from a recombinant plant or seed containing one or more of the nucleotide sequences of the present invention are specifically contemplated as embodiments of the present invention. A commodity product containing one or more of the sequences of the present invention is intended to include, but not be limited to, meals, oils, crushed or whole grains or seeds of a plant, or any food product comprising any meal, oil, or crushed or whole grain of a recombinant plant or seed containing one or more of the sequences of the present invention. The detection of one or more of the sequences of the present invention in one or more commodity or commodity products contemplated herein is *defacto* evidence that the commodity or commodity product is composed of a transgenic plant designed to express one or more of the nucleotides sequences of the present invention for the purpose of controlling plant disease using dsRNA mediated gene suppression methods.

E. Obtaining Nucleic acids

The present invention provides methods for obtaining a nucleic acid comprising a nucleotide sequence for producing a dsRNA including siRNA. In one embodiment, such a method comprises: (a) probing a cDNA or gDNA library with a hybridization probe comprising all or a portion of a nucleotide sequence or a homolog thereof from a targeted organism; (b) identifying a DNA clone that hybridizes with the hybridization probe; (c) isolating the DNA clone identified in step (b); and (d) sequencing the cDNA or gDNA fragment that comprises the clone isolated in step (c) wherein the sequenced nucleic acid molecule transcribes all or a substantial portion of the RNA nucleotide acid sequence or a homolog thereof.

In another embodiment, a method of the present invention for obtaining a nucleic acid fragment comprising a nucleotide sequence for producing a substantial portion of a dsRNA or siRNA comprises: (a) synthesizing first and a second oligonucleotide primers corresponding to a portion of one of the nucleotide sequences from a targeted organism; 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 transcribes a substantial portion of the a substantial portion of a dsRNA or siRNA of the present invention.

In practicing the present invention, a target gene may be derived from a pest or pathogen species that causes damage to the crop plants and subsequent yield losses. It is contemplated that several criteria may be employed in the selection of preferred target genes. The gene may be one whose protein product has a rapid turnover rate, so that dsRNA inhibition will result in a rapid decrease in protein levels. In certain embodiments it is advantageous to select a gene for which a small drop in expression level results in deleterious effects for the target organism. If it is desired to target a broad range of pest or pathogen species, a gene is selected that is highly conserved across these species. Conversely, for the purpose of conferring specificity, in certain

embodiments of the invention, a gene is selected that contains regions that are poorly conserved between individual species, or between the target and other organisms. In certain embodiments it may be desirable to select a gene that has no known homologs in other organisms. As used herein, the term "derived from" refers to a specified nucleotide sequence that may be obtained
5 from a particular specified source or species, albeit not necessarily directly from that specified source or species.

Other target genes for use in the present invention may include, for example, those that play important roles in the viability, growth, feeding, development, reproduction and infectivity of the target organism. These target genes may be one of the house keeping genes, transcription
10 factors and the like. Additionally, the nucleotide sequences for use in the present invention may also be derived from plant, viral, bacterial or insect genes whose functions have been established from literature and the nucleotide sequences of which share substantial similarity with the target genes in the genome of a target organism. According to one aspect of the present invention, the target sequences may essentially be derived from the targeted organism.

15 For the purpose of the present invention, the dsRNA or siRNA molecules, or polynucleotides that encode them, may be obtained by polymerase chain (PCR™) amplification of a target gene sequences derived from a gDNA or cDNA library or portions thereof. The DNA library may be prepared using methods known to the ordinary skilled in the art and DNA/RNA may be extracted. Genomic DNA or cDNA libraries generated from a target organism may be
20 used for PCR™ amplification for production of the dsRNA or siRNA. The target genes may be then be PCR™ amplified and sequenced using the methods readily available in the art. One skilled in the art may be able to modify the PCR™ conditions to ensure optimal PCR™ product formation. The confirmed PCR™ product may be used as a template for *in vitro* transcription to generate sense and antisense RNA with the included minimal promoters.

25 The present inventors contemplate that nucleic acid sequences identified and isolated from any pest or pathogen species may be used in the present invention for control of plant disease. In one aspect of the present invention, the nucleic acid may be derived from a Western Corn Rootworm (*Diabrotica virgifera virgifera*). The isolated nucleic acids may be useful, for example, in identifying a target gene and one or more sequences within the gene that encode
30 effective siRNA molecules. They may also be useful in constructing a recombinant vector according to the method of the present invention that produces stabilized dsRNAs or siRNAs of the present invention for protecting plants from the rootworm. Therefore, in one embodiment, the present invention comprises isolated and purified nucleotide sequences that may be used as plant pest or disease control agents.

35 The nucleic acids that may be used in the present invention may also comprise isolated and substantially purified Unigenes and EST nucleic acid molecules or nucleic acid fragment molecules thereof. EST nucleic acid molecules may encode significant portions of, or indeed

most of, the polypeptides. Alternatively, the fragments may comprise smaller oligonucleotides having from about 15 to about 250 nucleotide residues, and more preferably, about 15 to about 30 nucleotide residues. Alternatively, the nucleic acid molecules for use in the present invention may be from cDNA libraries from a target organism of interest.

5 Nucleic acid molecules and fragments thereof from a pest or pathogen species may be employed to obtain other nucleic acid molecules from other species for use in the present invention to produce desired dsRNA and siRNA molecules. Such nucleic acid molecules include the nucleic acid molecules that encode the complete coding sequence of a protein and promoters and flanking sequences of such molecules. In addition, such nucleic acid molecules include 10 nucleic acid molecules that encode for gene family members. Such molecules can be readily obtained by using the above-described nucleic acid molecules or fragments thereof to screen cDNA or genomic DNA libraries. Methods for forming such libraries are well known in the art.

As used herein, the phrase "coding sequence", "structural nucleotide sequence" or "structural nucleic acid molecule" refers to a nucleotide sequence that is translated into a 15 polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, genomic DNA, cDNA, EST and recombinant nucleotide sequences.

The term " recombinant DNA" or "recombinant nucleotide sequence" refers to DNA that 20 contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like.

For many of the pests and pathogens that are potential targets for control by the present invention, there may be limited information regarding the sequences of most genes or the phenotype resulting from mutation of particular genes. Therefore, it is contemplated that 25 selection of appropriate genes from pathogens for use in the present invention may be accomplished through use of information available from study of the corresponding genes in a model organism such in *Saccharomyces cerevisiae*, or in a nematode species such as *C. elegans*, in an insect species, or in a plant species, in which the genes have been characterized. In some cases it will be possible to obtain the sequence of a corresponding gene from a target pest or 30 pathogen by searching databases such as GenBank using either the name of the gene or the sequence from, for example, *Drosophila*, another insect, a nematode, or a plant from which the gene has been cloned. Once the sequence is obtained, PCR™ may be used to amplify an appropriately selected segment of the gene in the pathogen for use in the present invention.

In order to obtain a DNA segment from the corresponding gene, PCR™ primers may be 35 designed based on the sequence as found in another organism from which the gene has been cloned. The primers are designed to amplify a DNA segment of sufficient length for use in the present invention. DNA (either genomic DNA or cDNA) is prepared from the pathogen, and the

PCR™ primers are used to amplify the DNA segment. Amplification conditions are selected so that amplification will occur even if the primers do not exactly match the target sequence. Alternately, the gene (or a portion thereof) may be cloned from a gDNA or cDNA library prepared from the pathogen species, using the known gene as a probe. Techniques for performing
5 PCR™ and cloning from libraries are known. Further details of the process by which DNA segments from target pathogen species may be isolated based on the sequence of genes previously cloned from other species are provided in the Examples. One of ordinary skill in the art will recognize that a variety of techniques may be used to isolate gene segments from plant pest and pathogenic organisms that correspond to genes previously isolated from other species.

10

EXAMPLE 1

Effects of dsRNA presentation size on gene suppression in corn rootworm

Bio-assay of dsRNA constructs encoding portions of the western corn rootworm (*Diabrotica virgifera virgifera;* WCR) V-ATPase subunit A gene demonstrated efficacy in gene suppression. Additional work has determined that a 50 bp segment of the WCR V-ATPase
15 subunit A gene (SEQ ID NO:1), when presented as a dsRNA, is sufficient to elicit mortality when tandemly duplicated 5 times, but is ineffective as a 50 bp monomer (Table 1). The 50 bp segment embedded in a neutral carrier for a total dsRNA of 100 bp was also effective, indicating that there are size restrictions on efficient uptake of dsRNA into insects susceptible to RNAi.

Reduced efficacy of smaller unit sizes was also seen using a different gene sequence
20 consisting of 27bp derived from a *D. virgifera virgifera* sequence encoding Dv49 (SEQ ID NO:2), a putative ortholog of a Drosophila binding/carrier protein (FlyBase sequence CG8055 (SEQ ID NO:3)). A synthetic 27 bp dsRNA segment of Dv49 failed to show activity when fed to insects at 1 ppm (Table 2). The same 27 bp segment embedded in a vector backbone sequence to create a 50 bp dsRNA resulted in increased efficacy. However efficacy was still less than the same 27mer
25 embedded in a total of 206 bp of dsRNA (Table 2). Adjusting the concentration of dsRNA to achieve an equal molar ratio of 27mer sequence showed the 50mer caused no significant mortality (Table 2). Thus, two very different species, *C. elegans* and *D. virgifera,* exhibit an apparent need for dsRNA of minimum size to permit efficient uptake. This observation indicates the importance of ensuring the production of dsRNA *in planta* of sufficient size to enable uptake and subsequent
30 control of the targeted pest, rather than simply the production of smaller siRNAs that are less likely to be as effective when contacted by a target.

Table 1. Impact of dsRNA size on control of WCR in diet bio-assay fed at 1 ppm.

dsRNA	Mortality in WCR diet bio-assay [1]
Diabrotica virgifera V-ATPase subunit A, 50 bp segment	26.6 ± 4.9
Concatemer 3: 5 tandem copies of *Diabrotica virgifera* V-ATPase subunit A 50 bp segment (250 bp total	71.0 ± 11.8 *

1 Percent mortality and standard error of the means.
* significantly different from untreated control P value <0.05, Planned Contrasts..

5 **Table 2. Impact of dsRNA size on control of WCR in diet bio-assay using 27 bp of Dv49 target alone or embedded in neutral carrier and fed at 1 ppm final dsRNA concentration.**

dsRNA	Mortality in WCR diet bio-assay [1]
27 bp from WCR Dv49	6.19 ± 3.81
27 bp from WCR Dv49 plus 23 bp of vector sequence for total of 50 bp contiguous dsRNA	25.2 ± 6.7 *
27 bp from WCR Dv49 plus 179 bp of vector sequence for total of 206 bp contiguous dsRNA	100 *
0.1 ppm of 27 bp from WCR Dv49 + 0.9 ppm of vector sequence (non-contiguous to 27 bp of WCR sequence)	14.8 ± 4.2
0.2 ppm bp from WCR Dv49 plus 23 bp of vector sequence for total of 50 bp contiguous dsRNA + 0.9 ppm of vector sequence (non-contiguous to 50 bp of sequence)	11.2 ± 4.9

[1] Percent mortality and standard error of the means.
* significantly different from untreated control, P value <0.05, Planned Contrasts.

EXAMPLE 2

Fine mapping efficacious corn rootworm gene targets: 26-28mer analysis

10 Effective presentation of dsRNA sequences that are otherwise below efficient uptake size was accomplished by embedding segments down to the level of single siRNAs within "carrier" sequence. The WCR sequence Dv49 was chosen for further analysis due to high efficacy in previous insect bio-assays. A 100 bp fragment (SEQ ID NO:4) located 202 bp from the start of translation was synthesized by PCR, as follows:

15 The 100 bp segment of the Dv49 target was amplified, using cycling conditions described in Table 4, to produce an antisense template using oligonucleotides Dv49-1 (SEQ ID NO:5) and Dv49-2 (SEQ ID NO:6); and a separate sense template using oligonucleotides Dv49-3 (SEQ ID NO:7) and Dv49-4 (SEQ ID NO:8).

Table 3. Oligonucleotides used to clone and amplify 100 bp segment of Dv49 used in 26mer scan evaluation. T7 RNA polymerase promoters are shown in lower case (SEQ ID NO:5-8)

Name	Sequence	Target DNA	Orientation	Comments
Dv49-1	AAGAAGAAACGATTGGAAAAGAC	Dv49	sense	For synthesis of 100mer template for dsRNA production of anti-sense strand when used with Dv49-2.
Dv49-2	taatacgactcactataggCAGTATTTGTGCTAGCTCCTTC	Dv49	antisense	For synthesis of 100mer template for dsRNA production of anti-sense strand when used with Dv49-1.
Dv49-3	CAGTATTTGTGCTAGCTCCTTC	Dv49	antisense	For synthesis of 100mer template for dsRNA production of sense strand when used with Dv49-4.
Dv49-4	taatacgactcactataggAAGAAGAAACGATTGGAAAAGAC	Dv49	sense	For synthesis of 100mer template for dsRNA production of sense strand when used with Dv49-3.

Table 4. PCR conditions for amplifications of templates used in dsRNA synthesis.

Step	Temp (°C)	Time
1	94	2 minutes
2	94	30 seconds
3	52	30 seconds
4	72	30 seconds
5	go to step 2, 33 times	
6	72	2 minutes
7	hold at 10 forever	

5

The following reaction conditions were employed: 1X Sigma REDtaq buffer, 200 μM each dNTP, 0.4 μM each oligonucleotide primer, approximately 200 pg of pMON78428 template, and 2 U of REDtaq polymerase (Sigma, Cat. #D4309) in a 50 μl reaction volume. Five μl of each PCR reaction was used to produce a single stranded transcript with the MEGAshortscript™ kit

10 (Ambion, Cat. #1354) according to manufacturer's instructions. The sense and antisense reactions were mixed, heated to 75°C for 5 min and allowed to cool to room temperature. Further

purification of the annealed 100 bp dsRNA product was completed with the MEGAscript™ RNAi Kit (Ambion, Cat #1626) according to manufacture's instructions. This methodology produced a 100 bp product lacking the T7 promoter sequences.

The 100 bp fragment was used as a template for dsRNA synthesis, and the dsRNA was
5 subjected to insect bioassay. When fed at 0.2 ppm, mortality of WCR was 100% with the 100 bp dsRNA (Table 5). No mortality was observed when feeding dsRNA derived from the vector backbone (180 bp) by itself.

Table 5. Impact of dsRNA size on control of WCR in diet bio-assay using 26 bp Dv49 target embedded in vector sequence as carrier (206 bp final size). 1 ppm and 0.2 ppm assays were
10 **run at different times.**

dsRNA	**Mortality in WCR diet bio-assay fed at 1 ppm[1]**	**Mortality in WCR diet bio-assay fed at 0.2 ppm[1]**
Scan 0	60.1 ± 4.4 *	13.3 ± 9.7
Scan 1	36.4 ± 16.3 *	16.3 ± 4.3
Scan 2	35.8 ± 9.1 *	22.6 ± 3.3 *
Scan 3	85.7 ± 9.0 *	96.7± 3.30 *
Scan 4	75.0 ± 9.4 *	42.8 ± 3.8 *
Scan 5	65.4 ± 11.4 *	39.4 ± 10.7 *
Scan 6	92.5 ± 5.0 *	61.9 ± 8.5 *
Scan 7	94.6 ± 3.3 *	80.6 ± 9.4 *
Scan 8	91.0 ± 5.61 *	66.7± 10.0 *
Scan 9	41.4 ± 6.8 *	19.0 ± 7.5
Scan 10	7.9 ± 5.1	6.7 ± 4.1
Scan 11	39.3 ± 5.3 *	5.4 ± 3.3
Scan 12	37.9 ± 6.9 *	13.7 ± 6.9
Scan 13	61.2 ± 6.3 *	33.3 ± 12.6 *
Scan 14	70.6 ± 7.3 *	42.3 ± 7.8 *
100 bp Dv49 base sequence	100 *	100 *
Vector sequence only	NA	0.0 *

[1] Percent mortality and standard error of the means.
* significantly different from untreated control, P value <0.05, Planned Contrasts.
NA = not assayed

To define active 21 bp segments (siRNA-sized) and the effects of single nucleotide
15 polymorphisms (SNPs) on efficacy, 26 bp segments scanning through the 100mer base sequence in a 5 bp register were cloned as follows: 26bp segments derived from the 100 bp Dv49 test sequence were produced synthetically (Integrated DNA Technologies) as sense and antisense oligonucleotides. Pairs of oligonucleotides used in cloning (SEQ ID NO:9-38) were annealed and a 3' A-overhang was added by setting up the following reaction: 1X Sigma REDtaq buffer, 200
20 μM each dNTP, 0.4 μM each oligonucleotide primer and 2 U of REDtaq polymerase and incubation at 75°C for 2 minutes followed by 20 minutes at 50°C. Two μl of each PCR reaction was ligated into the PCR2.1-TOPO vector in a TOPO-TA cloning reaction (Invitrogen, Cat. #45-0641) according to manufacturer's instructions and transformed into *E. coli* TOP10 cells. White to light blue colonies were selected on LB plates containing 100 μg/ml carbenicillin and surface

treated with 40 μl of 50 mg/ml X-Gal. Colonies were screened for correct sequence and consistent sense orientation in the vectors. All are in the same relative orientation except for the Scan 7 segment (pMON98376) which is inverted relative to other cloned sequences.

Templates for RNA synthesis were prepared using oligonucleotides pCR2.1-5 and
5 pCR2.1-6 (SEQ ID NO:39-40), the cycling conditions in Table 4, and the same reaction conditions used to amplify the Dv49 100mer template. A blank vector (no corn rootworm sequence), pMON98397, was also amplified to serve as a control for the vector sequences. Fresh PCR product was amplified from verified clones for dsRNA synthesis. Amplifications were visualized on 1-3% agarose gels stained with ethidium bromide to ensure proper size and quality.
10 An aliquot of 5 μl was used in dsRNA synthesis directly from the PCR tube. Synthesis was carried out according to the MEGAscript™ RNAi Kit (Ambion, Cat #1626) with the following alterations: transcription was carried out at 37°C overnight in a convection oven. Final dsRNA products were quantified by absorption at 260nm, and visualized on a 1-3% agarose gel to ensure intactness of the product. All samples for insect bioassay were diluted to a final concentration
15 (e.g. 1 ppm) in 10 mM Tris pH 6.8. Twenty μl of each sample were applied to 200 μl of insect diet and allowed to absorb into the diet before addition of a WCR neonate. Stunting and mortality of larvae was scored at day 12.

dsRNA corresponding to the resulting fragments Scan 0 to Scan 14 (FIG. 1) was amplified in a larger neutral carrier (vector backbone sequence), using pCR2.1-5 and pCR2.1-6
20 oligonucleotides, and dsRNA was synthesized for a total dsRNA length of 206 bp. Since cloning into the pCR2.1-TOPO vector recapitulated the original Dv49 context for some of the cloned 26mer segments, the sequence interrogated for efficacy was actually 27-28 bp in size in some instances. When fed at 1 ppm, the dsRNAs synthesized from the 26mers resulted in a range of mortality from no significant difference from the untreated control to approximately 95%
25 mortality with the scan 7 segment (FIG. 7; Table 5). When fed at 0.2 ppm, the dsRNAs synthesized from the 26mers resulted in a range of mortality from no significant difference from the untreated control to 97% mortality with the scan 3 segment (FIG. 8; Table 5).

The lower dose tested proved useful in discriminating the most active segments. From the dsRNA of each cloned segment of Dv49, several 21 bp siRNAs could potentially result from
30 endogenous WCR DICER activity.

EXAMPLE 3

Fine mapping efficacious corn rootworm gene targets: 21mer analysis of Scan 14 region

Twenty one bp segments derived from Scan segment 14 of the 26mer analysis were synthesized as above except the ends were modified so that when annealed a Hind III restriction
35 site compatible overhang was created at the 5' end and an Spe I restriction site compatible overhang at the 3' end of each oligonucleotides (SEQ ID NO:41-54). These were ligated into a

Hind III/Spe I cut pCR2.1-TOPO backbone. Attempts were made to clone all seven possible 21mer sequences that could be produced from Scan 14. Cloning of Scan 15 failed and the cloned Scan 17 sequence was found to contain a point mutation that is likely responsible for its poor activity. Scan segments 16-21 were amplified to produce templates and dsRNA was prepared as
5 for the 26mer scan. The final size of each dsRNA was 184 bp. Samples were diluted, applied at 0.2 ppm and scored as above.

These 21 bp sub-sequences of Scan 14 (Scans 15-21) were tested and most were found to possess significant activity against WCR in diet bio-assay (Table 6; FIG. 9). Generally a higher positive Reynolds score (Reynolds *et al.* 2004) indicates a greater probability of gene suppression.
10 The noted discrepancies highlight the need for empirical testing in fine mapping efficacy against pest species such as rootworm.

Table 6. Impact of dsRNA size on control of WCR in diet bio-assay using 21 bp Dv49 target embedded in vector sequence as carrier. The parental embedded 26 bp sequence from Dv49 (Scan 14) and the 100 bp base sequence were also evaluated. 1 ppm and 0.2 ppm assays
15 **were run concurrently. Reynolds scores for 21 bp sequences are indicated.**

dsRNA	Reynolds score	Mortality in WCR diet bio-assay fed at 1 ppm[1]	Mortality in WCR diet bio-assay fed at 0.2 ppm[1]
Scan 14 parent		92.0 ± 8.0 *	77.3 ± 7.6 *
Scan 15	3	NA	NA
Scan 16	1	92.1 ± 5.1 *	53.2 ± 7.9 *
Scan 17	3	13.6 ± 6.0	0.0
Scan 18	4	77.8 ± 10.0 *	43.2 ± 9.2 *
Scan 19	6	73.3 ± 7.3 *	76.1 ± 9.6 *
Scan 20	8	85.3 ± 6.2 *	77.1 ± 7.1 *
Scan 21	9	5.0 ± 5.0	0.0
100 bp Dv49 base sequence		97.1 ± 2.9 *	NA
Vector sequence only		0.0	NA

[1] Percent mortality and standard error of the means.
* significantly different from untreated control, P value <0.05, Planned Contrasts. NA = not assayed

EXAMPLE 4

20 **Impact of Dv49 sequence polymorphism on efficacy**

The ability to finely map target genes allows an understanding of the impact of sequence variation on efficacy. In FIG. 1, a 100 bp segment of WCR Dv49 used in the 26 bp scan was compared to a number of related sequences from other species (Table 7; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:55-72). Sequences for the Dv49 orthologs among *Diabrotica sp.* were found
25 to be highly conserved. From the alignment it is possible to see variation at some locations (*e.g.* the highly efficacious scan 3 segment), that differs significantly between *Diabrotica* and all other species examined - even other beetle species such as *Tribolium castaneum*. Thus it is possible to

make novel chimeric sequences that incorporate small segments (down to siRNA-sized portions) that have high activity and conservation within target *Diabrotica* species but otherwise are poorly conserved outside of this taxonomic group. Such novel sequences could give high activity against *Diabrotica sp.*, but low activity against non-target species, even if a species is amenable to RNAi
5 through diet presentation. These may be arranged in novel concatemers that do not create fortuitous matches to other gene sequences via the juxtaposition of subunits (determined by bio-informatic evaluations).

Table 7. Gene sequences of animal species acquired from Genbank (accession number listed) or determined through sequencing efforts that have high identity to Dv49 at an amino
10 **acid level. Representative sequences (either cDNA or genomic) were used to prepare nucleotide alignments with Dv49. (SEQ ID NO:2; SEQ ID NO:3; SEQ ID NOs:55-72)**

Species	Common Name	Target	Source	SEQ ID NO:
Amphioxus floridae	Amphioxus	Af49	BW703594	55
Anopheles gambiae	mosquito	Ag49	CR528625	56
Acyrthosiphon pisum	pea aphid	Ap49	CN763091	57
Apis mellifera	honey bee	Am49	AADG05006126	
Bombyx mori	silkworm	Bm49	AADK01001496	
Canis familiaris	dog	Cf49_1	DN397962	58
Canis familiaris	dog	Cf49_2	DN434127	59
Ciona savignyi	sea squirt	Cs49	AACT01061660	
Danio rerio	zebra fish	Dr49	CAAK01000381	
Daphnia magna	water flea	Dmag49	BJ928947	60
Diabrotica balteata	banded cucumber beetle	Dbal49		61
Diabrotica barberi	northern corn rootworm	Db49		62
Diabrotica undecimpunctata	southern corn rootworm	Du49		63
Diabrotica virgifera virgifera	western corn rootworm	Dv49		2
Diabrotica virgifera zeae	mexican corn rootworm	Dz49		64
Drosophila melanogaster	fruitfly	Dm49	AABU01002766	3
Fugu rubripes	puffer fish	Fr49	BU807180	65
Gallus gallus	chicken	Gg49	AJ729228	66
Glossina morsitans	tsetse fly	Gm49	BX565926	67
Locusta migratoria	locust	Lm49	CO842932	68
Pan troglodytes	chimpanzee	Pt49_1	XM_528179	69
Pan troglodytes	chimpanzee	Pt49_2	XM_525305	70
Strongylocentrotus purpuratus	sea urchin	Sp49	CD309114	71
Tribolium castaneum	red flour beetle	Tc49	AAJJ01000852	
Xenopus laevis	African clawed frog	Xl49	BP672793	72

Small efficacious units such as the scan 3 segment could be vulnerable to nucleotide variation. Natural mutation or pre-existing allelic variation within or between species could reduce the ability to initiate gene suppression targeted against an organism. This potential impact
5 was examined using the sequence corresponding to Dv49, scan segment 3, from *Diabrotica barberi.* This species has a single nucleotide polymorphism when compared to all other *Diabrotica sp.* that were sequenced (FIG. 1). Assay of the Scan 3 segment from *Diabrotica barberi* (Db49 scan 3 segment) revealed it was much less effective than the native *Diabrotica virgifera* scan 3 segment in initiating WCR larval mortality (Table 8). Optimal sequences used
10 for pest RNAi should buffer this potential gene diversity by ensuring that sufficient numbers of highly effective siRNAs can be created from the transgenic construct to target the full range of intended species.

Table 8. Impact of Dv49 dsRNA single nucleotide polymorphism on a cloned 26 bp segment (Scan 3) from two Diabrotica species when assayed in western corn rootworm bio-assay.

dsRNA	Mortality in WCR diet bio-assay fed at 1 ppm[1]	Mortality in WCR diet bio-assay fed at 0.2 ppm[1]
Scan 3 from *Diabrotica virgifera*	86.3 ± 7.1 *	91.0 ± 5.6 *
Scan 3 from *Diabrotica barberi*	38.5 ± 5.7 *	7.3 ± 4.5

15 [1] Percent mortality and standard error of the means.

* significantly different from untreated control, P value <0.05, Planned Contrasts.

Inspection of alignments of Dv49-related sequences from the organisms listed in Table 7, combined with an analysis of regions within those sequences that may yield efficacious dsRNA (*e.g.* high Reynolds scores), allows the identification of segments that would likely yield
20 efficacious siRNAs in insect bioassays.

Desirable transgenic RNAi crops would specifically target certain pest species but minimize potential for interactions with unintended species. For instance, ideally one would have a single, simple dsRNA construct that targets a critical gene(s) from *Diabrotica virgifera virgifera* (western corn rootworm, WCR), *Diabrotica virgifera zeae* (Mexican corn rootworm, MCR), and
25 *Diabrotica barberi* (northern corn rootworm, NCR). Additional species, such as *Diabrotica undecimpunctata howardii* (southern corn rootworm, SCR), *Diabrotica undecimpunctata undecimpunctata* (western spotted cucumber beetle); *Diabrotica speciosa;* and *Diabrotica viridula* could also be included among the target species. Selection of gene sequences for inclusion in dsRNA constructs would be optimal with alignments of gene targets from multiple
30 species and populations and also pertinent non-target organisms. cDNA segments coding for Dv49 orthologs from a variety of organisms and populations were sequenced for comparison.

RT-PCR using RNA derived from adults and/or larvae served a source material for obtaining novel sequence. Depending on the target, specific or degenerate primer sets were used to amplify sequences based on information from internal WCR EST libraries and publicly

available insect sequences. At least two independent PCR products were examined to develop a consensus for each sequence.

In some instances, alleles were observable in the amplification products from multiple individuals. Alleles were also discernable from sequences present in the EST collections themselves when multiple overlapping ESTs were present for a given sequence. In these instances degenerate nucleotide designations were specified. These degeneracies do not denote ambiguous sequencing reads. Sequencing of target segments from multiple regional representatives of selected species may be performed in order to understand allelic variation on a regional scale.

In general, sequence identity corresponded to previously observed phylogenetic relationships (*e.g.* Clark *et al.*, 2001). WCR and MCR are closely related and NCR, also in the *virgifera* species group, bears many common stretches of identity. SCR and BCB are clearly more distinctive as members of the *fucata* species group. Each *Diabrotica* spp. exhibits unique small nucleotide polymorphisms (SNPs). If any of the SNPs fall into critical regions that give rise to efficacious siRNAs, they may affect efficacy of a given sequence used in a dsRNA construct. This may become important if a limited target sequence set is employed, for example on the order of one or a small number of efficacious siRNAs in a dsRNA construct. Having sequence available allows informed choices for target sequences in dsRNA constructs. These must however be validated for efficacy in bio-assay.

Examination of target sequences from related *Diabrotica* spp., such as BCB and SCR, may also help to determine likely polymorphic regions amongst relatively closely related species of diabroticine beetles when sequence information is not available.

EXAMPLE 5

Polymorphisms in other target sequences

Sequences from additional target genes were also obtained. These target sequences included putative orthologs of the following genes: mov34 (Flybase CG3416; SEQ ID NO:107-109); Na/K-exchanging ATPase (Flybase CG9261; SEQ ID NO:110-114); ribosomal protein L19 (Flybase CG2746; SEQ ID NO:115-118); RNA polymerase (Flybase CG3180; SEQ ID NO:119-121); ribosomal protein S9 (Flybase CG3395; SEQ ID NO:122-125); v-ATPase subunit 2 (Flybase CG3762; SEQ ID NO:126-135), in addition to carrier protein Flybase CG8055 orthologs (SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:61-64). Sequence comparisons were performed. The sequence relationships between orthologs of Flybase CG9261 in the different beetle species (FIG. 2) allowed a phylogenetic comparison (FIG. 3), which differentiates the *virgifera* group from the *fucata* group. These sequences extend the number of sequences that may be utilized in designing optimal segments for use in RNAi and other applications.

EXAMPLE 6

Mapping efficacious corn rootworm gene targets: 26mer analysis of V-ATPase subunit A

A 100 bp segment of *Diabrotica virgifera* V-ATPase subunit A was chosen for detailed efficacy mapping in a manner similar to that used to scan across a 100 bp segment of Dv49. This 100 bp segment was taken from a larger region that showed high efficacy at a discriminating dose (FIG. 6). This 100 bp region had multiple potential siRNAs with high predicted Reynolds scores and low secondary structure. Oligonucleotide pairs (vATP100-1 and vATP100-2; vATP100-3 and vATP100-4 (SEQ ID NO:73-76) were synthesized to allow amplification of template for sense or anti-sense strand transcripts. The transcript strands can then be annealed to create a 100 bp dsRNA.

Twenty-six bp segments were selected for fine mapping efficacy, tiling across the base sequence in 5 bp register. Oligonucleotides for each were synthesized as sense and anti-sense pairs (vATP_26-1 to vATP_26-30; SEQ ID NO:77-106). After annealing, the duplexes are cloned via sticky-end ligation using nucleotides added for annealing with Spe I / Eco RI cut vector (pCR2.1-TOPO). Once clones are sequence verified, templates for dsRNA synthesis are prepared using oligonucleotides pCR2.1-5 and pCR2.1-6, as for Dv49 scan in Example 2. The resulting embedded segments comprising candidate target sequences are assayed by WCR diet bio-assay for efficacy. Nucleotide sequences that encode potent siRNA derived from *Diabrotica virgifera* V-ATPase subunit A may be included with sequences derived, for instance, from *Diabrotica virgifera* Dv49, in an RNAi expression construct to yield a dsRNA-encoding construct which exhibits multiple modes of action in suppressing growth and development of the target organism.

Table 9. Oligonucleotides to allow amplification of a 100 bp segment of *Diabrotica virgifera* V-ATPase subunit A. T7 RNA polymerase promoters have been incorporated (lower case) (SEQ ID NOs:73-76).

Name	Sequence	Target DNA	Orientation	Comments
vATP100-1	taatacgactcactatagGACTTCAACCCAATCAAC	V-ATPAse subunit A	sense	for amplifying sense template to make 100mer segment of WCR V-ATPase
vATP100-2	GAATCATTTTGTGTTTGACAAGG	V-ATPAse subunit A	anti-sense	for amplifying sense template to make 100mer segment of WCR V-ATPase

Name	Sequence	Target DNA	Orientation	Comments
vATP100-3	GACTTCAACCCAATCAACATC	V-ATPAse subunit A	sense	for amplifying anti-sense template to make 100mer segment of WCR V-ATPase
vATP100-4	taatacgactcactatagGAATCATTTTGTGTTTGAC	V-ATPAse subunit A	anti-sense	for amplifying anti-sense template to make 100mer segment of WCR V-ATPase

Table 10. Oligonucleotides to allow cloning of 26 bp segments from *Diabrotica virgifera* V-ATPase subunit A (lower case). Upper case indicates restriction site overhangs incorporated to facilitate cloning (SEQ ID NOs:77-106).

Oligonucleotide	Sequence	Cloned Duplex Product	Orientation
vATP_26-1	CTAGTgacttcaacccaatcaacatcaagttG	Scan 1	sense
vATP_26-2	AATTCaacttgatgttgattgggttgaagtcA		anti-sense
vATP_26-3	CTAGTcaacccaatcaacatcaagttgggatG	Scan 2	sense
vATP_26-4	AATTCatcccaacttgatgttgattgggttgA		anti-sense
vATP_26-5	CTAGTcaatcaacatcaagttgggatctcacG	Scan 3	sense
vATP_26-6	AATTCgtgagatcccaacttgatgttgattgA		anti-sense
vATP_26-7	CTAGTaacatcaagttgggatctcacttaacG	Scan 4	sense
vATP_26-8	AATTCgttaagtgagatcccaacttgatgttA		anti-sense
vATP_26-9	CTAGTcaagttgggatctcacttaactggagG	Scan 5	sense
vATP_26-10	AATTCctccagttaagtgagatcccaacttgA		anti-sense
vATP_26-11	CTAGTtgggatctcacttaactggaggtgatG	Scan 6	sense
vATP_26-12	AATTCatcacctccagttaagtgagatcccaA		anti-sense
vATP_26-13	CTAGTtctcacttaactggaggtgatatataG	Scan 7	sense
vATP_26-14	AATTCtatatatcacctccagttaagtgagaA		anti-sense
vATP_26-15	CTAGTcttaactggaggtgatatatatggtcG	Scan 8	sense
vATP_26-16	AATTCgaccatatatatcacctccagttaagA		anti-sense
vATP_26-17	CTAGTctggaggtgatatatatggtctagttG	Scan 9	sense
vATP_26-18	AATTCaactagaccatatatatcacctccagA		anti-sense
vATP_26-19	CTAGTggtgatatatatggtctagttcatgaG	Scan 10	sense
vATP_26-20	AATTCtcatgaactagaccatatatatcaccA		anti-sense
vATP_26-21	CTAGTtatatatggtctagttcatgaaaacaG	Scan 11	sense
vATP_26-22	AATTCtgttttcatgaactagaccatatataA		anti-sense
vATP_26-23	CTAGTatggtctagttcatgaaaacacccttG	Scan 12	sense
vATP_26-24	AATTCaagggtgttttcatgaactagaccatA		anti-sense
vATP_26-25	CTAGTctagttcatgaaaacacccttgtcaaG	Scan 13	sense
vATP_26-26	AATTCttgacaagggtgttttcatgaactagA		anti-sense
vATP_26-27	CTAGTtcatgaaaacacccttgtcaaacacaG	Scan 14	sense
vATP_26-28	AATTCtgtgtttgacaagggtgttttcatgaA		anti-sense
vATP_26-29	CTAGTaaaacacccttgtcaaacacaaaatgG	Scan 15	sense
vATP_26-30	AATTCcattttgtgtttgacaagggtgttttA		anti-sense

EXAMPLE 7

Optimizing transgenes for gene suppression

Knowledge about variation within target and non-target species may also be incorporated to choose those siRNA-sized regions that most specifically target the pests of interest while
5 minimizing SNP variation that could reduce effectiveness. As plant produced siRNAs originating from known transgenes are cloned, and efficacy is confirmed by bioassay, any differences in effective siRNA production between crop and pest species given the same base target sequence may become apparent. Those sequences that effectively suppress gene expression in target insects, and have reduced capacity to initiate transgene suppression *in planta* (to help prevent
10 transgene silencing and dicing within the transgenic plant), may be selected for further analysis. Additionally, identification of effective and ineffective siRNAs allows further optimization of constructs. If UTRs or other expression elements are chosen for inclusion in a transgene construct coding for dsRNA, choosing those elements with minimal potential to produce effective siRNAs may be desired. This could be extended to coding regions when codon optimization is performed,
15 resulting in reduction in the potential for effective siRNA production or matches to endogenous miRNAs, unless such siRNA were desired.

EXAMPLE 8

Engineering stable expression of dsRNA

After selecting a pest RNAi target, one or more corresponding dsRNA segments is stably
20 expressed via a transgene *in planta*. The goal is production of a primary transcript that ultimately yields effective siRNAs when consumed by the targeted pest, but has a reduced propensity to undergo post-transcriptional gene silencing (PTGS) because the transgene has the sequences that give rise to siRNA disrupted through intron placement (*e.g.* illustrated in FIGs. 4-5).

Additional sequence such as 5' and 3' untranslated regions (UTRs) and "filler" (to make
25 exons of at least minimal required size for plant processing) can be produced by combining sequences (e.g. direct tandem sense sequence) that do not elicit effective siRNAs. The efficacy can be determined by practical evaluation of these in bio-assay or through the use of predictive tools (e.g. Reynolds scores) that consider biophysical parameters that a common to effective or ineffective siRNAs.

30 Such construct designs could result from identification of small regions exhibiting high efficacy against pest species. Regions that give rise to potent siRNAs may be disrupted by introns such as small segments of the natural gene target order or synthetic arrangements such as overlapping siRNAs as illustrated in FIG. 5. Additional exon sequences and UTRs could be created from sequence that does not give rise to productive siRNAs (*i.e.* those sequences shown in
35 bio-assay or via predictive algorithms to be poorly utilized by the RNA-induced silencing

complex (RISC) (Hammond *et al.*, 2000). Because the engineered transgene is distinct from the processed transcript as a result of disrupting the continuity of potential siRNAs, such an arrangement could result in a reduced potential to silence the transgene, including methylation and eventual transcriptional silencing via the RNA-induced initiation of transcriptional gene
5 silencing (RITS) complex (Verdel *et al.* 2004). The presence of introns in the primary transcript may also slow overall processing and potentially increase the longevity of the larger primary dsRNA transcript, thus enhancing uptake potential. Other designs for stabilizing "large" dsRNAs (*e.g.* inclusion of a nucleolar targeting sequence) would be compatible with this style of transgene construction.

10 Additional target sequences are added by extending the primary transcriptional unit with one or more additional introns and exons designed as above so that a longer dsRNA transcript could be created. Overlapping potent siRNAs and placing the intron within the overlap could expand the number of potential target sequences while minimizing the number of required introns within the construct.

15

REFERENCES

The references listed below are referred to, to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

US Patent 4,536,475; US Patent 4,693,977; US Patent 4,876,197; US Patent 4,880,734; US Patent 4,886,937; US Patent 4,940,838; US Patent 4,959,317; US Patent 5,015,580; US Patent 5,107,065; US Patent 5,110,732; US Patent 5,231,020; US Patent 5,283,184; US Patent 5,302,523; US Patent 5,384,253; US Patent 5,464,763; US Patent 5,464,765; US Patent 5,501,967; US Patent 5,508,184; US Patent 5,527,695; US Patent 5,538,880; US Patent 5,550,318; US Patent 5,563,055; US Patent 5,591,616; US Patent 5,593,874; US Patent 5,633,435; US Patent 5,693,512; US Patent 5,698,425; US Patent 5,712,135; US Patent 5,759,829; US Patent 5,780,708; US Patent 5,789,214; US Patent 5,804,693; US Patent 5,824,877; US Patent 5,837,848; US Patent 5,981,840; US Patent 6,118,047; US Patent 6,160,208; US Patent 6,326,193; US Patent 6,384,301; US Patent 6,399,861; US Patent 6,403,865; US Patent 6,506,599; US Patent 6,551,962

US Prov. Appln. 60/772,736

US Publn. 2003/0018993; US Publn. 2003/0061626; US Publn. 2003/0150017; US Publn. 2003/0175965; US Pubn. 2002/0048814

US Ser. No. 10/465,800

EP 0 120 516; EP 0 122 791; EP 0 127,328; EP 0 284044; EP 0 3215447

PCT Appln. WO 97/32016; PCT Appln. WO 99/49029; PCT Appln. WO 99/53050; PCT Appln. WO94/01550; PCT Appln. WO98/05770; PCT Appln. PCTUS2006033867

Altschul *et al., J. Mol. Biol.*, 215:403-410, 1990.

Arziman *et al.* 2005. *Nucl. Acids Res.* 33:W582-W588.

Ausubel *et al.* eds., *Protocols in Molecular Biology*, Second Edition, John Wiley & Sons, 1992

Bettencourt *et al.*, 2002. *Insect Mol. Biol.* 11:267-271.

Bevan *et al.* 1984. *Nucl. Acids Res.* 12:8711-8721

Bevan *et al., Nucleic Acids Res.*, 11(2):369-385, 1983.

Botstein *et al., Gene*, 8:17-24, 1979.

Brake *et al., Proc. Natl. Acad. Sci. USA*, 81(15):4642-4646, 1984.

Broothaerts *et al., Nature,* 433:629-633, 2005.

Brutlag *et al., Computers and Chemistry,* 17:203-207, 1993.

Bucher *et al.*, 2002. *Curr. Biol.* 12:R85-R86.

Busk *et al., Plant J.,* 11(6):1285-1295, 1997.

Carthew, 2001. *Curr. Opinion Genet. Devel.*, 13:244-248.

Clark *et al.*, 2001. *Insect Mol. Biol.* 10:303-314

Clark *et al., Blood,* 98:2887-2893, 2001.

Cogoni, and Macino. 2000. *Genes Dev* 10:638-643.

Current Protocols in Molecular Biology, Ausubel *et al.* (Eds.), Greene Publishing and Wiley-Interscience, NY, 1989.

Dellaporta *et al., In: Chromosome Structure and Function: Impact of New Concepts,* 18th Stadler Genetics Symposium, 11:263-282, 1988.

deMaagd *et al.,*2003. *Annu. Rev Genet* 37: 409-433.

Elbashir *et al., Methods*, 26:199-213, 2002.

Feinberg and Hunter. 2003. *Science*. 301:1545-7

Fire *et al.* 1998. *Nature.* 391:806–11

Gruber *et al.*, In: *Vectors for Plant Transformation*, Glick and Thompson (Eds.), CRC Press, Inc., Boca Raton, 89-119, 1993.

Hamilton and Baulcombe, *Science*, 286(5441):950-952, 1999.

Hammond *et al.*, 2000. *Nature* 404:293-296

Hannon *et al., J. Mol. Neurosci.,* 18(1-2):15-27, 2002.

Haymes *et al.*, In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, D.C., 1985

Herr 2005. *FEBS Lett.* 579:5879-88

Herrera-Estrella *et al., Nature*, 303:209-213, 1983.

Hirel *et al., Plant Molecular Biology,* 20:207-218, 1992.

Horsch *et al., Science*, 227:1229, 1985.

Ikatu *et al., Bio/Technol.*, 8:241-242, 1990.

Jefferson *et al., Biochem. Soc. Trans.*, 15:7-19, 1987.

Jefferson *et al., EMBO J.,* 6:3901-3907, 1987.

Kaeppler *et al., Plant Cell Reports,* 9:415-418, 1990.

Katz *et al., J. Gen. Microbiol.*, 129:2703-2714, 1983.

Klee *et al., Bio-Technology*, 3(7):637-642, 1985.

Miki *et al.*, In: *Methods in Plant Molecular Biology and Biotechnology*, Glick and Thompson (Eds.), CRC Press, 67–88, 1993.

Moloney *et al., Plant Cell Reports,* 8:238, 1989.

Myanohara *et al., Proc. Natl. Acad. Sci. USA*, 80(1):1-5, 1983.

Napoli *et al.*, 1990. *Plant Cell* 2:279-289.

Odell *et al., Nature,* 313:810-812, 1985.

Orr-Weaver *et al., Proc. Natl. Acad. Sci. USA*, 80(14):4417-4421, 1983.

Ow *et al., Science,* 234:856-859, 1986.

Padgette *et al.*, *Crop Sci.*, 35:1451-1461, 1995.

Potrykus *et al.*, *Mol. Gen. Genet.,* 199(2):169-177, 1985.

Reynolds *et al.*. 2004. *Nat Biotechnol.* 22:326-330.

Rine *et al., Proc. Natl. Acad. Sci. USA,* 80(22):6750-6754. 1983.

Sambrook *et al.* 1989. *Molecular Cloning: a Laboratory Manual: 2nd edition*, Cold Spring Harbor Laboratory Press

Stahl *et al., BMC Biotechnol.*, 4:31, 2004.

Stinchcomb *et al., J. Mol. Biol.*, 158(2):157-190, 1982.

Sutcliffe *et al.*, *Proc. Natl. Acad. Sci. USA,* 75:3737-3741, 1978.

Uhlirova *et al.,* 2003. *PNAS* 100: 15607-15613.

Van Heeke and Schuster, *J. Biol. Chem.,* 264(33):19475-19477, 1989.

Verdel *et al.,* 2004. *Science* 303:672-676.

Voinnet. 2005. *FEBS Lett.* 579:5858-71.

Wassenegger *et al.* 1994. *Cell* 76:567-576.

Wesley *et al.* 2001. *Plant J.* 27:581-590.

Winston, *et al.* 2002. *Science* 295: 2456-2459.

Yuan, *et al.*, 2004. *Nucl. Acids Res.* 32:W130-W134;

Zilberman *et al.,* 2004. *Curr. Biol.* 14:1214-1220.

Zukowsky *et al.*, *Proc. Natl. Acad. Sci. USA,* 80:1101-1105, 1983.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A method for managing crop pest resistance, comprising:

(a) preparing a nucleic acid by:

(a1) obtaining a starting nucleic acid molecule substantially complementary to a target pest gene wherein the target pest gene is a Western Corn Rootworm gene;

(a2) preparing a plurality of nucleic acid segments from the starting nucleic acid molecule;

(a3) assaying the nucleic acid segments for the ability to suppress expression of the target pest gene when expressed as a first dsRNA in a cell comprising the target pest gene;

(a4) identifying at least a first nucleic acid segment from the plurality of nucleic acid segments that suppresses the target pest gene when expressed as a dsRNA wherein the first nucleic acid segment comprises 19-50 contiguous nucleotides that are substantially complementary to the target pest gene; and

(a5) linking the first nucleic acid segment to a second nucleic acid segment to create a third nucleic acid segment that has 80 to 250 bases and is expressed as a third dsRNA, wherein the second nucleic acid segment is a neutral carrier sequence and does not inhibit feeding by the crop pest or progeny thereof when expressed as a dsRNA, and wherein the third dsRNA exhibits increased potency of inhibition of feeding by the crop pest or progeny thereof relative to the first dsRNA alone,

(b) contacting the crop pest with the nucleic acid and an additional agent selected from the group consisting of a patatin, a *Bacillus thuringiensis* insecticidal protein, a *Xenorhabdus* insecticidal protein, a *Photorhabdus* insecticidal protein, a *Bacillus laterosporus* insecticidal protein, a *Bacillus sphaericus* insecticidal protein, a biocontrol agent, and an insecticide;

wherein the crop pest is Western Corn Rootworm; and
wherein the target pest gene comprises SEQ ID NO: 1 or SEQ ID NO: 2.

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2. The method of claim 1, wherein the insecticide is selected from the group consisting of a carbaryl insecticide, fenvalerate, esfenvalerate, malathion, a carbofuran insecticide, chloropyrifos, fonophos, phorate, terbufos, permethrin, a neonicotinoid, and tefluthrin.

3. The method of claim 1, wherein the additional agent is provided as a seed treatment.

4. The method of claim 1, wherein the additional agent is a *Bacillus thuringiensis* insecticidal protein.

5. The method of claim 4, wherein the *Bacillus thuringiensis* insecticidal protein is selected from the group consisting of a Cryl, a Cry3, a T1C851, a CryET70, a Cry2, a ET29, a ET37, a CryET33, a CryET34, a CryET80, a CryET76, a TIC100, a TIC101, a TIC810, a T1C812, and a binary insecticidal protein PS149B1.

6. The method of claim 1, wherein the suppression is complete suppression of the target pest gene.

7. The method of claim 1, wherein the suppression is incomplete suppression of the target pest gene.

8. The method of claim 1, wherein assaying the nucleic acid segments for the ability to suppress the target gene comprises expressing the segments as the first dsRNA in a cell comprising the target pest gene and determining the level of suppression of the target pest gene.

9. The method of claim 1, wherein assaying the nucleic acid segments for the ability to suppress the target pest gene comprises expressing the segments as the first dsRNA in a cell; allowing a pest comprising the target pest gene to feed on the cell; and determining the level of suppression of the target pest gene.

10. The method of claim 1, wherein assaying the nucleic segments for the ability to suppress the target pest gene comprises calculating a Reynolds score for the nucleic acid segments.

11. The method of claim 1, wherein assaying the nucleic acid segments for the ability to suppress the target gene comprises providing said segments as dsRNA molecules in the diet of an organism comprising the target pest gene and determining the level of suppression of the target pest gene.

12. The method of claim 11, wherein determining the level of suppression of the target pest gene comprises observing morbidity, mortality, or stunting of said organism.

FIG. 1A

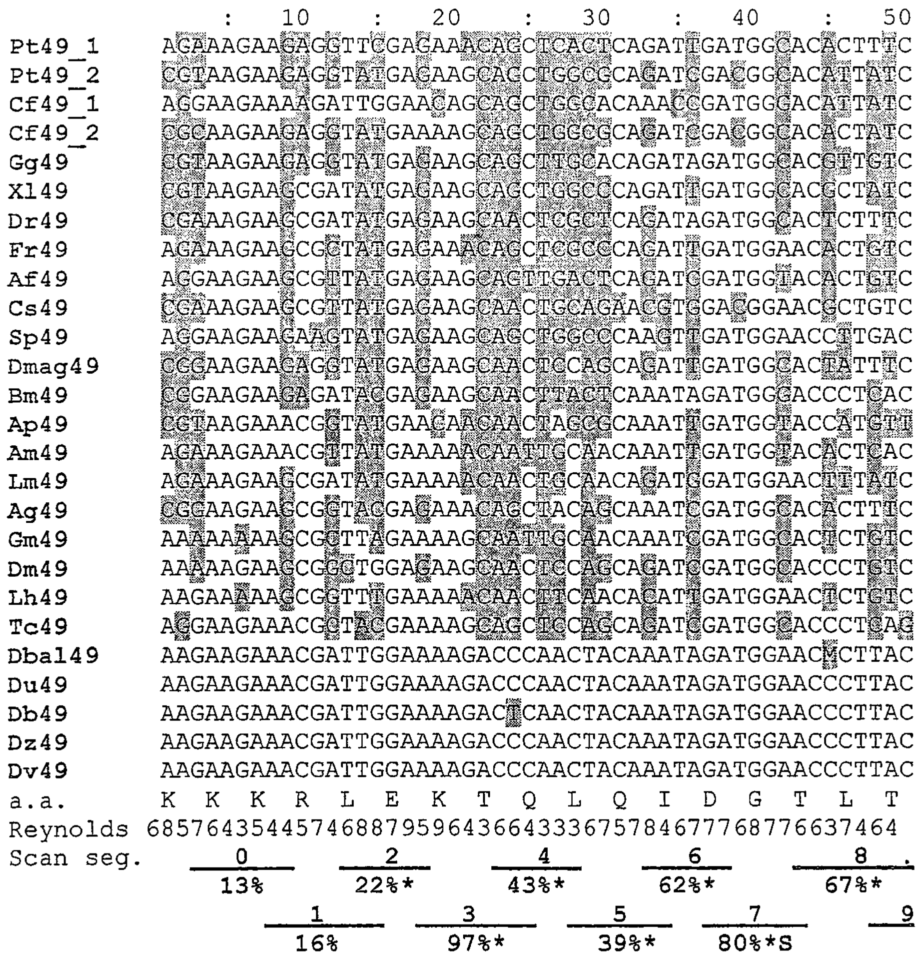

FIG. 1B

```
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Pt49_1     TACCATTGAGTTCCAGAGAGAAGCCCTGGAGAACTCACACACCAACACTG
Pt49_2     AACCATCGAGTTCCAGCGGGAGGCCCTGGAGAATGCCAACACCAACACCG
Cf49_1     CACCCTGGAGTTTCAGCGTGAGGCCATTGAGAATGCCACCACCAATGCAG
Cf49_2     AACCATCGAGTTTCAGCGGGAAGCCCTGGAGAATGCCAATACCAACACCG
Gg49       CACAATCGAATTTCAGAGGGAAGCCTTGGAGAATGCCAACACCAACACTG
Xl49       AACCATCGAATTCCAGAGGGAAGCCCTTGAAAATGCCAACACAAATACTG
Dr49       CACCATTGAGTTCCAGAGAGAAGCATTAGAAAATGCCAATACAAACACAG
Fr49       GACCATCGAGTTCCAGAGAGAAGCTTTAGAGAACGCCAACACCAGCACTGA
Af49       CACCATCGAGTTCCAGAGGGAAGCACTGGAGAATGCCAACACCAACACAG
Cs49       CACTGTTGAGTTCCAACTGGAAGCTTTACAAAATGCGCAATCAAATAAAC
Sp49       TACGATAGAAGCACAGAGGGAGGCGCTGGAGAATGCTAATACCAATGCAG
Dmag       AACTATTGAAATGCAAAGAGAAGCTTTGGAGGGAGCAAATACCAATACAG
Bm49       TCAGATTGAGCCCCAAAGGGAAGCGCTAGAAGGTGCCAATACCAATGCCC
Ap49       AACTATTGAACAACAGCGAGAGGCATTAGAAGGTGCTAACACAAATACAG
Am49       TACAATTGAAAGTCAAAGGGAAGCACTTGAATGTGCGAATACTAATACTG
Lm49       AACAATGGAGATGCAACGAGAAGCTCTTGAAGGAGCAAATACCAATACTG
Ag49       GACGATTGAAATGCAGCGAGAGGCGCTGGAGAATGCCAACACAAACGCCG
Gm49       AACAATTGAAATGCAACGAGAAGCATTGGAAAGTGCTAACACGAATACCG
Dm49       CACAATCGAAATGCAGCGCGAGGCTCTGGAGAGCGCCAACACAAACACTG
Lh49       GACGATTGAAATGCAGAGAGAAGTCTTGGAATCACCCAATACTAGTTCCA
Tc49       CACCATCGAGATGCAGCGGGAGGCCCTCGAGGGGGCCAACACCAACACAG
Dbal49     AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Du49       AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Db49       AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Dz49       AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Dv49       AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
a.a.        T  I  E  M  Q  R  E  A  L  E  G  A  S  T  N  T  A
Reynolds 22743221201351240134013134689565
Scan seg.    __10__     __12__     __14__.
               7%         14%        42%*
           _____    __11__     __13__.
            19%       5%         33%*
```

FIG. 2

WCR-Dv26	GTCGAGAACATTTTAAAATTTTTGGATAAGTACTACGTTCCCAGCAAAAT	50
MCR-Dv26	GTCGAGAACATTTTAAAATTTTTGGATAAGTACTACGTTCCCAGCAAAAT	50
NCR-Dv26	GTCGAGAACATTTTGAAATTTTTRGATAAGTACTACGTTCCCAGCAAAAT	50
SCR-Dv26	GTAGAGAACATTTTGAAATTTTTGGATAAGTACTATGTTCCCAGCAAAAT	50
BCB-Dv26	GTAGAGAACATTTTGAAATTTTTGGATAAGTACTACGTTCCCAGCAAAAT	50

WCR-Dv26	AGCTAAAGGAAATGGCCAAATWAAAACATGCTCTCATCACGACTATCCTA	100
MCR-Dv26	AGCTAAAGGAAATGGCCAAATAAAAACATGCTCTCATCACGACTATCCTA	100
NCR-Dv26	AGCTAAAGGAAATGGCCAAATAAAAACATGCTCTCATCACGACTATCCTA	100
SCR-Dv26	AGCTAAAGGAAATCGCCAAATAAAAACATGCTCTCATCACGACTATCCTA	100
BCB-Dv26	AGCTAAAGGAAATGGTCAAATAAAAACATGCTCTCATCACGACTATCCTA	100

WCR-Dv26	CTAGTGGAGAAGTATGCGAAGTCGATGTCAGAGATTGGGAAGAATGCAAC	150
MCR-Dv26	CTAGTGGAGAAGTATGCGAAGTCGATGTCAGAGATTGGGAAGAATGCAAC	150
NCR-Dv26	CTAGTGGAGAAGTATGCGAAGTCGATGTCAGAGATTGGGAAGAATGCAAC	150
SCR-Dv26	GCAGTGGAGAAGTATGCGAAGTTGATGTTAGAGATTGGGAAGAATGCAAT	150
BCB-Dv26	GCAGTGGAGAAGTATGCGAAGTTGATGTTAGAGATTGGGAGGAATGCAAT	150

WCR-Dv26	AGGGATCAATTCTTTAATTATCACAAGAATTCTCCATGTATTTTTATTAA	200
MCR-Dv26	AGGGATCAATTCTTTAATTATCACAAGAATTCTCCATGTATTTTTATTAA	200
NCR-Dv26	AGGGATCAATTCTTTAATTATCACAAGAATTCTCCATGTATTTTTATTAA	200
SCR-Dv26	AGGGATCAGTTCTTTAATTATCACAAGAATTCTCCATGTATCTTTATTAA	200
BCB-Dv26	AGGGATCAGTTCTTTAATTATCACAAGAATTCTCCATGTATTTTTATTAA	200

WCR-Dv26	ATTGAACAAAATATATAACTGGGAGCCAATRTATTACGATGATCCMTACG	250
MCR-Dv26	ATTGAACAAAATATATAACTGGGAGCCAATATATTACGATGATCCCTACG	250
NCR-Dv26	ATTGAACAAAATATATGACTGGGAGCCAGAATATTACGATGATCCCTACA	250
SCR-Dv26	ATTGAACAAAATATATGACTGGGAACCAATGTATTACGATGATCCCTATG	250
BCB-Dv26	ATTGAACAAAATATATGACTGGGAACCATTATATTACGACGATCCCTATA	250

WCR-Dv26	ATTTACCTGAGGAAATGCCGGATAATCTGAAGCAACATATAAGGGGTATC	300
MCR-Dv26	ATTTACCTGAGGAAATGCCGGATAATCTGAAGCAACATATAAGGGGTATC	300
NCR-Dv26	ATTTACCTGAGGAAATGCCGGATAATCTGAAGCAACATATAAGAAGTATC	300
SCR-Dv26	ATTTACCCGAGGAAATGCCTGATACTCTGAAGCAACATATAAGGAGTATC	300
BCB-Dv26	ATTTACCCGAGGAAATGCCTGATTGTCTGAAGCAACATATAAGAAGTATC	300

FIG. 3

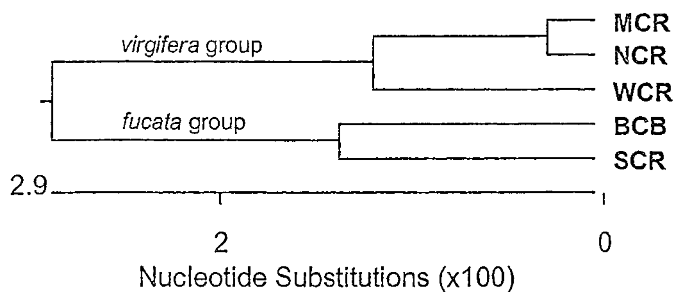


FIG. 4

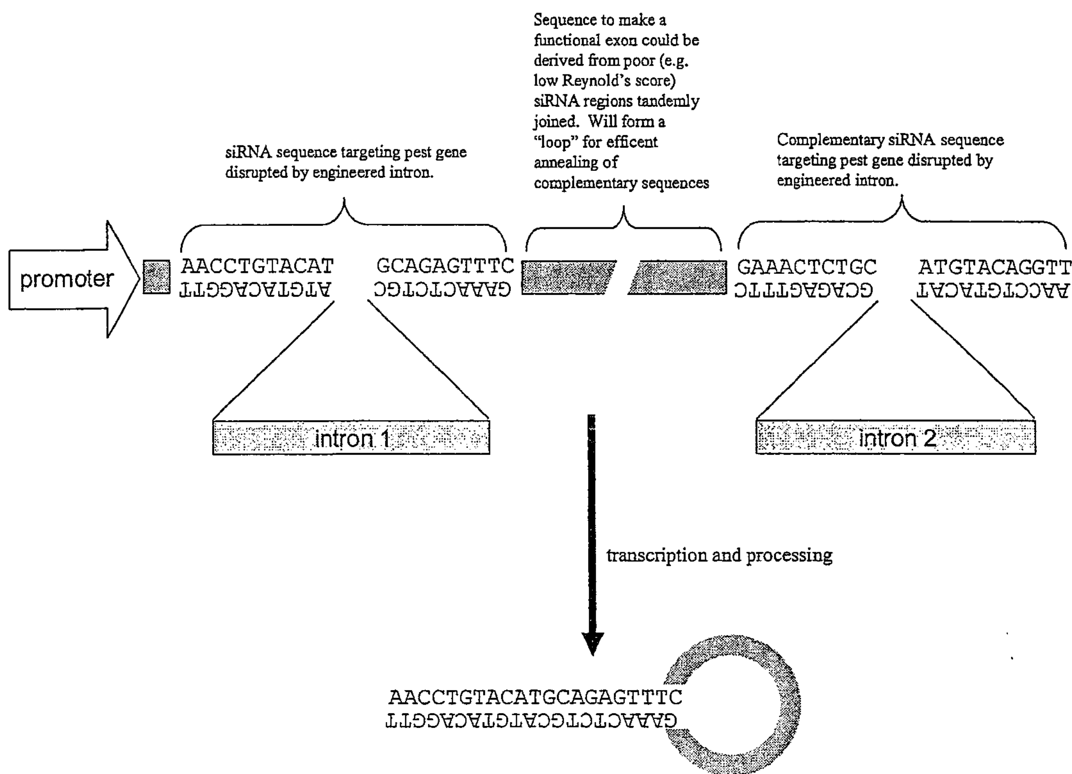


FIG. 5

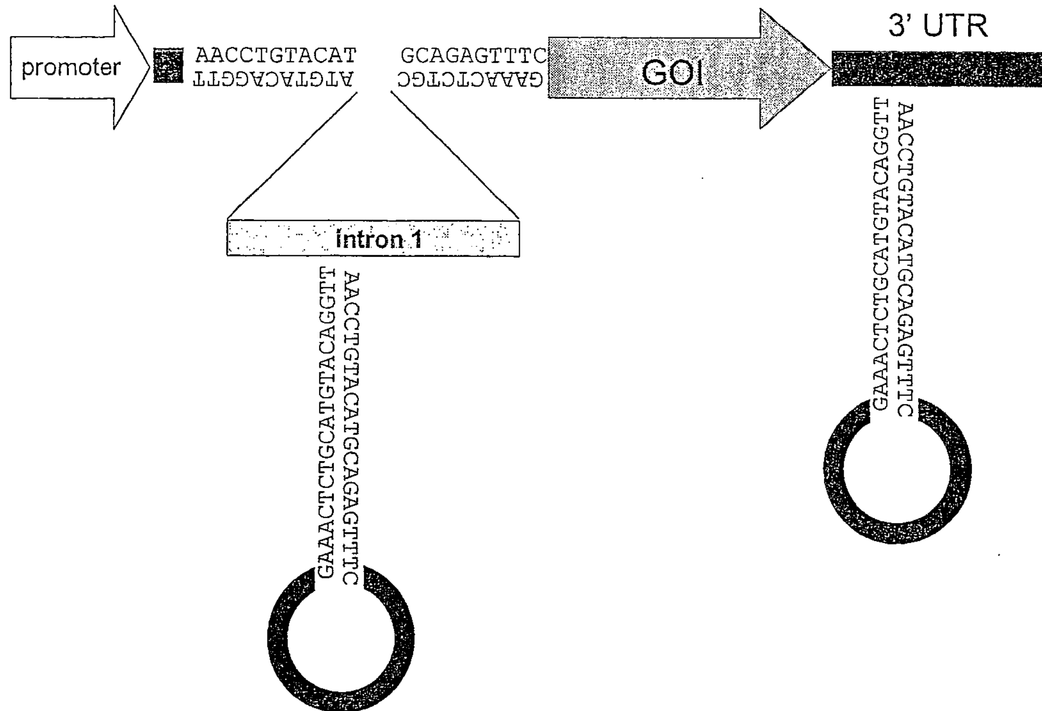
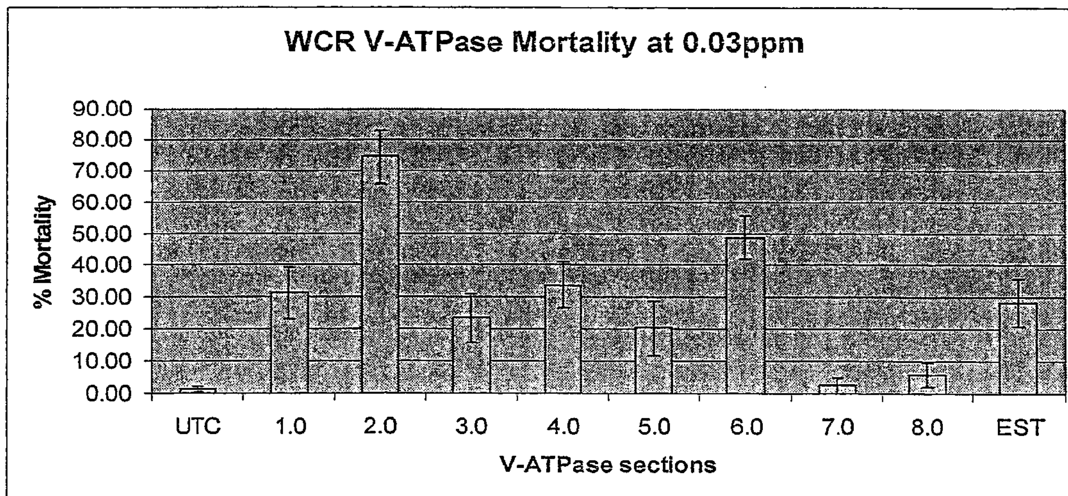


FIG. 6



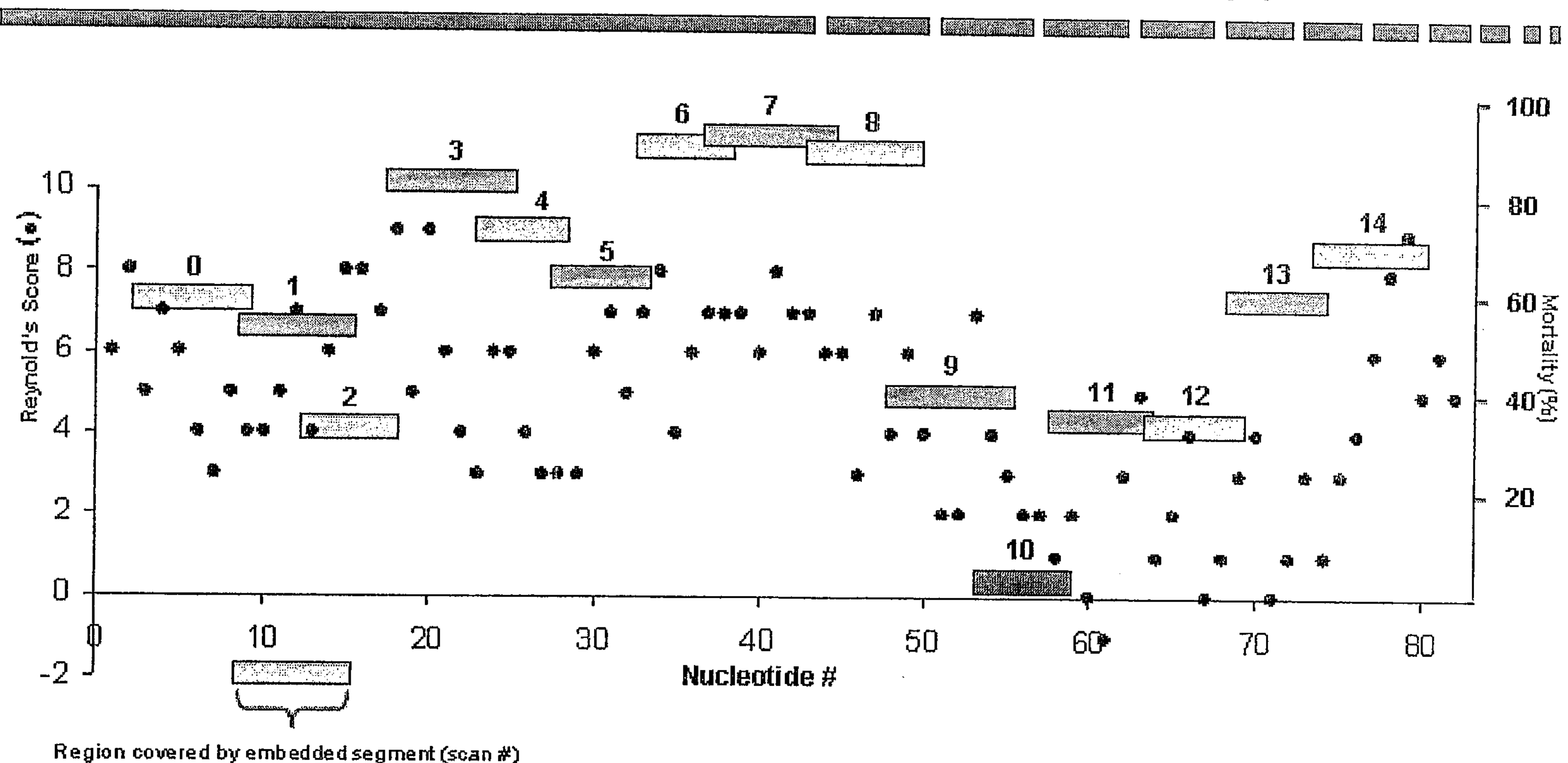


FIG. 7

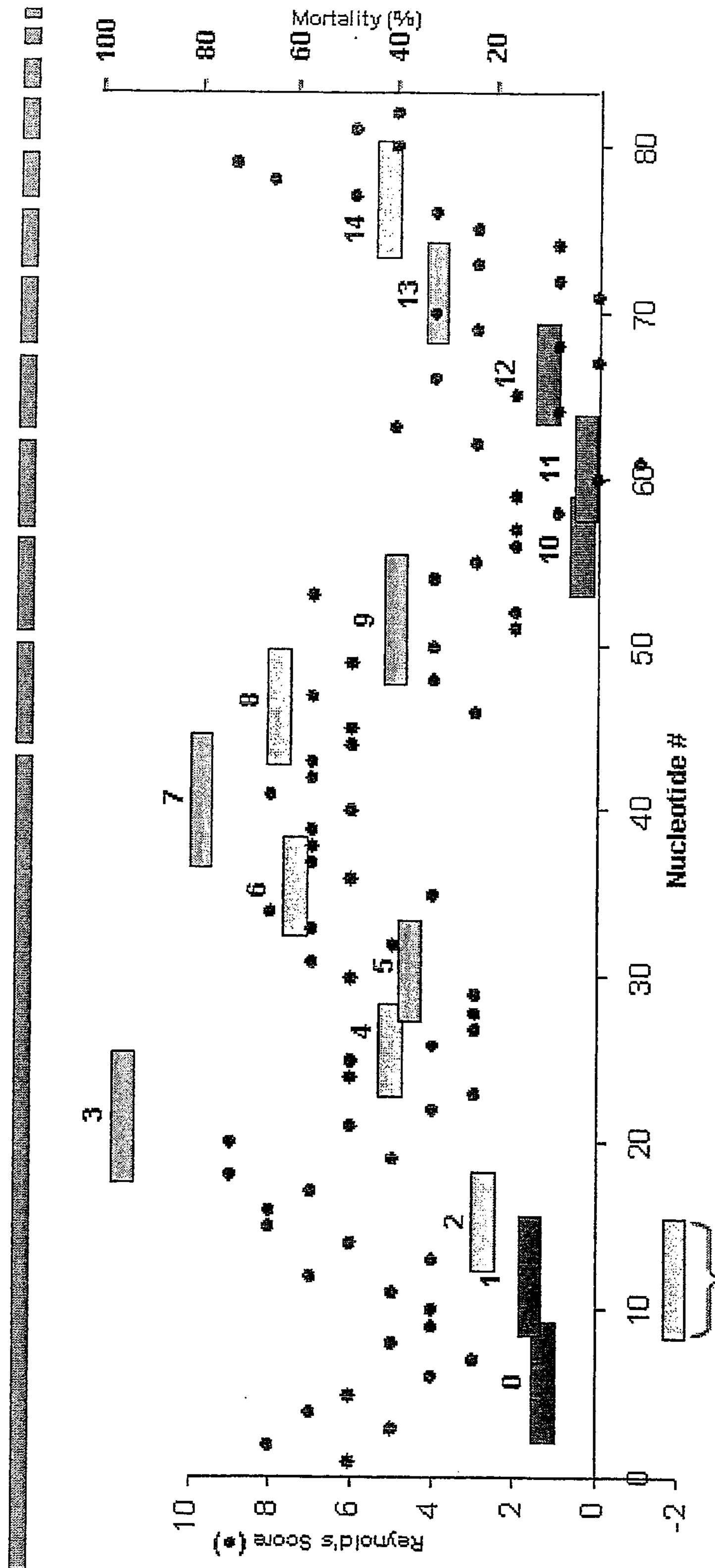


FIG. 8

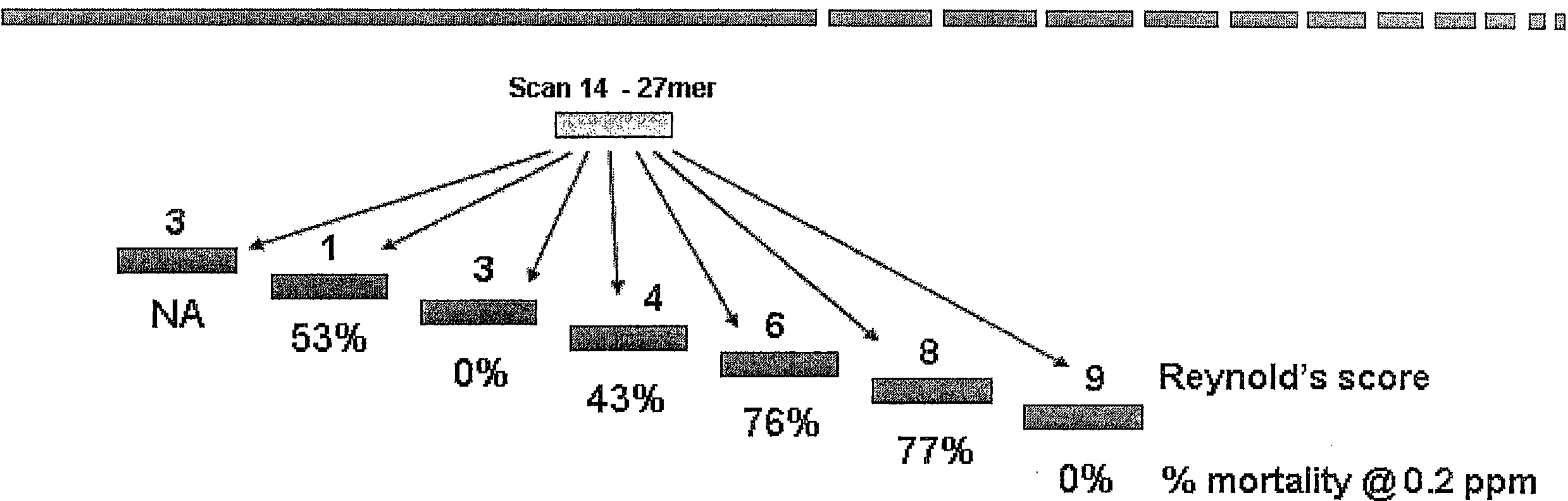


FIG. 9

A

```
               :    10    :    20    :    30    :    40    :    50
Pt49_1    AGGAAGAAGAGCTTTGAGAAAGCAGCTCAGCAGATTGATGGCACTCTTTC
Pt49_2    AGGAAGAAGAGCTTATGAGAAGCAGCTGCAGCAGATTGAGGGCACATTTC
Cf49_1    ACGAAGAAAAGATTGGAAGAGCAGCTGCACAAATTGATGGCACATTTTC
Cf49_2    AGGAAGAAGAGCTTATGAAAAGCAGCTGCGGCAGATTGAGGGCACCCTTTC
Gg49      AGGAAGAAGAGCTTATGAGAAGCAGCTGCACAGATAGATGGCACTCTTTC
Xl49      CGGAAGAAGCGATATGAGAAGCAGCTGCAGCAGATTGATGGCACCCTTTC
Dr49      CGAAAGAAGCGATTTGAGAAGCAGCTGCAGCAGATAGATGGCACCCTTTC
Fr49      AGGAAGAAGCGCTTTGAGAAGCAGCTGCAGCAGATTGATGGAACCCTGTC
Af49      AGGAAGAAGCGCTTTGAGAAGCAGCTGCAGCAGATTGATGGTACCCTGTC
Cs49      CGGAAGAAGCGCTTTGAGAAGCAGCTGCAGCAGCTTGAGGGAACCCTGTC
Sp49      AGGAAGAAGAGCTTTGAGAAGCAGCTGCAGCAAGTTGATGGAACCATGAC
Dmag49    CGGAAGAAGCGCTTTGAGAAGCAGCTGCAGCAGATTGATGGCACCATTTC
Bm49      CGGAAGAAGCGATTTGAGAAGCAGCTGCAGCAAATAGATGGCACCCTTAC
Ap49      CGAAAGAAACGCTTTGAAAAACAGCTCCAACAGCAAATTGATGGCACCTTAC
Am49      AGAAAGAAACGCTTTGAAAAACAGCTGCAACAAATTGATGGCACTCTTAC
Lm49      AGGAAGAAGCGATTTGAAAAACAGCTTCAACAGATTGATGGAACCATTTC
Ag49      AGGAAGAAGCGCTTCGAGAAACAGCTACAGCAAATTGATGGCACCCTTTC
Gm49      AAGAAGAAGCGCTTTGAAAAGCAGCTGCAACAAATTGATGGCACCCTTTC
Dm49      AAGAAGAAGCGCTTGGAGAAGCAGCTGCAGCAGATTGATGGCACCCTTTC
Lh49      AAGAAGAAGCGCTTTGAAAAACAGCTGCAACAGATTGATGGAACCCTTTC
Tc49      AGGAAGAAACGCTTTGAAAAGCAGCTGCAGCAGATTGATGGCACCCTTAG
Dbal49    AAGAAGAAACGATTGGAAAAGACCCAACTACAAATAGATGGAACCCTTAC
Du49      AAGAAGAAACGATTGGAAAAGACCCAACTACAAATAGATGGAACCCTTAC
Db49      AAGAAGAAACGATTGGAAAAGACCCAACTACAAATAGATGGAACCCTTAC
Dz49      AAGAAGAAACGATTGGAAAAGACCCAACTACAAATAGATGGAACCCTTAC
Dv49      AAGAAGAAACGATTGGAAAAGACCCAACTACAAATAGATGGAACCCTTAC
a.a.      K  K  K  R  L  E  K  T  Q  L  Q  I  D  G  T  L  T
Reynolds 68576435445746887959643664333675784677768776637464
Scan seg.      0          2          4          6          8
              13%        22%*       43%*       62%*       67%*
                    1          3          5          7          9
                   16%        97%*       39%*       80%*S
```

B

```
               :    60    :    70    :    80    :    90    :   100
Pt49_1    AACCATTGAGCTGCAGAGGGAAGCCCTGGAGAAAGCCGCCACCAAGACTG
Pt49_2    AACCATTGAGCTGCAGCGGGAAGCCCTGGAGAAGGCCAACACCAAGACGG
Cf49_1    AACCCTTGAGCTGCAGCGGGAGGCCCTTGAGAAGGCCAACACCAATGCGG
Cf49_2    AACCATTGAGCTGCAGCGGGAAGCCCTGGAGAAGGCCAACACCAAGACGG
Gg49      AACCATTGAATTGCAGAGGGAAGCCCTGGAGAAGGCCAACACCAAGACTG
Xl49      AACCATTGAATTGCAGAGGGAAGCCCTGGAAAAGGCCAACACAAATACTG
Dr49      AACCATTGAGCTGCAGAGGGAAGCCTTGGAAAAGGCCAACACAAAGACGG
Fr49      AACCATTGAGCTGCAGAGGGAAGCCTTGGAGAAGGCCAACACCAAGACTGA
Af49      AACCATTGAGCTGCAGAGGGAAGCACTGGAGAAGGCCAACACCAAGACGG
Cs49      AACTATTGAGCTGCAGCGGGGAAGCCTTGGAAAAGGCCAACACAAATACGC
Sp49      AACCATTGAAGCTCAGAGGGAAGCGCTTGAGAAAGCTAATACCAATGCGG
Dmag      AACTATTGAAATGCAGAGGGAAGCATTGGAGGGAGCTAATACCAATACGG
Bm49      AACCATTGAGCTGCAGAGGGAAGCCCTGGAAGGTGCTAATACCAATGCGG
Ap49      AACTATTGAAGCTCAGAGGGAGGCATTGGAAGGAGCTAACACAAATACGG
Am49      AACCATTGAAAGCCAGAGGGAAGCACTGGAAGGAGCCAATACCAATACTG
Lm49      AACCATTGAGATGCAGCGGGAAGCACTGGAAGGAGCCAATACCAATACTG
Ag49      AACCATTGAAATGCAGCGCGAGGCGCTGGAGGCGGCCAACACAAAGACGG
Gm49      AACCATTGAAATGCAGCGGGAAGCCTTGGAAGGCGCTAACACCAATACGG
Dm49      AACCATTGAAATGCAGCGCGAGGCGCTGGAGGGCGCCAACACAAAGACTG
Lh49      AACCATTGAAATGCAGAGCGAAGCCCTGGAAGGAGCCAATACCAATACCG
Tc49      AACCATTGAGATGCAGCGGGAGGCCCTCGAGGGCGCCAACACCAAGACGG
Dbal49    AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Du49      AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Db49      AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Dz49      AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
Dv49      AACTATTGAAATGCAGAGGGAAGCCCTCGAAGGAGCTAGCACAAATACTG
a.a.       T  I  E  M  Q  R  E  A  L  E  G  A  S  T  N  T  A
Reynolds 22743221201351240134013134689565
Scan seg.      10         12          14
               7%         14%         42%*
          ___       11          13
          19%       5%          33%*
```