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(54) Title: COMPOSITIONS AND METHODS FOR THE SUPPRESSION OF TARGET POLYNUCLEOTIDES FROM THE FAMILY APHIDIDAE

(57) Abstract: Methods and compositions are provided which employ a silencing element that, when ingested by a pest, such as a pest from the Aphididae family, they are capable of decreasing the expression of a target sequence in the pest. In specific embodiments, the decrease in expression of the target sequence controls the pest and thereby the methods and compositions are capable of limiting damage to a plant. The present invention provides target polynucleotides for specific protein classes and also target polynucleotides as set forth in SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or active variants or fragments thereof, wherein a decrease in expression of one or more the sequences in the target pest controls the pest (i.e., has insecticidal activity). Further provided are silencing elements which when ingested by the pest decrease the level of the target polypeptide and thereby control the pest. In specific embodiment, the pest is Aphis glycines. Plants, plant part, bacteria and other host cells comprising the silencing elements or an active variant or fragment thereof of the invention are also provided.

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COMPOSITIONS AND METHODS FOR THE SUPPRESSION OF TARGET POLYNUCLEOTIDES FROM THE FAMILY APHIDIDAE

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

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

BRIEF SUMMARY OF THE INVENTION

30 Methods and compositions are provided which employ a silencing element that,
when ingested by a pest, such as a pest from the Aphididae family, is capable of

decreasing the expression of a target sequence in the pest. In specific embodiments, the decrease in expression of the target sequence controls the pest and thereby the methods and compositions are capable of limiting damage to a plant or plant part. The present invention provides various target polynucleotides from specific polypeptide families as disclosed herein and various target polynucleotides as set forth in SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or active variants or fragments thereof, wherein a decrease in expression of one or more the sequences in the target pest controls the pest (i.e., has insecticidal activity). Further provided are silencing elements, which when ingested by the pest, decrease the level of expression of one or more of the target polynucleotides. In specific embodiment, the pest that is controlled is *Aphis glycines*. Plants, plant parts, bacteria and other host cells comprising the silencing elements or an active variant or fragment thereof are also provided.

A method for controlling a pest, such as a pest from the Aphididae family, is provided. The method comprises feeding to a pest a composition comprising a silencing element, wherein the silencing element, when ingested by the pest, reduces the level of a target sequence in the pest and thereby controls the pest. Further provided are methods to protect a plant from a pest. Such methods comprise introducing into the plant or plant part a silencing element of the invention. When the plant or plant part expressing the silencing element is ingested by the pest, the level of the target sequence is decreased, and the pest is controlled.

DETAILED DESCRIPTION OF THE INVENTION

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

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

herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

I. Overview

5 Methods and compositions are provided which employ a silencing element that, when ingested by a pest, such as a pest from the Aphididae family, is capable of decreasing the expression of a target sequence in the pest. In specific embodiments, the decrease in expression of the target sequence controls the pest and thereby the methods and compositions are capable of limiting damage to a plant or plant part. In specific
10 embodiments, the present invention provides target polynucleotides which encode a cuticle polypeptide, a dolichyl-di-phosphooligosaccharide-protein glycotransferase, a myosin polypeptide, a proteosome, a tousel-like kinase, a translation initiation factor 4A, and a Sar1 polypeptide. In other embodiments, the target polynucleotides encode an elongation factor, Gq-like G protein alpha subunit, or a translation initiation factor 1A. In other
15 embodiments the target polynucleotides are set forth in SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or active variants and fragments thereof. Silencing elements designed in view of these target polynucleotides are provided which, when ingested by the pest, decrease the expression of one or more of the target sequences and thereby controls the pest (i.e., has insecticidal activity).

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

 By “disease resistance” is intended that the plants avoid the disease symptoms that are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen is minimized or lessened.

30 Reducing the level of expression of the target polynucleotide or the polypeptide encoded thereby, in the pest results in the suppression, control, and/or killing the invading pathogenic organism. Reducing the level of expression of the target sequence of the pest will reduce the disease symptoms resulting from pathogen challenge by at least about 2% to at least about 6%, at least about 5% to about 50%, at least about 10% to about 60%, at

least about 30% to about 70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by pests from the Aphididae family.

5 Assays that measure the control of a pest are commonly known in the art, as are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. See, for example, Thomma *et al.* (1998) *Plant*
10 *Biology* 95:15107-15111, herein incorporated by reference. See, also the examples below.

The invention is drawn to compositions and methods for protecting plants from a plant pest, such as pests from the Aphidoidea superfamily, or inducing resistance in a plant or plant part to a plant pest, such as pests from the Aphidoidea superfamily. More specifically, members of the family Aphididae comprise an industrially significant group
15 of pests which may limit the growth and seed production of agriculturally important plants as well as serve as vectors for plant associated viruses. As used herein, the term “Aphididae” or “Aphid” is used to refer to any member of the Aphididae family.

Accordingly, the compositions and methods are useful in protecting plants against any Aphididae including, for example, peach-potato aphid *Myzus persicae*, the bean aphid
20 *Aphis fabae*, the pea aphid *Acyrtosiphum pisum*, the cabbage aphid *Brevicoryne brassicae*, the grain aphid *Sitobion avenae*, the rose-grain aphid *Metopolophium dirhodum*, the Russian wheat aphid *Diuraphis noxia* (Mordvilko), the English grain aphid *Macrosiphum avenae*, the greenbug aphid *Schizaphis graminum* (Rondani), the carrot aphid *Cavariella aegopodii*, the potato aphid *Macrosiphum euphorbiae*, the groundnut aphid *Aphis*
25 *craccivora*, the cotton aphid *Aphis gossypii*, the black citrus aphid *Toxoptera aurantii*, the brown citrus aphid *Toxoptera cidius*, the willow aphid *Cavariella* spp., the corn leaf aphid *Rhopalosiphum maidis*, the aphid *Rhopalosiphum padi*, the willow leaf aphids *Chaitophorus* spp., the black pine aphids *Cinara* spp., the sycamore aphid *Drepanosiphum platanoides*, the spruce aphids *Elatobium* spp., *Aphis citricola*, *Lipaphis pserudobrassicae*
30 (turnip aphid), *Nippolachnus piri*, the foxglove aphid *Aulacorthum solani*, the asparagus aphid *Brachycorynella asparagi*, the brown ambrosia aphid *Uroleucon ambrosiae*, the buckthorn aphid *Aphis nasturtii*, the corn root aphid *Aphis maidiradicis*, the crescentmarked lily aphid *Neomyzus circumflexes*, the goldenglow aphid *Dactynotus rudbeckiae*, the honeysuckle and parsnip aphid *Hyadaphis foeniculi*, the leafcurl plum aphid *Brachycaudus*

helichrysi, the lettuce root aphid *Pemphigus bursarius*, the mint aphid *Ovatus crataegarius*, the artichoke aphid *Capitophorus elaeagni*, the onion aphid *Neotoxoptera formosana*, the pea aphid *Macrosiphum pisi*, the rusty plum aphid *Hysteroneura setariae*, the shallot aphid *Myzus ascalonicus*, the solanum root aphid *Smynthuroides betae*, the sugarbeet root aphid *Pemphigus betae*, the tulip bulb aphid *Dysaphis tulipae*, the western aster root aphid *Aphis armoraciae*, the white aster root aphid *Prociphilus erigeronensis*. In particular embodiments, methods control the soybean aphid *Aphis glycines*. In still other embodiments, the pest of interest comprises a species from *Homoptera*, such as, white flies.

10 II. Target Sequences

As used herein, a “target sequence” or “target polynucleotide” comprises any sequence in the pest that one desires to reduce the level of expression. In specific embodiments, decreasing the level of the target sequence in the pest controls the pest. For instance the target sequence can be essential for growth and development. While the target
15 sequence can be expressed in any tissue of the pest, in specific embodiments of the invention, the sequences targeted for suppression in the pest are expressed in cells of the gut tissue of the pest, cells in the midgut of the pest, and cells lining the gut lumen or the midgut. Such target sequences can be involved in gut cell metabolism, growth or differentiation.

20 In one embodiment, the target sequence comprises a polynucleotide encoding a polypeptide belonging to one or more classes of polypeptides such as a cuticle polypeptide, a dolichyl-di-phosphooligosaccharide-protein glycotransferase, a myosin polypeptide, a proteosome, a tousel-like kinase, a translation initiation factor 4A, and a Sar1 polypeptide. In other embodiments, the target polynucleotides encode an elongation
25 factor, a Gq-like G protein alpha subunit, or a translation initiation factor 1A.

Non-limiting examples of target sequences of the invention include a polynucleotide set forth in SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or a biologically active variant or fragment thereof. As exemplified elsewhere herein, decreasing the level of expression of these target sequence in Aphididae controls the pest.

30

III. Silencing Elements

By “silencing element” is intended a polynucleotide which when ingested by a pest, is capable of reducing or eliminating the level or expression of a target polynucleotide or the polypeptide encoded thereby. The silencing element employed can

reduce or eliminate the expression level of the target sequence by influencing the level of the target RNA transcript or, alternatively, by influencing translation and thereby affecting the level of the encoded polypeptide. Methods to assay for functional silencing elements that are capable of reducing or eliminating the level of a sequence of interest are disclosed elsewhere herein. A single polynucleotide employed in the methods of the invention can comprises one or more silencing elements to the same or different target polynucleotides.

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

As discussed in further detail below, silencing elements can include, but are not limited to, a sense suppression element, an antisense suppression element, a double stranded RNA, a miRNA, or a hairpin suppression element. Non-limiting examples of silencing elements that can employed to decrease expression of these target Aphididae sequences comprise or consists of fragments and variants of the sense or antisense sequence of the sequence set forth in SEQ ID NO: 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, 89, 12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27, 28, 30, 31, 33, 34, 36, 37, 39, 40, 42, 43, 45, 46, 48, 49, 51, 52, 54, 55, 57, 58, 60, 61, 63, 64, 66, 67, 69, 70, 72, 73, 75, 76, 78, 79, 81, 82, 84, 85, 87, 88, 90, 91 or a biologically active variant or fragment thereof.

In specific embodiments, the silencing element comprises or consists of at least one of the sequences set forth in any one of SEQ ID NO:11-91. In some embodiments, the silencing element further comprises at least one additional thymine residue on the 3' end. Such residues can aid in stabilization. In specific embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more thymine residues can be added to the 3' end of the silencing elements. In further embodiments, silencing elements comprise a polynucleotide having SEQ ID NO: 12 and 13; 15 and 16; 18 and 19; 21 and 22; 24 and 25; 27 and 28; 30 and 31; 33 and 34; 36 and 37; 39 and 40; 42 and 43; 45 and 46; 48 and 49; 51 and 52; 54 and 55; 57 and 58; 60 and 61; 63 and 64; 66 and 67; 69 and 70; 72 and 73; 75 and 76; 78 and 79; 81 and 82; 84 and 85; 87 and 88; and/or 90 and 91. In other embodiments, the silencing element is designed to target the sequence set forth in SEQ ID NO: 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, or 89.

By "reduces" or "reducing" the expression level of a polynucleotide or a polypeptide encoded thereby is intended to mean, the polynucleotide or polypeptide level of the target sequence is statistically lower than the polynucleotide level or polypeptide

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

i. Sense Suppression Elements

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

Typically, a sense suppression element has substantial sequence identity to the target polynucleotide, typically greater than about 65% sequence identity, greater than about 85% sequence identity, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference. The sense suppression element can be any length so long as it allows for the suppression of the targeted sequence. The sense suppression element can be, for example, 15, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 900 or longer.

ii. Antisense Suppression Elements

As used herein, an “antisense suppression element” comprises a polynucleotide which is designed to express an RNA molecule complementary to all or part of a target messenger RNA. Expression of the antisense RNA suppression element reduces or eliminates the level of the target polynucleotide. The polynucleotide for use in antisense

suppression may correspond to all or part of the complement of the sequence encoding the target polynucleotide, all or part of the complement of the 5' and/or 3' untranslated region of the target polynucleotide, all or part of the complement of the coding sequence of the target polynucleotide, or all or part of the complement of both the coding sequence and the untranslated regions of the target polynucleotide. In addition, the antisense suppression element may be fully complementary (i.e., 100% identical to the complement of the target sequence) or partially complementary (i.e., less than 100% identical to the complement of the target sequence) to the target polynucleotide. In specific embodiments, the antisense suppression element comprises at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence complementarity to the target polynucleotide. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, U.S. Patent No. 5,942,657. Furthermore, the antisense suppression element can be complementary to a portion of the target polynucleotide. Generally, sequences of at least 25, 50, 100, 200, 300, 400, 450 nucleotides or greater may be used. Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu *et al* (2002) *Plant Physiol.* 129:1732-1743 and U.S. Patent Nos. 5,759,829 and 5,942,657, each of which is herein incorporated by reference.

iii. Double Stranded RNA Silencing Element

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

The dsRNA can reduce or eliminate the expression level of the target sequence by influencing the level of the target RNA transcript, by influencing translation and thereby

affecting the level of the encoded polypeptide, or by influencing expression at the pre-transcriptional level (i.e., via the modulation of chromatin structure, methylation pattern, etc., to alter gene expression). See, for example, Verdel *et al.* (2004) *Science* 303:672-676; Pal-Bhadra *et al.* (2004) *Science* 303:669-672; Allshire (2002) *Science* 297:1818-1819; Volpe *et al.* (2002) *Science* 297:1833-1837; Jenuwein (2002) *Science* 297:2215-2218; and Hall *et al.* (2002) *Science* 297:2232-2237. Methods to assay for functional iRNA that are capable of reducing or eliminating the level of a sequence of interest are disclosed elsewhere herein. Accordingly, as used herein, the term “dsRNA” is meant to encompass other terms used to describe nucleic acid molecules that are capable of mediating RNA interference or gene silencing, including, for example, short-interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), hairpin RNA, short hairpin RNA (shRNA), post-transcriptional gene silencing RNA (ptgsRNA), and others.

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

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

The “second segment” of the hairpin comprises a “loop” or a “loop region.” These terms are used synonymously herein and are to be construed broadly to comprise any nucleotide sequence that confers enough flexibility to allow self-pairing to occur between complementary regions of a polynucleotide (i.e., segments 1 and 3 which form the stem of the hairpin). For example, in some embodiments, the loop region may be substantially single stranded and act as a spacer between the self-complementary regions of the hairpin stem-loop. In some embodiments, the loop region can comprise a random or nonsense nucleotide sequence and thus not share sequence identity to a target polynucleotide. In

other embodiments, the loop region comprises a sense or an antisense RNA sequence or fragment thereof that shares identity to a target polynucleotide. See, for example, International Patent Publication No. WO 02/00904, herein incorporated by reference. In specific embodiments, the loop region can be optimized to be as short as possible while still providing enough intramolecular flexibility to allow the formation of the base-paired stem region. Accordingly, the loop sequence is generally less than 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 25, 20, 15, 10 nucleotides or less.

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

The first and the third segment are at least about 1000, 500, 400, 300, 200, 100, 50, 40, 30, 25, 20, 15 or 10 nucleotides in length. In specific embodiments, the length of the first and/or the third segment is about 10-100 nucleotides, about 10 to about 75 nucleotides, about 10 to about 50 nucleotides, about 10 to about 40 nucleotides, about 10 to about 35 nucleotides, about 10 to about 30 nucleotides, about 10 to about 25 nucleotides, about 10 to about 20 nucleotides. In other embodiments, the length of the first and/or the third segment comprises at least 10-20 nucleotides, 20-35 nucleotides, 30-45 nucleotides, 40-50 nucleotides, 50-100 nucleotides, or 100-300 nucleotides. See, for example, International Publication No. WO 0200904. In specific embodiments, the first and the third segment comprises at least 20 nucleotides having at least 85% complementary to the first segment. In still other embodiments, the first and the third segments which form the stem-loop structure of the hairpin comprises 3' or 5' overhang regions having unpaired nucleotide residues.

In specific embodiments, the sequences used in the first, the second, and/or the third segments comprise domains that are designed to have sufficient sequence identity to a target polynucleotide of interest and thereby have the ability to decrease the level of

expression of the target polynucleotide. The specificity of the inhibitory RNA transcripts is therefore generally conferred by these domains of the silencing element. Thus, in some embodiments of the invention, the first, second and/or third segment of the silencing element comprise a domain having at least 10, at least 15, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 500, at least 1000, or more than 1000 nucleotides that share sufficient sequence identity to the target polynucleotide to allow for a decrease in expression levels of the target polynucleotide when expressed in an appropriate cell. In other embodiments, the domain is between about 15 to 50 nucleotides, about 20-35 nucleotides, about 25-50 nucleotides, about 20 to 75 nucleotides, about 40-90 nucleotides about 15-100 nucleotides.

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

The amount of complementarity shared between the first, second, and/or third segment and the target polynucleotide or the amount of complementarity shared between the first segment and the third segment (i.e., the stem of the hairpin structure) may vary depending on the organism in which gene expression is to be controlled. Some organisms or cell types may require exact pairing or 100% identity, while other organisms or cell types may tolerate some mismatching. In some cells, for example, a single nucleotide mismatch in the targeting sequence abrogates the ability to suppress gene expression. In these cells, the suppression cassettes of the invention can be used to target the suppression of mutant genes, for example, oncogenes whose transcripts comprise point mutations and

therefore they can be specifically targeted using the methods and compositions of the invention without altering the expression of the remaining wild-type allele.

Any region of the target polynucleotide can be used to design the domain of the silencing element that shares sufficient sequence identity to allow expression of the hairpin transcript to decrease the level of the target polynucleotide. For instance, the domain can be designed to share sequence identity to the 5' untranslated region of the target polynucleotide(s), the 3' untranslated region of the target polynucleotide(s), exonic regions of the target polynucleotide(s), intronic regions of the target polynucleotide(s), and any combination thereof. In specific embodiments a domain of the silencing element shares sufficient homology to at least about 15 consecutive nucleotides from about nucleotides 1-50, 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 550-600, 600-650, 650-700, 750-800, 850-900, 950-1000, 1000-1050, 1050-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800, 1800-1900, 1900-2000 of the target sequence. In some instances to optimize the siRNA sequences employed in the hairpin, the synthetic oligodeoxyribonucleotide/RNase H method can be used to determine sites on the target mRNA that are in a conformation that is susceptible to RNA silencing. See, for example, Vickers *et al.* (2003) *J. Biol. Chem* 278:7108-7118 and Yang *et al.* (2002) *Proc. Natl. Acad. Sci. USA* 99:9442-9447, herein incorporated by reference. These studies indicate that there is a significant correlation between the RNase-H-sensitive sites and sites that promote efficient siRNA-directed mRNA degradation.

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

In specific embodiments, the silencing element comprising the hairpin comprises a sequence selected from the group consisting of SEQ ID NO: 12 and 13; 15 and 16; 18 and 19; 21 and 22; 24 and 25; 27 and 28; 30 and 31; 33 and 34; 36 and 37; 39 and 40; 42 and 43; 45 and 46; 48 and 49; 51 and 52; 54 and 55; 57 and 58; 60 and 61; 63 and 64; 66 and 67; 69 and 70; 72 and 73; 75 and 76; 78 and 79; 81 and 82; 84 and 85; 87 and 88; and/or 90 and 91.

In addition, transcriptional gene silencing (TGS) may be accomplished through use of a hairpin suppression element where the inverted repeat of the hairpin shares sequence

identity with the promoter region of a target polynucleotide to be silenced. See, for example, Aufsatz *et al.* (2002) *PNAS* 99 (Suppl. 4):16499-16506 and Mette *et al.* (2000) *EMBO J* 19(19):5194-5201.

In other embodiments, the dsRNA can comprise a small RNA (sRNA). sRNAs can
5 comprise both micro RNA (miRNA) and short-interfering RNA (siRNA) (Meister and Tuschl (2004) *Nature* 431:343-349 and Bonetta *et al.* (2004) *Nature Methods* 1:79-86). miRNAs are regulatory agents comprising about 19 ribonucleotides which are highly efficient at inhibiting the expression of target polynucleotides. See, for example Javier *et al.* (2003) *Nature* 425: 257-263, herein incorporated by reference. For miRNA
10 interference, the silencing element can be designed to express a dsRNA molecule that forms a hairpin structure containing a 19-nucleotide sequence that is complementary to the target polynucleotide of interest. The miRNA can be synthetically made, or transcribed as a longer RNA which is subsequently cleaved to produce the active miRNA. Specifically, the miRNA can comprise 19 nucleotides of the sequence having homology to a target
15 polynucleotide in sense orientation and 19 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence.

When expressing an miRNA, it is recognized that various forms of an miRNA can be transcribed including, for example, the primary transcript (termed the “pri-miRNA”) which is processed through various nucleolytic steps to a shorter precursor miRNA
20 (termed the “pre-miRNA”); the pre-miRNA; or the final (mature) miRNA is present in a duplex, the two strands being referred to as the miRNA (the strand that will eventually basepair with the target) and miRNA*. The pre-miRNA is a substrate for a form of dicer that removes the miRNA/miRNA* duplex from the precursor, after which, similarly to siRNAs, the duplex can be taken into the RISC complex. It has been demonstrated that
25 miRNAs can be transgenically expressed and be effective through expression of a precursor form, rather than the entire primary form (Parizotto *et al.* (2004) *Genes & Development* 18 :2237-2242 and Guo *et al.* (2005) *Plant Cell* 17:1376-1386).

The methods and compositions of the invention employ silencing elements that when transcribed “form” a dsRNA molecule. Accordingly, the heterologous
30 polynucleotide being expressed need not form the dsRNA by itself, but can interact with other sequences in the plant cell or in the pest gut after ingestion to allow the formation of the dsRNA. For example, a chimeric polynucleotide that can selectively silence the target polynucleotide can be generated by expressing a chimeric construct comprising the target sequence for a miRNA or siRNA to a sequence corresponding to all or part of the gene or

genes to be silenced. In this embodiment, the dsRNA is "formed" when the target for the miRNA or siRNA interacts with the miRNA present in the cell. The resulting dsRNA can then reduce the level of expression of the gene or genes to be silenced. See, for example, U.S. Provisional Application No. 60/691,613, filed June 17, 2005, entitled "Methods and Compositions for Gene Silencing, herein incorporated by reference. The construct can be designed to have a target for an endogenous miRNA or alternatively, a target for a heterologous and/or synthetic miRNA can be employed in the construct. If a heterologous and/or synthetic miRNA is employed, it can be introduced into the cell on the same nucleotide construct as the chimeric polynucleotide or on a separate construct. As discussed elsewhere herein, any method can be used to introduce the construct comprising the heterologous miRNA.

IV. Variants and Fragments

By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the native protein. Alternatively, fragments of a polynucleotide that are useful as a silencing element do not need to encode fragment proteins or variant polypeptides that retain biological activity. Thus, fragments of a nucleotide sequence may range from at least about 10, about 15, about 20 nucleotides, about 50 nucleotides, about 75 nucleotides, about 100 nucleotides, about 200 nucleotides, about 300 nucleotides, about 400 nucleotides, about 500 nucleotides, about 600 nucleotides, about 700 nucleotides and up to the full-length polynucleotide (i.e., the target polynucleotide) employed in the invention. Methods to assay for the activity of a desired silencing element or suppressor enhancer element are described elsewhere herein.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides employed in the invention. Variant polynucleotides also include synthetically derived polynucleotide, such as those generated,

for example, by using site-directed mutagenesis, but continue to retain the desired activity. Generally, variants of a particular polynucleotide of the invention (i.e., a silencing element) will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

5 Variants of a particular polynucleotide of the invention (i.e., the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides employed in the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity. A silencing element or suppressor enhancer element of a variant target sequence need not encode a protein, but rather will have the ability to reduce the level of expression of the target sequence.

10 "Variant" protein is intended to mean a protein derived from the native protein by deletion or addition of one or more amino acids at one or more internal sites in the native protein and/or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, as discussed elsewhere herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native protein will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

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

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

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

Unless otherwise stated, sequence identity/similarity values provided herein refer
15 to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any
20 sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

(c) As used herein, "sequence identity" or "identity" in the context of two
25 polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other
30 amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for

making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

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

V. DNA constructs

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

The polynucleotide encoding the silencing element employed in the methods and compositions of the invention can be provided in expression cassettes for expression in a plant or organism of interest. It is recognized that multiple silencing elements including multiple identical silencing elements, multiple silencing elements targeting different regions of the target sequence, or multiple silencing elements from different target sequences can be used. In this embodiment, it is recognized that each silencing element can be contained in a single or separate cassette, DNA construct, or vector. As discussed, any means of providing the silencing element is contemplated. A plant or plant cell can be

transformed with a single cassette comprising DNA encoding one or more silencing elements or separate cassettes comprising each silencing element can be used to transform a plant or plant cell or host cell. Likewise, a plant transformed with one component can be subsequently transformed with the second component. One or more silencing elements
5 can also be brought together by sexual crossing. That is, a first plant comprising one component is crossed with a second plant comprising the second component. Progeny plants from the cross will comprise both components.

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

The expression cassette can include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a polynucleotide comprising the silencing element employed in the methods and compositions of the invention, and a transcriptional and translational termination region (i.e., termination
25 region) functional in plants. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotides employed in the invention may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the polynucleotide employed in the invention may be heterologous to the host cell or to each other. As used herein, "heterologous" in
30 reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are

substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

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

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

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as
25 appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

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

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No.

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

Possible promoters also include the Black Cherry promoter for Prunasin Hydrolase (PH DL1.4 PRO) (US Patent No. 6,797, 859), Thioredoxin H promoter from cucumber and rice (Fukuda A *et al.* (2005). *Plant Cell Physiol.* 46(11):1779-86), Rice (RSs1) (Shi, T. Wang *et al.* (1994). *J. Exp. Bot.* 45(274): 623-631) and maize sucrose synthase -1 promoters (Yang., N-S. *et al.* (1990) *PNAS* 87:4144-4148), PP2 promoter from pumpkin Guo, H. *et al.* (2004) *Transgenic Research* 13:559-566), At SUC2 promoter (Truernit, E. *et al.* (1995) *Planta* 196(3):564-70., At SAM-1 (S-adenosylmethionine synthetase) (Mijnsbrugge KV. *et al.* (1996) *Planr. Cell. Physiol.* 37(8): 1108-1115), and the Rice tungro bacilliform virus (RTBV) promoter (Bhattacharyya-Pakrasi *et al.* (1993) *Plant J.* 4(1):71-79).

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

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

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

10 Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in
 15 the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the
 20 glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

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

(1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

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

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β -glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2):343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

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

Expression of a phloem-specific promoter need not be exclusively in phloem cells, expression in other cell types or tissues, e.g., xylem tissue, is possible. In one embodiment of this invention, a phloem-specific promoter is a plant-expressible promoter at least expressed in phloem cells, wherein the expression in non-phloem cells is more limited (or absent) compared to the expression in phloem cells. Examples of suitable vascular-specific or phloem-specific promoters in accordance with this invention include but are not limited to the promoters selected from the group consisting of: the SCSV3, SCSV4, SCSV5, and SCSV7 promoters (Schunmann *et al.* (2003) *Plant Functional Biology* 30:453-60; the rolC gene promoter of *Agrobacterium rhizogenes* (Kiyokawa *et al.* (1994) *Plant Physiology* 104:801-02; Pandolfini *et al.* (2003) *BioMedCentral (BMC) Biotechnology* 3:7, (www.biomedcentral.com/1472-6750/3/7); Graham *et al.* (1997) *Plant Mol. Biol.* 33:729-35; Guivarc'h *et al.* (1996); Almon *et al.* (1997) *Plant Physiol.* 115:1599-607; the rolA gene promoter of *Agrobacterium rhizogenes* (Dehio *et al.* (1993) *Plant Mol. Biol.* 23:1199-210); the promoter of the *Agrobacterium tumefaciens* T-DNA gene 5 (Korber *et al.* (1991) *EMBO J.* 10:3983-91); the rice sucrose synthase RSs1 gene promoter (Shi *et al.* (1994) *J. Exp. Bot.* 45:623-31); the CoYMV or Commelina yellow mottle badnavirus promoter (Medberry *et al.* (1992) *Plant Cell* 4:185-92; Zhou *et al.* (1998) *Chin. J. Biotechnol.* 14:9-16); the CFDV or coconut foliar decay virus promoter (Rohde *et al.* (1994) *Plant Mol. Biol.* 27:623-28; Hehn and Rhode (1998) *J. Gen. Virol.* 79:1495-99); the RTBV or rice tungro bacilliform virus promoter (Yin and Beachy (1995) *Plant J.* 7:969-80; Yin *et al.* (1997) *Plant J.* 12:1179-80); the pea glutamin synthase GS3A gene (Edwards *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:3459-63; Brears *et al.* (1991) *Plant J.* 1:235-44); the inv CD111 and inv CD141 promoters of the potato invertase genes (Hedley *et al.* (2000) *J. Exp. Botany* 51:817-21); the promoter isolated from *Arabidopsis* shown to have phloem-specific expression in tobacco by Kertbundit *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5212-16); the VAHOX1 promoter region (Tornerio *et al.* (1996)

Plant J. 9:639-48); the pea cell wall invertase gene promoter (Zhang *et al.* (1996) *Plant Physiol.* 112:1111-17); the promoter of the endogenous cotton protein related to chitinase of US published patent application 20030106097, an acid invertase gene promoter from carrot (Ramloch-Lorenz *et al.* (1993) *The Plant J.* 4:545-54); the promoter of the sulfate transporter gene Sultr1; 3 (Yoshimoto *et al.* (2003) *Plant Physiol.* 131:1511-17); a promoter of a sucrose synthase gene (Nolte and Koch (1993) *Plant Physiol.* 101:899-905); and the promoter of a tobacco sucrose transporter gene (Kuhn *et al.* (1997) *Science* 275:1298-1300).

The expression cassette can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D).

Additional selectable markers include phenotypic markers such as β -galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su *et al.* (2004) *Biotechnol Bioeng* 85:610-9 and Fetter *et al.* (2004) *Plant Cell* 16:215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science* 117:943-54 and Kato *et al.* (2002) *Plant Physiol* 129:913-42), and yellow florescent protein (PhiYFP™ from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science* 117:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschmidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919;

Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

5

VI. Compositions Comprising Silencing Elements

One or more of the polynucleotides comprising the silencing element and/or suppressor enhancer element can be provided as an external composition such as a spray or powder to the plant, plant part, seed, a pest, or an area of cultivation. In another example,
10 a plant is transformed with a DNA construct or expression cassette for expression of at least one silencing element. In either compositions, the silencing element, when ingested by an insect, can reduce the level of a target pest sequence and thereby control the pest (i.e., any pest from the Aphididae family, such as, *Aphis glycines*). It is recognized that the composition can comprise a cell (such as plant cell or a bacterial cell), in which a
15 polynucleotide encoding the silencing element is stably incorporated into the genome and operably linked to promoters active in the cell. Compositions comprising a mixture of cells, some cells expressing at least one silencing element are also encompassed. In other embodiments, compositions comprising the silencing elements are not contained in a cell. In such embodiments, the composition can be applied to an area inhabited by a pest. In
20 one embodiment, the composition is applied externally to a plant (i.e., by spraying a field or area of cultivation) to protect the plant from the pest.

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

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

It is recognized that the polynucleotides comprising sequences encoding the silencing element can be used to transform organisms to provide for host organism

production of these components, and subsequent application of the host organism to the environment of the target pest(s). Such host organisms include baculoviruses, bacteria, and the like. In this manner, the combination of polynucleotides encoding the silencing element may be introduced via a suitable vector into a microbial host, and said host

5 applied to the environment, or to plants or animals.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be stably incorporated into the genome of the cell (e.g., chromosome, plasmid, 10 plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

Microbial hosts that are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest may be selected. These microorganisms are selected so as to be capable of successfully competing 15 in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the sequences encoding the silencing element, and desirably, provide for improved protection of the components from environmental degradation and inactivation.

Such microorganisms include bacteria, algae, and fungi. Of particular interest are 20 microorganisms such as bacteria, e.g., *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylius*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*, fungi, particularly yeast, e.g., *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such 25 phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacteria*, *Rhodopseudomonas spheroides*, *Xanthomonas campestris*, *Rhizobium melioli*, *Alcaligenes entrophus*, *Clavibacter xyli* and *Azotobacter vinlandir*, and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C. diffluens*, *C. laurentii*, 30 *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces rosues*, *S. odoros*, *Kluyveromyces veronae*, and *Aureobasidium pollulans*. Of particular interest are the pigmented microorganisms.

A number of ways are available for introducing the polynucleotide comprising the silencing element into the microbial host under conditions that allow for stable

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

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

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

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

Host organisms of particular interest include yeast, such as *Rhodotorula spp.*, *Aureobasidium spp.*, *Saccharomyces spp.*, and *Sporobolomyces spp.*, phylloplane organisms such as *Pseudomonas spp.*, *Erwinia spp.*, and *Flavobacterium spp.*, and other

such organisms, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Bacillus thuringiensis*, *Escherichia coli*, *Bacillus subtilis*, and the like.

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

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

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

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

Such compositions disclosed above may be obtained by the addition of a surface-active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protectant, a buffer, a flow agent or fertilizers, micronutrient donors, or other preparations that influence plant growth.

One or more agrochemicals including, but not limited to, herbicides, insecticides, fungicides, bactericides, nematocides, molluscicides, acaricides, plant growth regulators, harvest aids, and fertilizers, can be combined with carriers, surfactants or adjuvants customarily employed in the art of formulation or other components to facilitate product handling and application for particular target pests. Suitable carriers and adjuvants can be

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

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

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

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

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

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

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

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

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

14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

In specific embodiments, the silencing element sequences of the invention can be provided to a plant using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the protein or variants and fragments thereof directly into the plant or the introduction of the transcript into the plant. Such methods include, for example, microinjection or particle bombardment. See, for example, Crossway *et al.* (1986) *Mol Gen. Genet.* 202:179-185; Nomura *et al.* (1986) *Plant Sci.* 44:53-58; Hepler *et al.* (1994) *Proc. Natl. Acad. Sci.* 91: 2176-2180 and Hush *et al.* (1994) *The Journal of Cell Science* 107:775-784, all of which are herein incorporated by reference. Alternatively, polynucleotides can be transiently transformed into the plant using techniques known in the art. Such techniques include viral vector system and the precipitation of the polynucleotide in a manner that precludes subsequent release of the DNA. Thus, the transcription from the particle-bound DNA can occur, but the frequency with which it is released to become integrated into the genome is greatly reduced. Such methods include the use of particles coated with polyethylimine (PEI; Sigma #P3143).

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

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

recombinogenic recombination sites that correspond to the sites of the transfer cassette. An appropriate recombinase is provided and the transfer cassette is integrated at the target site. The polynucleotide of interest is thereby integrated at a specific chromosomal position in the plant genome.

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

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

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

comosus), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). In specific embodiments, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean plants are optimal, and in yet other embodiments corn plants are optimal.

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

VIII. Methods of Use

Methods for controlling a pest (i.e., pest from the Aphididae family, such as, *Aphis glycines*) are provided. The method can comprise feeding to a pest a composition comprising a silencing element of the invention, wherein said silencing element, when

ingested by a pest (i.e., pests from the Aphididae family, such as, *Aphis glycines*), reduces the level of a target polynucleotide of the pest and thereby controls the pest. The pest can be fed the silencing element in a variety of ways. For example, in one embodiment, the polynucleotide comprising the silencing element is introduced into a plant or plant cell. As the aphid feeds on the plant, part thereof, or plant cell expressing these sequences, the silencing element is delivered to the pest. When the silencing element is delivered to the plant in this manner, it is recognized that the silencing element can be expressed constitutively or alternatively, it may be produced in a stage-specific manner by employing the various inducible or tissue-preferred or developmentally regulated promoters that are discussed elsewhere herein. In one embodiment, expression in phloem companion cells occurs. Such expression could be achieved employing a tissue specific promoter or a constitutive promoter.

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

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

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

The polynucleotides of the present invention can also be stacked with traits desirable for disease or herbicide resistance (e.g., fumonisin detoxification genes (U.S. Patent No. 5,792,931); avirulence and disease resistance genes (Jones *et al.* (1994) *Science* 266:789; Martin *et al.* (1993) *Science* 262:1432; Mindrinis *et al.* (1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)); and traits desirable for processing or process products such as high oil (e.g., U.S. Patent No. 6,232,529); modified oils (e.g., fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE), and starch debranching enzymes (SDBE)); and polymers or bioplastics (e.g., U.S. Patent No. 5,602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert *et al.* (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)); the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides providing agronomic traits such as male sterility (e.g., see U.S. Patent No. 5,583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (e.g., WO 99/61619, WO 00/17364, and WO 99/25821); the disclosures of which are herein incorporated by reference.

These stacked combinations can be created by any method including, but not limited to, cross-breeding plants by any conventional or TopCross methodology, or genetic transformation. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced

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

Methods and compositions are further provided which allow for an increase in RNAi produced from the silencing element. In such embodiments, the methods and
15 compositions employ a first polynucleotide comprising a silencing element for a target pest sequence operably linked to a promoter active in the plant cell; and, a second polynucleotide comprising a suppressor enhancer element comprising the target pest sequence or an active variant or fragment thereof operably linked to a promoter active in the plant cell. The combined expression of the silencing element with suppressor enhancer
20 element leads to an increased amplification of the inhibitory RNA produced from the silencing element over that achievable with only the expression of the silencing element alone. In addition to the increased amplification of the specific RNAi species itself, the methods and compositions further allow for the production of a diverse population of RNAi species that can enhance the effectiveness of disrupting target gene expression. As
25 such, when the suppressor enhancer element is expressed in a plant cell in combination with the silencing element, the methods and compositions can allow for the systemic production of RNAi throughout the plant; the production of greater amounts of RNAi than would be observed with just the silencing element construct alone; and, the improved loading of RNAi into the phloem of the plant, thus providing better control of phloem
30 feeding insects by an RNAi approach. Thus, the various methods and compositions provide improved methods for the delivery of inhibitory RNA to the target organism. See, for example, U.S. Provisional Application No. 61/021,676, entitled "*Compositions and Methods for the Suppression of Target Polynucleotides*", filed January 17, 2008 and herein incorporated by reference in its entirety.

As used herein, a “suppressor enhancer element” comprises a polynucleotide comprising the target sequence to be suppressed or an active fragment or variant thereof. It is recognized that the suppressor enhancer element need not be identical to the target sequence, but rather, the suppressor enhancer element can comprise a variant of the target sequence, so long as the suppressor enhancer element has sufficient sequence identity to the target sequence to allow for an increased level of the RNAi produced by the silencing element over that achievable with only the expression of the silencing element. Similarly, the suppressor enhancer element can comprise a fragment of the target sequence, wherein the fragment is of sufficient length to allow for an increased level of the RNAi produced by the silencing element over that achievable with only the expression of the silencing element. Thus, in specific embodiments, the suppressor enhancer element comprises a fragment or a variant of a polynucleotide encoding a juvenile hormone polypeptide, a vacuolar polypeptide, a cadherin polypeptide, a cuticle polypeptide, a translation initiation factor, a SAR1 polypeptide, an elongation factor, a phosphooligosaccharide, a myosin polypeptide, a potassium channel amino acid transporter, a potassium inwardly rectifier polypeptide, an amino acid transporter, a tubulin polypeptide, a ubiquitin polypeptide, and small nuclear ribonucleoprotein. In still other embodiments, the suppressor enhancer element comprises a polynucleotide set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or an active variant or fragment thereof.

It is recognized that multiple suppressor enhancer elements from the same target sequence or from different target sequences, or from different regions of the same target sequence can be employed. For example, the suppressor enhancer elements employed can comprise fragments of the target sequence derived from different region of the target sequence (i.e., from the 3'UTR, coding sequence, intron, and/or 5'UTR). Further, the suppressor enhancer element can be contained in an expression cassette, as described elsewhere herein, and in specific embodiments, the suppressor enhancer element is on the same or on a different DNA vector or construct as the silencing element. The suppressor enhancer element can be operably linked to a promoter as disclosed herein. It is recognized that the suppressor enhancer element can be expressed constitutively or alternatively, it may be produced in a stage-specific manner employing the various inducible or tissue-preferred or developmentally regulated promoters that are discussed elsewhere herein.

In specific embodiments, employing both a silencing element and the suppressor enhancer element results in the systemic production of RNAi throughout the entire plant.

In further embodiments, the plant or plant parts of the invention have improved loading of RNAi into the phloem of the plant over what is observed with the expression of the silencing element construct alone and, thus provide better control of phloem feeding insects by an RNAi approach. In specific embodiments, the plants, plant parts, and plant cells of the invention can further be characterized as allowing for the production of a diversity of RNAi species that can enhance the effectiveness of disrupting target gene expression. Thus, in specific embodiments, the combined expression of the silencing element and the suppressor enhancer element increases the concentration of the inhibitory RNA in the plant cell, plant, plant part, plant tissue or phloem over the level that is achieved when the silencing element is expressed alone.

As used herein, an "increased level of inhibitory RNA" comprises any statistically significant increase in the level of RNAi produced in a plant having the combined expression when compared to an appropriate control plant. For example, an increase in the level of RNAi in the plant, plant part or the plant cell can comprise at least about a 1%, about a 1%-5%, about a 5% -10%, about a 10%-20%, about a 20%-30%, about a 30%-40%, about a 40%-50%, about a 50%-60%, about 60-70%, about 70%-80%, about a 80%-90%, about a 90%-100% or greater increase in the level of RNAi in the plant, plant part, plant cell, or phloem when compared to an appropriate control. In other embodiments, the increase in the level of RNAi in the plant, plant part, plant cell, or phloem can comprise at least about a 1 fold, about a 1 fold-5 fold, about a 5 fold -10 fold, about a 10 fold-20 fold, about a 20 fold -30 fold, about a 30 fold -40 fold, about a 40 fold-50 fold, about a 50 fold-60 fold, about 60 fold -70 fold, about 70 fold-80 fold, about a 80 fold-90 fold, about a 90 fold-100 fold or greater increase in the level of RNAi in the plant, plant part, plant cell or phloem when compared to an appropriate control. Methods to assay for an increase in the level of RNAi are discussed elsewhere herein.

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

EXPERIMENTAL

Example 1. Specific Target Genes and Silencing Elements that Cause Insecticidal Activity Against *Aphis glycines*.

Disruption of insect gene function via RNAi can produce specific activity against target insects. This specificity is enhanced by delivery of the dsRNAs via transgenic plants. Identification of gene function in insects via RNAi has been largely limited to

injection of dsRNAs. In fact, past experiments have indicated that insects are not capable of systemic RNAi response based on exposure to dsRNAs.

As described below, we have demonstrated acute activity of numerous dsRNA pairs through injection experiments and additionally have demonstrated insect antagonism through ingestion of dsRNAs. This evidence identifies several gene/primer pair combinations with clear insecticidal properties. The use of dsRNAs in transgenic plants also addresses the potential complication of heterologous protein expression and the possible risks of allergic reaction, non-target activity, and environmental- or bioaccumulation. The data presented below represents the first test of disruption of these particular genes resulting in insecticidal activity in whole organisms and the first report of insecticidal activity of dsRNAs against *A. glycines*.

The invention describes specific target genes and the dsRNA sequences causing insecticidal activity against the soybean aphid *Aphis glycines* through RNA interference of the target gene's expression. Disruption of the genes targeted by the dsRNA sequences may be broadly insecticidal in numerous species. The specific dsRNA sequences display insecticidal activity upon ingestion indicating they can be utilized with a transgenic plant mode of delivery. Table 1 provides the polynucleotide of each target sequence from *Aphis glycines*, a brief description of the function of the protein encoded by the target sequence, and a SEQ ID NO. Table 2 and Table 3 provide a summary of primers used to suppress the target polynucleotides. Various assays to test for the pesticidal activity of dsRNA are described below.

Bioassay testing pesticidal activity of dsRNA in solution against aphids

This assay can be used for a variety of homopterans. The assay involves trapping the sample dsRNA between two layers of stretched parafilm which act as a sachet on top of a small vessel containing the insect of choice.

The assay is prepared as follows: 1cm diameter polystyrene tubing is cut into 1cm lengths. One end of the tube is then capped with parafilm or a fine mesh screen. The desired number of aphids is then added to the chamber, after which the remaining open end of the chamber is sealed with a finely stretched layer of parafilm. 20 ul of dsRNA resuspended in a solution of 20% sucrose and 0.1% green food coloring (McCormick and Company, Inc.) is added to the top of the parafilm. A second layer of parafilm is then stretched by hand and placed over the sample. The sample is spread between the two layers of parafilm to make a continuous sachet on which the insects feed. The sachet is

then covered tightly with a piece of plastic food film to prevent evaporation and produce a slightly pressurized sample.

Bioassay testing pesticidal activity of dsRNA expressed by plants against aphids

- 5 Aphid assays can be run in individual enclosures or in open systems if movement between plants can be prevented. Alternatively, a single leaf may be detached and enclosed in a container such as a Petri dish or microtiter plate. The preferred method is a whole plant assay in individual containers. When transgenic soybeans are used as the sample plant, the chamber should allow for sufficient growth for the duration of the assay.
- 10 The assay is prepared as follows: T0 transgenic events are transferred to 4 in. square pots and grown to a height of 6-8 inches. A mylar sheet is formed into a 4 in. diameter x 16 in. high cylinder and embedded into the soil of the pot to a depth of 1 inch. 10 adult aphids are then transferred either individually or as a leaf clipping to the inside of the cylinder and the cylinder is sealed with 2 tissue papers secured with two rubber bands.
- 15 After 1 week, the assay is scored for aphid mortality and/or reproduction.

Soybean aphid feeding assay

- Soybean aphids were assayed through the traditional parafilm satchet methodology. The sample of interest was added to 20% sucrose 0.1% green food coloring (McCormick and Company inc.). Data from this assay is shown in tables 2 and 3.
- 20 1. The sample was resuspended in 20% sucrose 0.1% green food coloring (McCormick and Company inc.) solution.
2. One end of 1 cm long x 1 cm wide section of polystyrene tubing was sealed with parafilm or a fine mesh.
- 25 3. The desired number of aphids was added to the polystyrene tubing.
4. The open end of the tubing with a section of finely stretched parafilm was added.
5. A 20ul droplet of sample was added to the top of the finely stretched parafilm.
6. A second piece of parafilm was held and stretched between one's hands.
7. This second parafilm membrane was placed over the sample thereby capturing the sample between the two parafilm membranes and creating the satchet.
- 30 8. The satchet was sealed with a piece of saran wrap to prevent evaporation.
9. After 5 days the number of aphids was counted and reproduction and mortality was determined.

Whole plant Aphid Assay

Aphid assays can be run in individual enclosures or in open systems if movement between plants can be prevented. A third alternative is to detach a single leaf and enclose the leaf in a container such as a Petri dish or microtiter plate. The preferred method is a whole plant assay in individual containers. For transgenic soybeans the chamber should allow for sufficient growth for the duration of the assay. The method is described below.

1. T₀ transgenic events are transferred to 4" square pots (soil, fertilizer, light, temp humidity).
2. Plants are assayed when they reach a height of 6-8 inches.
3. A mylar sheet is formed into a 4dx 16h" cylinder and embedded in the 4" pot to a depth of 1 inch.
4. 10 adult aphids are then transferred either individually or as a leaf clipping to the container.
5. The top of the cylinder is sealed with 2 tissue papers held in place with two rubber bands.
6. The assay is scored for aphid mortality and or reproduction after 1 week.
7. Upon completion of the bioassay, plants are fumigated and held for production of T1 seed.

Table 1. Target Polynucleotides from *Aphis glycines*.

SEQ ID NO:1
 >iag1c.pk005.i6
 TATTTTATTTTGCTCATAGTCCATAGATTTTATTTATCATTAAATTTAGTTTAGGTATATTGTGTACATAATATA
 AATTATCTGTTTTCTACTACGACGCTTCGTTAGGGACTTTGTTGGTAACGTAGCTGTCTTCACTTTTCGTCGGC
 CCAGACGCACAGTGAGAATCGAATTGTGGCCAACAATAATTGTTACGACATCAAACTTTTACCAATGAACGAC
 ATTAAGTTGTGACACTGATTTACTGTACATATTACTATTTTTAAAGATAATTTTCATCAAAAAATGTTTCTTTGGG
 ATTGGGTTACTGGCGTTCTGGGATATTTAGGACTGTGAAAAATCAGGAAAACTTCTATTCCCTGGTTTAGATAA
 TGCTGGAAAACTACTTTATTACATATGTTGAAAGATGATCGTTTGGCACAACACACTCCAACATTACATCCAAC
 ATCTGAGGAATTGTCTGTTGGAAACATAAAATTCACAACGTTTGATTGGGTGGCCATTCAACAAGCAAGAAAAGT
 ATGGAAAGATTATTTCCCTGCTGTAGATGCAATTGTATTTTGGTAGACGCTTGTGACAAATCTAGAATTATGGA
 AAGTAAAAATGAATTAGATTCATTATTGCTTGATGAATCCTTAnnnnACTGCCCCnTACTCGTTCTTGGGnnnnA
 AAT

SEQ ID NO:2
 >iag1c.pk007.p22
 CTTATTTATTTTATTAATAATATGAGCCCTACAGATTAATAATTGTAATTAATAAAGCAAAGTAATACTATT
 CTTGATGTTATGTATAATTGAGTACTGGATGAAGGTGTTGATTTTATTGCTGTGTCATCTACTGGAATACTTGAT
 GATGTCGAAATTAGTTGTGAGTGAGTACAATTGTGGCACCAGGACTTAGATTCAACATTCTGTAGTTGATATC
 GAGTTTCCAGAATTGAAATTCGAGTTTTCGGATCCAACAACAATAGTTAAAGTATCATGCAAATTTGATACTTGG
 TCTGATAAACGAACACGCTGTGTATAGTTTGTATCCATATTGATCATTATGTGTAGTTCTCATGTCCATGCAAA

ACAAACGAAGAACTGAAACACCAATTAAGCAGTCAACAGAAAAACAATCGAACAGCACAAAGTCACACATAAATAAG
TGCATTGAAGTGGTGAAGAAGCTATTGAAAGAAAAGTCAAATATTGAAAAAAGAAGCAAGACAGCGGTGTATG
CAAAACAGACTTCGGCTTGCCAGTTTGTAACGCAAAGGGTTGGTGCACAATTTCAAGAAAATTGGACTGATGGC
TATGCGTTTCAAGAATTGTCTAGAAGACAAGAAGAAATAACGTCAGAAAGAGAAGAGATTGACCGACAGAAAAAG
ATGTTGGTCAAAAAACGCCATCAAACAGTGAAACTGGTGGACGCAAACGAGCTAGTAGTCAGTCGGGTACAGGA
AGTAGTAGTTCGAGTACTAACAGTGTACCAATCAATTCGACGCCTTCAGTTTCTACACCACCTATAACGCTACCC
AGTGCAAGTATCAACAATAACCAAGTTACTGGTGCTAACAGGCACGGTCTTGACAACGGCACTGTAGCCCCA
TCGTCCGCCTTGACACAGCAACGTTCTAAAGCCAGAAGCTGTTCCGGGTTTATCGTGGAAGAGTATTACGAA
GCAG

SEQ ID NO:7

>iag1c.pk001.b9

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ACCTGGTACCATGGATTCCGTCAGATCTGGACCATTCCGGTCAAATTTTCAGACCAGACAACCTTTGTCTTCGGACA
GTCTGGTGCTGGAAACAATTGGGCCAAAGGTCATTACACAGAAGGTGCTGAGCTTGTAGATTCACTATTAGATGT
TGTCAGGAAAGAAGCTGAGAGCTGTGATTGTCTTCAAGGTTTCCAATTGACACATTCCTTGGGTGGTGGTACCGG
TTCTGGTATGGGAACCTTATTGATCTCCAAAATCCGTGAAGAATACCCTGACAGAATTATGAACACATACTCTGT
TGTACCCTCTCCTAAAGTATCAGACACTGTTGTAGAACCCTACAATGCTACTCTTTCAGTTCACCAATTGGTTGA
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ACCAACATATGGTGACTTAAACCACTTGGTC

SEQ ID NO:8

>iag1c.pk003.g7

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TTGTAAATCGGTCCGAAATGGCATCGGGAGTAACCGTAGCGGATGCGTGCAAAAAGGTCTACGAGGAGATCAAAA
AGGACAAGAAGCACAGGTACGTGGTGTTCACATCAAGGACGAGAAGCAGATCGACATCGAAGTCATCGGCGAGC
GTAATCTACTTATGACCTGTTCTAGAAAGACCTACAAAAGGCCGGCCGCAAGAATGCCGTTACGGTCTATTTG
ACTTTGAGTACACTCACCAGTGTCAAGGCACGTCCGAGAGCTCAAAGAAGCAAAAGCTCTTCTTGCTTTGCTGGT
GCCCAGACACAGCTAAAGTAAAGAAGAAGATGGTCTACTCATCCAGCTACGACGCGCTCAAGAAATCACTGGTTG
GCGTACACAAGGCGTTCCAGGCTACTGACCATTCCGAAGCTTCCCAAGAAGTCATCGAGGAGAAGCTCAGGTCCA
CCGACAGACAGTAAA

SEQ ID NO:9

>iag1c.pk001.j9

CGGTCCGCTTCGATGTATATACGCGCAACCGGCGGTGTTAGTGCGGTTTTTTTTATCGTTTTAACGTATATATA
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ATATAATTGTATTATATAAATATATACAGCCAGTGTTTTAAATACCGATCTACCGTCGCAAGAATCGTAAAAAG
AAAAATTGTTTTTCATTGAAAAATATTCGGTACATTTTTCTATGGTGCACCGTCCGGTAGTTGTAGTACCGTC
GAGATAACTCCTTTGAATATCGAACTCTAAACGCATTCTGTCGACGACCGTCGCGAAGTAATAAAACACCCGCGAGT
GCTGTTTCCAAATGATGACCATGAACAACCATTGGAACGACATCGACTACAGTAACGGATACATGGAACCGGAAG
AGGAATGGGAACGGGAAGGGCTGTTGGACCCAGCATGGGAAAAACAACAGAAAAAGACCTTTACAGCATGGGGCA
ACTCGCACTTACGTAAAGCAGGTACAGCCATTGAGAACATTGAAGAGGACTTCCGCAATGGGCTCAAACCTCATGT
TGCTCCTTGAnn

SEQ ID NO:10

>iag1c.pk001.l15

TAGGACCGTAGTGCCGAAACCGTTTAGTGACTACTAACTAGTAAATATGACAATTTTGTTATGCTAGTAAATACT
AAGTAGGTCCACAAGTACAAGTAACATAGTTGGAACGAACGAAATACGATGACTCAATTTCTACCACCCAATTTG
TTGGCGCTTTTCGCGCCGAGGGATCCAGTCCCTTACCTGCCGCCAGTTGCCAAATTACCGCACGAGAAGAAGACT
CGCGGGTATCTGGGCGTCGGAAGTTTTATGGATTTATTGGAAGATCCCGAAGACACTCCTCCTCCAACAAAAATT
GAAACCCGAGAAGAACGACTAGAACGCCGTCGTCGAGAGAAGGCTGAACAGGTAGCATACAAGTTAGAACAAGAA
ATTGCTTTATGGGAACCATTCAGTTGCAAACGCTACTACAGATCCATTCAAAACATTATTTGTTGCTAGAATT
AATTATGATACATCTGAATCAAAATTACGAAGAGAATTTGAACTTTACGGTCCTATAAAAAAGATTGTTGTCACA
CATAATAAGATTGATGGTAAACCGAGAGGGTATGCTTTCATTGAGTACGAATATGAACGTGATATGCATTCTGCA

TATAAACATGCTGATGGAAnnn

Table 2 (Note: the sense RNA primer sequence and the antisense RNA primer sequences shown in table 4 were generated having 2 thymine residues at the 3' end.)

Gene	id	Targeted region 5'AACTATCAAAGTAGACACCAT 3'	sense strand	antisense strand	SEQ ID NO
Cuticle protein	iag1c.pk003.c6	active	ACUAUCAAAGUAGACACCA	UGGUGUCUACUUUGAUAGU	11/12/13
dolichyl-di-phosphooligosaccharide-protein glycotransferase	iag1c.pk007.f19	active	AATCAGCACGATGACCCCTACATT	UGUAGGGUACAUCGUGCUGA	14/15/16
elongation factor	iag1c.pk003.n7	active	AAGCCAATGTAGTGTGAGACTT	GUCUACAACACUACAUUGGC	17/18/19
		active	AACTCCAAACGTTTCAGCTATCTT	GAUAGCUGAACGCUUUGGAG	20/21/22
			AATGAAATGCATAAGTTCTCCTT	GGAGAACUUUAUGCAUUUCA	23/24/25
Gq-like G protein alpha subunit	iag1c.pk007.e11		AAGGTACCCACGACGGAGCATT	UGCUCCGUCGUGGGUACCC	26/27/28
			AAGAAATTCGGTTTAGGATGTT	CCAUCUAAACCGAAUUUC	29/30/31
Myosin	iag1c.pk007.o21	active	AAAGGAAACACGTGTGCTTTCTT	GAAAGCACACGUGUUUUCU	32/33/34
		active	AAGAGAACACTGCAAAATGAGTT	CUCAUUUUGCAGUGUUUCUC	35/36/37
			AACAAGATTGGAAGACCCCTTATT	UAAGGGUCUUUCGAAUCUUG	38/39/40
proteasome	iag1c.pk006.o6		AATTGCACATGGAGATGATCTT	GAUCAUCUCCAUGUGCAA	41/42/43
			AACGTCCATATGGAGTTGGTCTT	GACCAACUCCAUAUGGACG	44/45/46
			AAGGAAGCAAGTTTGGAGGAATT	UUCUCCAAACUUGCUUCC	47/48/49
tousled-like kinase	iag1c.pk001.c6		AACGAAGAACTGAAACACCAATT	UUGGUGUUUUCAGUUCUUCG	50/51/52
			AACAGACTTCGGCTTGGCCAGTT	CUGGCCAAGCCGAAAGUCUG	53/54/55
			AATTGGACTGATGGCTATGCGTT	CGCAUAGCCCAUCAGUCCAA	56/57/58
			AATCAATTCGACGCCCTTCAGTTT	ACUGAAGGCGUCGAAUUGA	59/60/61
translation initiation factor 1A	iag1c.pk007.b12		AAAAGATACTAGCTAGTGAGTT	CUCACUAGCUCAGUAUCUU	62/63/64
		active	AAAGATACTAGCTAGTGAGTTT	ACUCACUAGCUCAGUAUCU	65/66/67
		active	AATGAAACAGAAAAAGCGTGAGTT	CUCACGCUUUUCUGUUUCA	68/69/70
			AATGCTCAAGTTACCAAAATGTT	CAUUUGGUAACUUGAGCA	71/72/73
			AAGATACAAAAGCCGATGTAATT	UUACAUCGGCUUUUGUAUC	74/75/76
translation initiation factor 4A	iag1c.pk002.a3	Active	AAATACAGCGTTCAAAAACGATT	UCGUUUUUUGAACGCUGUAU	77/78/79

putative Sar1 protein	iag1c.pk005.i6	active	AATGATTTCCTTGGGATTGGGTT	UGAUUUCUUUUGGGAUUGGG	CCCAAUCCCCAAAGAAAUCA	80/81/82
			AATCAGGAAACTTCTATTCTT	UCAGGAAAACUUCUUAUCC	GGAAUAGAAGUUUUCUGA	83/84/85
		active	AACGTTTGATTGGTGCCATT	CGUUUGAUUUGGGUGGCCA	UGGCCACCCCAAUCAAACG	86/87/88
			AATTGTATTTTGGTAGACGCTT	UUGUAUUUUUUGGUAGACGC	GCGUCUACCAAAAAUACAA	89/90/91

Table 3. Data from the Soybean Aphid Assay described above.

dsRNA #	Target	id	Assay 1 1.0uM	Assay 2 1.0uM	Assay 3 1.0uM	Assay 4 1.0uM
sucrose control	SBA		no	no	no	no
Tris control	SBA		no	no	no	no
0001	SBA	iag1c.pk003.c6	no	no	no	moderate
0002	SBA	iag1c.pk007.f19	no	no	no	moderate
0003	SBA	iag1c.pk003.n7	no	no	no	no
0004	SBA	iag1c.pk003.n7	moderate	no	no	no
0005	SBA	iag1c.pk003.n7	no	no	moderate	moderate
0006	SBA	iag1c.pk007.e11	moderate	no	no	moderate
0007	SBA	iag1c.pk007.e11	no	no	severe	severe
0008	SBA	iag1c.pk007.o21	no	no	no	no
0009	SBA	iag1c.pk007.o21	no	no	no	no
0010	SBA	iag1c.pk007.o21	no	no	severe	no
0011	SBA	iag1c.pk006.o6	no	no	no	no
0012	SBA	iag1c.pk006.o6	no	no	no	no
0013	SBA	iag1c.pk006.o6	no	no	severe	no
0014	SBA	iag1c.pk001.c6	severe	no	no	severe
0015	SBA	iag1c.pk001.c6	severe	severe	severe	severe
0016	SBA	iag1c.pk001.c6	no	severe	severe	severe
0017	SBA	iag1c.pk001.c6	severe	severe	no	no
0018	SBA	iag1c.pk007.b12	moderate	severe	no	no
0019	SBA	iag1c.pk007.b12	severe	severe	no	severe
0020	SBA	iag1c.pk007.b12	moderate	no	no	no
0021	SBA	iag1c.pk007.b12	no	severe	no	severe
0022	SBA	iag1c.pk007.b12	no	no	severe	severe
0023	SBA	iag1c.pk002.a3	no	no	no	no
0024	SBA	iag1c.pk005.i6	severe	no	no	severe
0025	SBA	iag1c.pk005.i6	moderate	no	no	severe
0026	SBA	iag1c.pk005.i6	moderate	severe	no	severe
0027	SBA	iag1c.pk005.i6	severe	no	severe	severe
0028	lamin		severe	severe	severe	no
0029	lamin		severe	severe	no	severe
0030	SBA	iag1c.pk007.b12	no	no	no	no
0031	SBA	iag1c.pk001.b9	no	severe	severe	severe
0032	SBA	iag1c.pk001.b9	no	no	severe	severe
0033	SBA	iag1c.pk001.b9	no	no	severe	no
0034	SBA	<u>iag1c.pk001.i24</u>	no	no	no	severe
0035	SBA	<u>iag1c.pk001.i24</u>	no	no	no	severe
0036	SBA	<u>iag1c.pk003.g7</u>	no	no	no	severe
0037	SBA		no	no	no	no
0038	SBA	<u>iag1c.pk003.g7</u>	nt	severe	no	severe
0039	SBA	<u>iag1c.pk003.g7</u>	nt	no	severe	severe
0040	SBA	<u>iag1c.pk003.g7</u>	nt	no	severe	no

0041	SBA	<u>iag1c.pk003.g7</u>	nt	no	no	no
0042	SBA	<u>iag1c.pk003.g7</u>	nt	no	no	no
0043	SBA	<u>iag1c.pk003.g7</u>	nt	no	no	no
0044	SBA	<u>iag1c.pk003.g7</u>	nt	no	no	no
0045	SBA	iag1c.pk007.h4	nt	no	no	no
0046	SBA	iag1c.pk007.i11	nt	no	no	no
0047	SBA	iag1c.pk001.j11	nt	no	no	no
0048	SBA	iag1c.pk001.j11	nt	no	no	no
0049	SBA	iag1c.pk001.j11	nt	no	no	severe
0050	SBA	iag1c.pk001.k11	nt	no	no	no
0051	SBA	iag1c.pk001.k11	nt	no	no	severe
0052	SBA	<u>iag1c.pk014.m6</u>	nt	no	no	severe
0053	SBA		nt	no	no	severe
0054	SBA		nt	no	no	no
0055	SBA		nt	no	no	no
0056	SBA	iag1c.pk004.n16	nt	no	no	no
0057	SBA		nt	no	no	no
0058	SBA		nt	no	no	no
0059	SBA		nt	no	no	no
0060	SBA		nt	no	no	severe
0061	SBA	<u>iag1c.pk007.p22</u>	nt	no	no	severe
0062	SBA		nt	no	no	severe
0063	SBA		nt	no	no	severe
0064	SBA		nt	severe	severe	severe
0065	SBA	iag1c.pk001.j9	nt	no	severe	severe
0066	SBA	iag1c.pk007.h2	nt	severe	severe	severe
0067	SBA		nt	no	severe	severe
0068	SBA		nt	no	no	severe
0069	SBA	iag1c.pk001.l15	nt	no	severe	severe
0070	SBA		nt	no	severe	severe
0071	SBA		nt	no	no	severe
0072	SBA	iag1c.pk002.c12	nt	no	no	no
0073	SBA	iag1c.pk001.n13	nt	no	no	no
0074	SBA		nt	no	no	no

Table 3 provides are two designations for activity; severe, and moderate. Severe activity is defined as >90% mortality in original aphids and any offspring. Moderate activity is defined as reduced reproduction combined with moderate mortality in original aphids and offspring.

5 Some surviving aphids can be found with sample demonstrating moderate activity.

Example 2. Transformation of Maize

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the silencing element of the invention operably linked to a PP2 promoter from
10 pumpkin and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

15 The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5cm target zone in preparation for bombardment.

20 A plasmid vector comprising the silencing element of interest operably linked to a PP2 promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl_2 precipitation procedure as follows: 100 μl prepared tungsten particles in water; 10 μl (1 μg) DNA in Tris EDTA buffer (1 μg total DNA); 100 μl 2.5 M CaCl_2 ; and, 10 μl 0.1 M spermidine.

25 Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 μl 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 μl 100% ethanol is added to the final tungsten
30 particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 μl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

The sample plates are bombarded at level #4 in a particle gun. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for the appropriate marker.

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H₂O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H₂O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCl, 0.10 g/l pyridoxine HCl, and 0.40 g/l glycine brought to volume with polished D-I H₂O) (Murashige and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H₂O after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling

to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCl, 0.10 g/l pyridoxine HCl, and 0.40 g/l glycine brought to volume with polished D-I H₂O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H₂O after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H₂O), sterilized and cooled to 60°C.

Various feeding assays that you would use to show that plants ingested by the *Aphids* have insecticidal activity are described above.

10 Example 3. *Agrobacterium*-mediated Transformation of Maize

For *Agrobacterium*-mediated transformation of maize with a silencing element of the invention, the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the polynucleotide comprising the silencing element to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). The immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). The immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). The immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 4: Soybean Embryo Transformation

Culture Conditions

Soybean embryogenic suspension cultures (cv. Jack) are maintained in 35 ml liquid medium SB196 (see recipes below) on rotary shaker, 150 rpm, 26°C with cool white fluorescent lights on 16:8 hr day/night photoperiod at light intensity of 60-85 $\mu\text{E}/\text{m}^2/\text{s}$. Cultures are subcultured every 7 days to two weeks by inoculating approximately 35 mg of tissue into 35 ml of fresh liquid SB196 (the preferred subculture interval is every 7 days).

Soybean embryogenic suspension cultures are transformed with the plasmids and DNA fragments described in the following examples by the method of particle gun bombardment (Klein *et al.* (1987) *Nature*, 327:70).

Soybean Embryogenic Suspension Culture Initiation

Soybean cultures are initiated twice each month with 5-7 days between each initiation.

Pods with immature seeds from available soybean plants 45-55 days after planting are picked, removed from their shells and placed into a sterilized magenta box. The soybean seeds are sterilized by shaking them for 15 minutes in a 5% Clorox solution with 1 drop of ivory soap (95 ml of autoclaved distilled water plus 5 ml Clorox and 1 drop of soap). Mix well. Seeds are rinsed using 2 1-liter bottles of sterile distilled water and those less than 4 mm are placed on individual microscope slides. The small end of the seed are cut and the cotyledons pressed out of the seed coat. Cotyledons are transferred to plates containing SB1 medium (25-30 cotyledons per plate). Plates are wrapped with fiber tape and stored for 8 weeks. After this time secondary embryos are cut and placed into SB196 liquid media for 7 days.

Preparation of DNA for Bombardment

Either an intact plasmid or a DNA plasmid fragment containing the genes of interest and the selectable marker gene are used for bombardment. Plasmid DNA for bombardment are routinely prepared and purified using the method described in the PromegaTM Protocols and Applications Guide, Second Edition (page 106). Fragments of the plasmids carrying the silencing element of interest are obtained by gel isolation of double digested plasmids. In each case, 100 μg of plasmid DNA is digested in 0.5 ml of the specific enzyme mix that is

appropriate for the plasmid of interest. The resulting DNA fragments are separated by gel electrophoresis on 1% SeaPlaque GTG agarose (BioWhitaker Molecular Applications) and the DNA fragments containing silencing element of interest are cut from the agarose gel. DNA is purified from the agarose using the GELase digesting enzyme following the manufacturer's protocol.

A 50 µl aliquot of sterile distilled water containing 3 mg of gold particles (3 mg gold) is added to 5 µl of a 1 µg/µl DNA solution (either intact plasmid or DNA fragment prepared as described above), 50 µl 2.5M CaCl₂ and 20 µl of 0.1 M spermidine. The mixture is shaken 3 min on level 3 of a vortex shaker and spun for 10 sec in a bench microfuge. After a wash with 400 µl 100% ethanol the pellet is suspended by sonication in 40 µl of 100% ethanol. Five µl of DNA suspension is dispensed to each flying disk of the Biolistic PDS1000/HE instrument disk. Each 5 µl aliquot contains approximately 0.375 mg gold per bombardment (i.e. per disk).

Tissue Preparation and Bombardment with DNA

Approximately 150-200 mg of 7 day old embryonic suspension cultures are placed in an empty, sterile 60 x 15 mm petri dish and the dish covered with plastic mesh. Tissue is bombarded 1 or 2 shots per plate with membrane rupture pressure set at 1100 PSI and the chamber evacuated to a vacuum of 27-28 inches of mercury. Tissue is placed approximately 3.5 inches from the retaining / stopping screen.

Selection of Transformed Embryos

Transformed embryos were selected either using hygromycin (when the hygromycin phosphotransferase, HPT, gene was used as the selectable marker) or chlorsulfuron (when the acetolactate synthase, ALS, gene was used as the selectable marker).

Hygromycin (HPT) Selection

Following bombardment, the tissue is placed into fresh SB196 media and cultured as described above. Six days post-bombardment, the SB196 is exchanged with fresh SB196 containing a selection agent of 30 mg/L hygromycin. The selection media is refreshed weekly. Four to six weeks post selection, green, transformed tissue may be observed growing

from untransformed, necrotic embryogenic clusters. Isolated, green tissue is removed and inoculated into multiwell plates to generate new, clonally propagated, transformed embryogenic suspension cultures.

5 *Chlorsulfuron (ALS) Selection*

Following bombardment, the tissue is divided between 2 flasks with fresh SB196 media and cultured as described above. Six to seven days post-bombardment, the SB196 is exchanged with fresh SB196 containing selection agent of 100 ng/ml Chlorsulfuron. The selection media is refreshed weekly. Four to six weeks post selection, green, transformed
10 tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated, green tissue is removed and inoculated into multiwell plates containing SB196 to generate new, clonally propagated, transformed embryogenic suspension cultures.

Regeneration of Soybean Somatic Embryos into Plants

15 In order to obtain whole plants from embryogenic suspension cultures, the tissue must be regenerated.

Embryo Maturation

Embryos are cultured for 4-6 weeks at 26°C in SB196 under cool white fluorescent
20 (Phillips cool white Econowatt F40/CW/RS/EW) and Agro (Phillips F40 Agro) bulbs (40 watt) on a 16:8 hr photoperiod with light intensity of 90-120 uE/m²s. After this time embryo clusters are removed to a solid agar media, SB166, for 1-2 weeks. Clusters are then subcultured to medium SB103 for 3 weeks. During this period, individual embryos can be removed from the clusters and screened for the appropriate marker or the ability of the plant,
25 when ingested by Aphididae, to control the Aphididae.

Embryo Desiccation and Germination

Matured individual embryos are desiccated by placing them into an empty, small petri dish (35 x 10 mm) for approximately 4-7 days. The plates are sealed with fiber tape (creating
30 a small humidity chamber). Desiccated embryos are planted into SB71-4 medium where they were left to germinate under the same culture conditions described above. Germinated

plantlets are removed from germination medium and rinsed thoroughly with water and then planted in Redi-Earth in 24-cell pack tray, covered with clear plastic dome. After 2 weeks the dome is removed and plants hardened off for a further week. If plantlets looked hardy they are transplanted to 10" pot of Redi-Earth with up to 3 plantlets per pot.

5

Media Recipes

SB 196 - FN Lite liquid proliferation medium (per liter) -

	MS FeEDTA - 100x Stock 1	10 ml
	MS Sulfate - 100x Stock 2	10 ml
10	FN Lite Halides - 100x Stock 3	10 ml
	FN Lite P,B,Mo - 100x Stock 4	10 ml
	B5 vitamins (1ml/L)	1.0 ml
	2,4-D (10mg/L final concentration)	1.0 ml
	KNO ₃	2.83 gm
15	(NH ₄) ₂ SO ₄	0.463 gm
	Asparagine	1.0 gm
	Sucrose (1%)	10 gm
	pH 5.8	

20 FN Lite Stock Solutions

	<u>Stock #</u>	<u>1000ml</u>	<u>500ml</u>
	1	MS Fe EDTA 100x Stock	
		Na ₂ EDTA *	3.724 g
		FeSO ₄ - 7H ₂ O	1.862 g
			2.784 g
25		* Add first, dissolve in dark bottle while stirring	
	2	MS Sulfate 100x stock	
		MgSO ₄ - 7H ₂ O	37.0 g
			18.5 g
		MnSO ₄ - H ₂ O	1.69 g
			0.845 g
		ZnSO ₄ - 7H ₂ O	0.86 g
			0.43 g
30		CuSO ₄ - 5H ₂ O	0.0025 g
			0.00125 g

3 FN Lite Halides 100x Stock

CaCl ₂ - 2H ₂ O	30.0 g	15.0 g
KI	0.083 g	0.0715 g
CoCl ₂ - 6H ₂ O	0.0025 g	0.00125 g

5 4 FN Lite P,B,Mo 100x Stock

KH ₂ PO ₄	18.5 g	9.25 g
H ₃ BO ₃	0.62 g	0.31 g
Na ₂ MoO ₄ - 2H ₂ O	0.025 g	0.0125 g

SB1 solid medium (per liter) comprises: 1 pkg. MS salts (Gibco/ BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 31.5 g sucrose; 2 ml 2,4-D (20mg/L final concentration); pH 5.7; and, 8 g TC agar.

SB 166 solid medium (per liter) comprises: 1 pkg. MS salts (Gibco/ BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 60 g maltose; 750 mg MgCl₂ hexahydrate; 5 g activated charcoal; pH 5.7; and, 2 g gelrite.

15 SB 103 solid medium (per liter) comprises: 1 pkg. MS salts (Gibco/BRL - Cat# 11117-066); 1 ml B5 vitamins 1000X stock; 60 g maltose; 750 mg MgCl₂ hexahydrate; pH 5.7; and, 2 g gelrite.

SB 71-4 solid medium (per liter) comprises: 1 bottle Gamborg's B5 salts w/ sucrose (Gibco/BRL - Cat# 21153-036); pH 5.7; and, 5 g TC agar.

20 2,4-D stock is obtained premade from Phytotech cat# D 295 – concentration is 1 mg/ml.

B5 Vitamins Stock (per 100 ml) which is stored in aliquots at -20C comprises: 10 g myo-inositol; 100 mg nicotinic acid; 100 mg pyridoxine HCl; and, 1 g thiamine. If the solution does not dissolve quickly enough, apply a low level of heat via the hot stir plate.

25 Chlorsulfuron Stock comprises 1mg / ml in 0.01 N Ammonium Hydroxide

Example 5. Examples of Suppression Constructs of Interest

Soybean aphid miRNA constructs were generated as described below. The miRNA sequence of interest along with the NOS terminator, XmaI, and EcoR1 sites were synthesized by DNA2.0. GUS; NOS cassettes of ubiq3 vector (PHP17474) was substituted with our cassette by SmaI and EcorR1 digests.

Construct #1

```

LOCUS      PHP35696                      5270 bp    DNA    circular
DEFINITION UBi3 pro;gmirl68.iagic.pk005.i6;NOS term.
5  SOURCE
   ORGANISM
COMMENT    Component Fragments
          #1: FRAGMENT of PHP17474
          parent position: from 6394 to 4240
10          original length: 4853
          molecule position: from 1 to 4853
          Left Terminus
             EcoRI site #1
          Right Terminus
15          SmaI site #1
          #2: FRAGMENT of gmir 168c-iaglc.pk005.i6 with R.Sites
          parent position: from 4 to 420
          original length: 417
          molecule position: from 4854 to 5270
20          Left Terminus
             SmaI site #1
          Right Terminus
             EcoRI site #1

25  COMMENT    This file is created by Vector NTI
             http://www.invitrogen.com/
   COMMENT    ORIGDB|GenBank
   COMMENT    VNTDATE|472664005|
   COMMENT    VNTDBDATE|473182290|
30  COMMENT    LSOWNER|
   COMMENT    VNTNAME|PHP35696|
   COMMENT    VNTAUTHORNAME|Mani Muthalagi|
   COMMENT    VNTAUTHORTEL|5-1975|
   COMMENT    VNTAUTHOREML|mani.muthalagi@cgr.dupont.com|
35  COMMENT    VNTREPLTYPE|Plasmid
   COMMENT    VNTEXTCHREPL|Bacteria
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/note="synthetic sequence complimentary to sequence
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45 Construct #2

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LOCUS      PHP35697                      5270 bp    DNA      circular
DEFINITION UBI3 PRO;gmir 168c-iaglc.pk007.b12;NOS term.
SOURCE
  ORGANISM
50 COMMENT  Component Fragments
           #1: FRAGMENT of PHP17474
           parent position: from 6394 to 4240
           original length: 4853
           molecule position: from 1 to 4853
55          Left Terminus

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        EcoRI site #1
        Right Terminus
        SmaI site #1
#2: FRAGMENT of gmir 168c-iaglc.pk007.bl2 with R.Sites
5   parent position: from 4 to 420
    original length: 417
    molecule position: from 4854 to 5270
    Left Terminus
10   SmaI site #1
    Right Terminus
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COMMENT      This file is created by Vector NTI
              http://www.invitrogen.com/
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    COMMENT   VNTDBDATE|473182320|
    COMMENT   LSOWNER|
    COMMENT   VNTNAME|PHP35697|
20  COMMENT   VNTAUTHORNAME|Mani Muthalagi|
    COMMENT   VNTAUTHORTEL|5-1975|
    COMMENT   VNTAUTHOREML|mani.muthalagi@cgr.dupont.com|
FEATURES
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30 Construct #3

LOCUS PHP35698 5270 bp DNA circular
 DEFINITION UBI3 PROO gmir168c-iaglc.pk001.c6. miRNA construct of AG-TIK
 for SOY.

SOURCE

35 ORGANISM

COMMENT Component Fragments

#1: FRAGMENT of PHP17474

parent position: from 6394 to 4240

original length: 4853

40 molecule position: from 1 to 4853

Left Terminus

EcoRI site #1

Right Terminus

SmaI site #1

45 #2: FRAGMENT of gmir168c-iaglc.pk001.c6 with R.Sites

parent position: from 4 to 420

original length: 417

molecule position: from 4854 to 5270

Left Terminus

50 SmaI site #1

Right Terminus

EcoRI site #1

COMMENT This file is created by Vector NTI

55 <http://www.invitrogen.com/>

COMMENT ORIGDB|GenBank

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COMMENT      VNTDBDATE|473182342|
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5 COMMENT     VNTNAME|PHP35698|
COMMENT      VNTAUTHORNAME|Mani Muthalagi|
COMMENT      VNTAUTHORTEL|5-1975|
COMMENT      VNTAUTHOREML|mani.muthalagi@cgr.dupont.com|
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               /note="The first intron at 5'-end of gene"
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25    misc_RNA   4877..4897
               /vntifkey="53"
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               /note="Synthetic sequence complementary to tousled
like kinase from Aphis glycines. This is an artificial microRNA."
30    misc_RNA   complement(4944..4964)
               /vntifkey="53"
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               /note="Synthetic sequence complimentary to sequence
AG-MTIK to silence the expression of tousled like kinase in Aphis
35 glycines.This sequence forms the hairpin in an artificial microRNA
precursor"
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 15 5221 caaactagga taaattatcg cgcgcggtgt catctatgtt actagatcgg

The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least
 20 one) of the grammatical object of the article. By way of example, "an element" means one or
 more element.

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

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

THAT WHICH IS CLAIMED:

1. A plant cell having stably incorporated into its genome a heterologous polynucleotide comprising a silencing element, wherein said silencing element, when ingested
5 by a pest, reduces the level of a target sequence in said pest, wherein said pest is from the family Aphididae and said silencing element comprises

a) a polynucleotide comprising the sense or antisense sequence of the sequence set forth in SEQ ID NO:11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, or 89;

10 b) a polynucleotide comprising the sense or antisense sequence of a sequence having at least 95% sequence identity to the sequence set forth in SEQ ID NO: 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, or 89;

c) a polynucleotide comprising the sequence set forth in SEQ ID NO:12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27, 28, 30, 31, 33, 34, 36, 37, 39, 40, 42, 43, 45, 46, 48, 49, 51, 52, 15 54, 55, 57, 58, 60, 61, 63, 64, 66, 67, 69, 70, 72, 73, 75, 76, 78, 79, 81, 82, 84, 85, 87, 88, 90 or 91; and

d) a polynucleotide comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27, 28, 30, 31, 33, 34, 36, 37, 39, 40, 42, 43, 45, 46, 48, 49, 51, 52, 54, 55, 57, 58, 60, 61, 63, 64, 66, 67, 69, 70, 72, 20 73, 75, 76, 78, 79, 81, 82, 84, 85, 87, 88, 90 or 91.

2. The plant cell of claim 1, wherein said pest comprises *Aphis glycines*.

3. The plant cell of claim 1 or 2, wherein the reduction of the level of target pest
25 sequence controls the pest from the family Aphididae.

4. The plant cell of any one of claims 1-3, wherein said silencing element comprises a hairpin RNA.

30 5. The plant cell of claim 1, 2, or 3, wherein said polynucleotide comprising the silencing element comprises, in the following order, a first segment, a second segment, and a third segment, wherein

- a) said first segment comprises the nucleotide sequence of claim 1(d);
- b) said second segment comprises a loop of sufficient length to allow the silencing element to be transcribed as a hairpin RNA; and,
- c) said third segment comprises a nucleotide sequence having at least 85% complementary to the first segment.

6. The plant cell of any one of claims 1-5, wherein said silencing element is operably linked to a heterologous promoter.

7. The plant cell of any one of claims 1-6, wherein said plant cell has stably incorporated into its genome a second polynucleotide comprising a suppressor enhancer element comprising the target pest sequence or an active variant or fragment thereof, wherein the combined expression of the silencing element and the suppressor enhancer element increases the concentration of an inhibitory RNAi specific for the pest target sequence in said plant cell.

8. The plant cell of any one of claims 1-7, wherein said plant cell is from a monocot.

9. The plant cell of claim 8, wherein said monocot is maize, barley, millet, wheat or rice.

10. The plant cell of any one of claims 1-7, wherein said plant is from a dicot.

11. The plant cell of claim 10, wherein said plant cell is from soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

12. A plant or plant part comprising a plant cell of any one of claims 1-11.

13. The plant or plant part of claim 12, wherein the combined expression of said silencing element and the suppressor enhancer element increases the concentration of an inhibitory RNA specific for the pest target sequence in the phloem of said plant or plant part.

14. A transgenic seed from the plant of any one of claims 12 or 13.

15. A method for controlling Aphididae comprising feeding to a Aphididae a
5 composition comprising a silencing element, wherein said silencing element, when ingested
by said Aphididae, reduces the level of a target Aphididae sequence and thereby controls the
Aphididae, wherein the silencing element comprises

a) a polynucleotide comprising the sense or antisense sequence of the sequence
set forth in SEQ ID NO:11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62,
10 65, 68, 71, 74, 77, 80, 83, 86, or 89;

b) a polynucleotide comprising the sense or antisense sequence of a sequence
having at least 95% sequence identity to the sequence set forth in SEQ ID NO: 11, 14, 17, 20,
23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59, 62, 65, 68, 71, 74, 77, 80, 83, 86, or 89;

c) a polynucleotide comprising the sequence set forth in SEQ ID NO:12, 13, 15,
15 16, 18, 19, 21, 22, 24, 25, 27, 28, 30, 31, 33, 34, 36, 37, 39, 40, 42, 43, 45, 46, 48, 49, 51, 52,
54, 55, 57, 58, 60, 61, 63, 64, 66, 67, 69, 70, 72, 73, 75, 76, 78, 79, 81, 82, 84, 85, 87, 88, 90
or 91; and

d) a polynucleotide comprising a nucleotide sequence having at least 95%
sequence identity to SEQ ID NO: 12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27, 28, 30, 31, 33, 34,
20 36, 37, 39, 40, 42, 43, 45, 46, 48, 49, 51, 52, 54, 55, 57, 58, 60, 61, 63, 64, 66, 67, 69, 70, 72,
73, 75, 76, 78, 79, 81, 82, 84, 85, 87, 88, 90 or 91.

16. The method of claim 15, wherein said composition comprises a plant or plant
part having stably incorporated into its genome a polynucleotide comprising said silencing
25 element.

17. The method of claim 15 or 16, wherein said pest comprises *Aphis glycines*.

18. The method of claim 15, 16, or 17, wherein said polynucleotide comprising the
30 silencing element comprising, in the following order, a first segment, a second segment, and a
third segment, wherein

a) said first segment comprises the nucleotide sequence of claim 15(d);

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

c) said third segment having at least 85% complementary to the first segment.

5

19. The method of any one of claims 15-18, wherein said silencing element is operably linked to a heterologous promoter.

20. The method of any one of claims 15-19, wherein said plant cell has stably incorporated into its genome a second polynucleotide comprising a suppressor enhancer element comprising the target pest sequence or an active variant or fragment thereof, wherein the combined expression of the silencing element and the suppressor enhancer element increases the concentration of an inhibitory RNAi specific for the pest target sequence in said plant cell.

15

21. The method of claim 20, wherein the combined expression of said silencing element and the suppressor enhancer element increases the concentration of an inhibitory RNA specific for the pest target sequence in the phloem of said plant or plant part.

20

22. The method of anyone of claims 15-21, wherein said plant is a monocot.

23. The method of claim 22, wherein said monocot is maize, barley, millet, wheat or rice.

25

24. The method of anyone of claims 15-21, wherein said plant is a dicot.

25. The method of claim 24, wherein said plant is soybean, canola, alfalfa, sunflower, safflower, tobacco, *Arabidopsis*, or cotton.

30