



- (51) International Patent Classification:
C12N 15/82 (2006.01)
- (21) International Application Number:
PCT/IL2013/051083
- (22) International Filing Date:
30 December 2013 (30.12.2013)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
- | | | |
|------------|-------------------------------|----|
| 61/748,095 | 1 January 2013 (01.01.2013) | US |
| 61/748,101 | 1 January 2013 (01.01.2013) | US |
| 61/748,094 | 1 January 2013 (01.01.2013) | US |
| 61/748,099 | 1 January 2013 (01.01.2013) | US |
| 61/814,888 | 23 April 2013 (23.04.2013) | US |
| 61/814,892 | 23 April 2013 (23.04.2013) | US |
| 61/814,899 | 23 April 2013 (23.04.2013) | US |
| 61/814,890 | 23 April 2013 (23.04.2013) | US |
| 61/908,855 | 26 November 2013 (26.11.2013) | US |
| 61/908,965 | 26 November 2013 (26.11.2013) | US |
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).
- Published:
- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
 - with sequence listing part of description (Rule 5.2(a))

(54) Title: ISOLATED dsRNA MOLECULES AND METHODS OF USING SAME FOR SILENCING TARGET MOLECULES OF INTEREST

dsRNA CONSTRUCT #1 - EXOGENOUS TRIGGER CONTROL



FIG. 2B

(57) Abstract: An isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA sequence is flanked by two complementary sites to an smRNA or smRNAs expressed in the plant and wherein the dsRNA molecule further comprises a helicase binding site positioned so as to allow unwinding of the strands of the isolated dsRNA molecule to single stranded RNA (ssRNA) and recruitment of an RNA-dependent RNA Polymerase so as to amplify the dsRNA molecule in the plant cell and generate secondary siRNA products of the dsRNA sequence.



WO 2014/106837 A2

ISOLATED dsRNA MOLECULES AND METHODS OF USING SAME FOR
SILENCING TARGET MOLECULES OF INTEREST

SEQUENCE LISTING STATEMENT

5 The ASCII file, entitled 58068 P34097-12-22-2013_ST25.txt, created on
29 December 2013, comprising 64,103 bytes, submitted concurrently with the filing of
this application.

FIELD OF THE DISCLOSURE

10 The present disclosure provides for, and includes, methods and compositions for
silencing target molecules of plants and plant pathogens. Also provided are plants,
plant parts and seeds having dsRNAs and methods of introducing dsRNAs into seeds.

BACKGROUND

 The present disclosure, in some embodiments thereof, relates to isolated dsRNA
molecules and methods of using same for silencing target molecules of interest.

15 RNA interference (RNAi) has been shown effective in silencing gene expression
in a broad variety of species, including plants, with wide ranging implications for
cancer, inherited disease, infectious disease in plants and animals. Studies in a variety
of organisms have shown that effectors of RNAi include dsRNA and related small
interfering RNAs (siRNAs; also called “short interfering RNAs” and “silencing
20 RNAs”). Studies have also shown in a variety of organisms that dsRNA or their siRNA
derivatives can be used to arrest, retard or even prevent a variety of pathogens, most
notably viral diseases (see, for example, PCT Patent Application Publication No.
WO/2003/004649).

25 It has been shown in some species that RNAi mediated interference spreads
from the initial site of dsRNA delivery, producing interference phenotypes throughout
the injected animal. Recently the same spreading effect of dsRNA has been
demonstrated in bee larva. In addition, homologs of transmembrane proteins called
systemic RNA interference defective proteins (SID) have been detected in, for example,
humans, mouse and *C. elegans*. It is thought that SID transmembrane channels are
30 responsible for endocytic uptake and spreading effect of dsRNA (Aronstein *et al.*, J.
Apic Res and Bee World, 2006; 45:20-24; *see also* van Roessel P, Brand AH.,
“Spreading silence with Sid,” Genome Biol. 5(2):208 (2004)).

Application of RNA interference technology for insects that are plant pests and other plant pests has been suggested. Moderate RNAi-type silencing of insect genes by feeding has been demonstrated (Turner *et al.*, *Insect Mol Biol* 2006; 15:383; and Araujo *et al.*, *Insect Mol. Biol* 2006; 36:683). Various publications have since then focused on the incorporation of dsRNA in plants as pesticides. Such incorporation methods can be divided into transgenic gene expression and coating such as a seed coating.

U.S. Patent No. 6,326,193 refers to the use of recombinant insect viruses such as baculoviruses expressing dsRNA to silence selected insect genes for pest control. PCT Patent Application Publication No. WO 99/32619 describes the use of dsRNA for reducing crop destruction by plant pathogens or pests such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. PCT Patent Application Publication No. WO 2004/005485 describes RNAi sequences and transgenic plants designed to control plant-parasitic nematodes.

U.S. Patent Application Publication No. 20030154508 describes pest control with a dsRNA against a cation-amino acid transporter/channel protein. PCT Patent Application Publication No. WO 02/14472 describes an inverted repeat and a sense or antisense nucleic acids for inhibiting target gene expression in a sucking insect. U.S. Patent Application Publication No. 20030150017 describes the use of RNA molecules homologous or complementary to a nucleotide sequence of a plant pest such as nematodes and insects.

Raemakers *et al.* (PCT Patent Application Publication Nos. WO 2007/080127 and WO 2007/080126) have disclosed transgenic plants expressing RNAi for controlling pest infestation by insects, nematodes, fungus and other plant pests. Among the sequences taught are sequences targeting essential genes of insects. Waterhouse *et al.* (U.S. Patent Application Publication No. 20060272049) and Van De Craen (U.S. Patent Application Publication No. 2010068172) also disclosed transgenic plants expressing dsRNA directed to essential genes of plant insect pests, for use as pesticides and insecticides. Boukharov *et al.* (U.S. Patent Application Publication No. 20070250947) disclosed dsRNA in transgenic plants for targeting plant parasitic nematodes.

U.S. Patent Application Publication No. 20080022423 describes the control of fungal and oomycete plant pathogens by inhibiting one or more biological functions.

The disclosure provides methods and compositions for such control. By feeding one or more recombinant double stranded RNA molecules provided by the disclosure to the pathogen, a reduction in disease may be obtained through suppression of gene expression. The disclosure is also directed to methods for making transgenic plants that
5 express the double stranded RNA molecules, and to particular combinations of transgenic agents for use in protecting plants from pathogen infection. Also described is a seed coating with the dsRNA anti-pathogenic compositions.

PCT Patent Application Publication No. WO 2011112570 describes a method of
10 regulating target endogenous gene expression in growing plants/plant organs involving topically coating onto plants/organs, a composition comprising polynucleotide having sequence of specific contiguous nucleotides, and a transferring agent.

U.S. Patent No. 8,143,480 refers to methods for knock-down of a target genes in plants, particularly efficient and specific methods for knock-down of a target gene in plants. This disclosure also relates to methods for silencing endogenous plant genes or
15 plant pathogen genes. It further relates to nucleic acid constructs (DNA, RNA) which comprise a nucleic acid sequence that corresponds to a target gene or fragment thereof flanked by two complementary sites to an smRNA, *e.g.*, a miRNA (one complementary site is on either side of the nucleic acid sequence), resulting in, for example the configuration: complementary site--nucleic acid sequence that corresponds to a target
20 gene--complementary site. Axtell and Bartell describe siRNA biogenesis in *Arabidopsis* (Axtell and Bartel Cell. 2006 Nov 3;127(3):565-77.).

It has been reported that an autonomous dsRNA sequence derived from endovirus is found in every tissue of an infected plant and at every developmental stage. Thus, in 1993 Fukuhara *et al.* (Plant Mol. Biol. 21(6):1121-1130) identified a linear, 16
25 kb, dsRNA in symptomless *Japonica* rice that is not found in *Indica* rice. The dsRNA was detected in every tissue and at every developmental stage and its copy number was approximately constant (about 20 copies/cell). A sequence of about 13.2 kb of the dsRNA was determined and two open reading frames (ORFs) were found. The larger ORF (ORF B) was more than 12,351 nucleotides long and encoded a polypeptide of
30 more than 4,117 amino acid residues having an RNA helicase-like domain followed by an RNA dependent RNA Polymerase-like domain, as characterized in subsequent works published as Fukuhara *et al.* 1995 J. Biol. Chem. 270(30):18147-18149; and Moriyama

et al. 1995 Mol. Gen. Genet. 248(3):364-369.

While not limited by theory, during RNA silencing, RNAs of about 21 to 24 nucleotides (nt) in length are generated, which are incorporated into a protein complex where they serve as guide RNAs to direct the down-regulation of gene expression at the transcriptional or posttranscriptional level. These small interfering RNAs, small silencing RNAs, or short interfering RNAs are called “siRNAs” or “microRNAs”, depending upon their biogenesis: endogenous siRNAs derive from long double-stranded RNA and miRNAs derive from local hairpin structures within longer transcripts.

RNA silencing occurs in plants, insects, nematodes and other animals. In addition, new compositions (*e.g.*, nucleic acid constructs) and methods of achieving RNA-based silencing would be useful, and plants in which expression of one or more genes of interest is modulated, *e.g.*, inhibited, would be of great use. New compositions and rapid cost-effective methods of achieving RNA-based silencing by directly manipulating the plant seed are highly desirable.

SUMMARY OF THE INVENTION

The present disclosure provides for, and includes, methods and compositions for the regulation of gene expression in plants.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant and a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in a plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant) and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a helicase binding sequence and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA

molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant and a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant) and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence and the first heterologous RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in a plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a helicase binding sequence and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence and the first heterologous RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in said plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a helicase binding sequence and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence and the first heterologous RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in said plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a helicase binding sequence and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence, the first heterologous RNA sequence, and helicase binding sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for

regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a second heterologous RNA sequence corresponding to a second smRNA expressed in the plant (*e.g.*, a second heterologous smRNA-binding sequence for binding a second smRNA expressed in a plant), where the first heterologous smRNA and said second heterologous smRNA flank the at least one antisense RNA sequence, and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a second heterologous RNA sequence corresponding to a second smRNA expressed in the plant (*e.g.*, a second heterologous smRNA-binding sequence for binding a second smRNA expressed in a plant), where the first heterologous smRNA and said second heterologous smRNA flank the at least one antisense RNA sequence, and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence, and first heterologous RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a second heterologous RNA sequence corresponding to a second smRNA expressed in the plant (*e.g.*, a second heterologous smRNA-binding sequence for binding a second smRNA expressed in a plant), where the first heterologous smRNA and said second heterologous smRNA flank the at least one antisense RNA sequence, and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence, first heterologous RNA sequence, and second heterologous RNA sequence.

The present disclosure provides for, and includes, isolated double-stranded RNA molecules having a first RNA strand of at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of a plant, a helicase binding sequence, a first heterologous RNA sequence corresponding to a first small RNA (smRNA) expressed in the plant (*e.g.*, a first heterologous smRNA-binding sequence for binding a first smRNA expressed in a plant), a second heterologous RNA sequence corresponding to a second smRNA expressed in the plant (*e.g.*, a second heterologous smRNA-binding sequence for binding a second smRNA expressed in a plant), where the first heterologous smRNA and said second heterologous smRNA flank the at least one antisense RNA sequence, and a second RNA strand that is a reverse complement of the at least one antisense RNA sequence, first heterologous RNA sequence, and second heterologous RNA sequence.

According to some embodiments of the present disclosure there is provided an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA sequence is flanked by two complementary sites to an smRNA or smRNAs expressed in the plant and wherein the dsRNA molecule further comprises a helicase binding site positioned so as to allow unwinding of the strands of the isolated dsRNA molecule to single stranded RNA (ssRNA) and recruitment of an RNA-dependent RNA Polymerase so as to amplify the dsRNA molecule in the plant cell and generate secondary siRNA products of the dsRNA sequence.

According to some embodiments of the present disclosure there is provided an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA sequence is flanked by two complementary sites to an smRNA or smRNAs expressed in the plant.

According to an embodiment of some embodiments of the present disclosure there is provided an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA molecule further comprises a complementary site to an smRNA expressed in the plant located upstream or downstream the dsRNA.

According to some embodiments of the disclosure, the isolated dsRNA molecule further comprises a helicase binding site positioned so as to allow unwinding of the

strands of the isolated dsRNA molecule to single stranded RNA (ssRNA) and recruitment of an RNA-dependent RNA Polymerase so as to amplify the dsRNA molecule in the plant cell.

5 According to some embodiments of the disclosure, the complementary site to the smRNA is located downstream of the dsRNA sequence.

According to some embodiments of the disclosure, the complementary site to the smRNA is located upstream of the dsRNA sequence.

10 According to some embodiments of the disclosure, one of the two complementary sites to the smRNA or smRNAs comprises a mutation rendering it resistant to cleavage by the complementary smRNA.

According to some embodiments of the disclosure, the helicase binding site is positioned upstream of the dsRNA sequence.

15 According to some embodiments of the disclosure, wherein the helicase binding site is position in the dsRNA sequence for regulating a target gene of interest in the plant or the phytopathogen of the plant.

According to some embodiments of the disclosure, the helicase binding site is positioned upstream of the dsRNA sequence and the two complementary sites to the smRNA or smRNAs flank the helicase binding site.

20 According to some embodiments of the disclosure, the smRNA or smRNAs is selected from the group consisting of a miRNA and a siRNA.

According to some embodiments of the disclosure, the smRNA or smRNAs is a miRNA.

According to some embodiments of the disclosure, the miRNA is smRNA390.

25 According to some embodiments of the disclosure, the plant comprises a TAS locus that has a second smRNA complementary site.

According to some embodiments of the disclosure, the first and second complementary sites are naturally found flanking the TAS locus in the plant.

According to some embodiments of the disclosure, the smRNA is an smRNA for which complementary sites are naturally found flanking a TAS locus in a plant.

30 According to some embodiments of the disclosure, the two complementary sites are complementary sites for the same smRNA.

According to some embodiments of the disclosure, the two complementary sites

comprise difference sequences.

According to some embodiments of the disclosure, the two complementary sites comprise the same sequence.

5 According to some embodiments of the disclosure, the smRNAs are non-identical.

According to some embodiments of the disclosure, the smRNA or smRNAs is selected from the group consisting of miR390, miR161.1, miR168, miR393, miR828 and miR173.

According to some embodiments of the disclosure, the plant is a crop plant.

10 According to an embodiment of some embodiments of the present disclosure there is provided a method of silencing expression of a target gene of interest in a plant, the method comprising introducing the isolated dsRNA molecule, and wherein the dsRNA sequence is for silencing the target gene of interest in the plant, thereby silencing expression of the target gene of interest in the plant.

15 According to an embodiment of some embodiments of the present disclosure there is provided a method of introducing dsRNA molecule into a seed, the method comprising contacting the seed with the isolated dsRNA molecule under conditions which allow penetration of the dsRNA molecule into the seed, thereby introducing the dsRNA molecule into the seed.

20 According to an embodiment of some embodiments of the present disclosure there is provided an isolated seed comprising the isolated dsRNA molecule.

According to some embodiments of the disclosure, the isolated seed is devoid of a heterologous promoter for driving expression of the dsRNA molecule in the plant.

25 According to an embodiment of some embodiments of the present disclosure there is provided a seed comprising the isolated dsRNA molecule and the secondary siRNA products.

According to an embodiment of some embodiments of the present disclosure there is provided a plant or plant part generated from the seed.

30 According to an embodiment of some embodiments of the present disclosure there is provided a seed containing device comprising a plurality of the seeds.

According to an embodiment of some embodiments of the present disclosure there is provided a sown field comprising a plurality of the seeds.

According to an embodiment of some embodiments of the present disclosure there is provided a method of producing a plant the method comprising: (a) providing the seed; and (b) germinating the seed so as to produce the plant.

5 According to an embodiment of some embodiments of the present disclosure there is provided a method of modulating gene expression in a plant, the method comprising: (a) contacting a seed of the plant with the dsRNA molecule, under conditions which allow penetration of the dsRNA molecule into the seed, thereby introducing the dsRNA molecule into the seed; and optionally (b) generating a plant of the seed.

10 According to some embodiments of the disclosure, the penetration is to an endosperm and alternatively or additionally an embryo of the seed.

According to an embodiment of some embodiments of the present disclosure there is provided a method of silencing expression of a target gene in a phytopathogenic organism, the method comprising providing to the phytopathogenic organism the plant or plant part, thereby silencing expression of a target gene in the phytopathogenic organism.

15 According to some embodiments of the disclosure, the phytopathogenic organism is selected from the group consisting of a fungus, a nematode, an insect, a bacteria and a virus.

20 According to an embodiment of some embodiments of the present disclosure there is provided a kit for introducing a dsRNA molecule to seeds comprising: (i) the dsRNA molecule; and (ii) a priming solution.

According to some embodiments of the disclosure, the dsRNA molecule and the priming solution are comprised in separate containers.

25 According to an embodiment of some embodiments of the present disclosure there is provided a pesticidal composition comprising the isolated dsRNA molecule.

According to some embodiments of the disclosure, the contacting is effected by inoculating the seed with the dsRNA molecule.

30 According to some embodiments of the disclosure, the method further comprises priming the seed prior to the contacting.

According to some embodiments of the disclosure, the priming is effected by: (i) washing the seed prior to the contacting; and (ii) drying the seed following step (i).

According to some embodiments of the disclosure, the washing is effected in the presence of double deionized water.

According to some embodiments of the disclosure, the washing is effected for 2-6 hours.

5 According to some embodiments of the disclosure, the washing is effected at 4-28 °C.

According to some embodiments of the disclosure, the drying is effected at 25-30 °C for 10 - 16 hours.

10 According to some embodiments of the disclosure, the contacting is effected in a presence of the dsRNA molecule at a final concentration of 0.1-100 µg/µl.

According to some embodiments of the disclosure, the contacting is effected in a presence of the dsRNA molecule at a final concentration of 0.1-0.5 µg/µl.

15 According to some embodiments of the disclosure, the method further comprises treating the seed with an agent selected from the group consisting of a pesticide, a fungicide, an insecticide, a fertilizer, a coating agent and a coloring agent following the contacting.

According to some embodiments of the disclosure, the treating comprises coating the seed with the agent.

20 According to some embodiments of the disclosure, the conditions allow accumulation of the dsRNA molecule in the endosperm and alternatively or additionally embryo of the seed.

25 According to some embodiments of the disclosure, a concentration of the dsRNA molecule is adjusted according to a parameter selected from the group consisting of, seed size, seed weight, seed volume, seed surface area, seed density and seed permeability.

According to some embodiments of the disclosure, the contacting is effected prior to breaking of seed dormancy and embryo emergence.

According to some embodiments of the disclosure, the seed is a primed seed.

30 According to some embodiments of the disclosure, the seed comprises RNA dependent RNA polymerase activity for amplifying expression of the dsRNA molecule.

According to some embodiments of the disclosure, the seed is a hybrid seed.

According to an aspect of some embodiments of the present disclosure there is

provided an isolated dsRNA molecule comprising a nucleic acid sequence which comprises in a sequential order from 5' to 3', an endovirus 5' UTR, an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence, an endovirus 3' UTR and a multiple cloning site flanked by the RDRP and the 3' UTR.

5 According to an aspect of some embodiments of the present disclosure there is provided an isolated dsRNA molecule comprising a nucleic acid sequence which comprises in a sequential order from 5' to 3', an endovirus 5' UTR, an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence, an endovirus 3' UTR and a nucleic acid sequence for regulating a target gene flanked by the RDRP and the 3' UTR.

10 According to some embodiments of the disclosure, the endovirus 5' UTR, endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence and the endovirus 3' UTR are selected capable of autonomous replication in the plant cell.

 According to some embodiments of the disclosure, the 5' UTR is as set forth in SEQ ID NO: 14.

15 According to some embodiments of the disclosure, the 3' UTR is as set forth in SEQ ID NO: 22.

 According to some embodiments of the disclosure, the endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence is as set forth in SEQ ID NO: 23.

20 According to some embodiments of the disclosure, the nucleic acid sequence for regulating a target gene is 17-600 bp long.

 According to some embodiments of the disclosure, the nucleic acid sequence for regulating a target gene is selected from the group consisting of a miRNA and a siRNA.

 According to some embodiments of the disclosure, the nucleic acid sequence for regulating a target gene is a miRNA.

25 Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the disclosure, examples of methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials,
30 methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the disclosure are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the disclosure. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the disclosure may be practiced.

FIG. 1 presents fluorescent images of siGLO-treatment rice seeds over a 24 hour period according to embodiment of the present disclosure.

FIG. 2A presents a schematic representation of the *Solanum Lycopersicum* (*Lycopersicon esculentum*) TAS3 gene according to an embodiment of the present disclosure. Mir390BS is displayed in a darker gray box. The light gray box represents the 5' Mut Mir390BS.

FIG. 2B presents a schematic representation of dsRNA construct #1 having an exogenous trigger control according to an embodiment of the present disclosure. The construct includes a 234bp exogenous sequence provided in Table 5 (*e.g.*, Trigger #1).

FIG. 2C presents a schematic representation of dsRNA construct #2 having a dual Mir390BS sequence on the sense strand and an exogenous sequence according to an embodiment of the present disclosure. The construct is comprises 3 parts from 5' to 3': a 5' Mut Mir390BS sequence, a 234bp exogenous sequence in reverse complement orientation, and a 3' Mir390BS. The sequences are presented in Table 5 (*e.g.*, Trigger #2).

FIG. 3 presents a schematic representation of dsRNA construct#3 having a dual Mir390BS on both on the sense and antisense strands. The construct is composed of 5 parts from 5' to 3': 3' Mir390BS in the reverse complement orientation, 5' Mut Mir390BS, a 234bp exogenous sequence in the reverse complement orientation, 5' Mut Mir390BS in the reverse complement orientation and 3' Mir390BS. For sequence, see Table 5 (Trigger #3).

FIG. 4 presents a schematic representation of dsRNA construct #4 having miR390S as overhangs. This construct is composed of two different strands. The sense strand is composed of 3 parts from 5' to 3': 5' Mut Mir390BS, a 234bp exogenous sequence in the reverse complement orientation, 3' Mir390BS. The antisense is composed of only one part: a 234bp exogenous sequence in the sense orientation. For

sequences, see Table 5 (Sense-Trigger#4, Antisense-Trigger #5).

FIG. 5 presents a schematic representation of dsRNA construct #5 having miR390BS and a helicase binding sequence (Helicase BS). This construct is composed of 4 parts from 5' to 3': 5' Mut Mir390BS, a 234bp exogenous sequence in the reverse complement orientation, Helicase BS in the reverse complement orientation, 3' Mir390BS. For sequence see Table 5 (Trigger #6).

FIG. 6 presents a schematic representation of dsRNA construct #6 having Mir390BS on both strands and Helicase BS as an overhang. This construct is composed of two different strands. The sense strand is composed of 5 parts from 5' to 3': '3' Mir390BS in the reverse complement orientation, 5' Mut Mir390BS, a 234bp exogenous sequence in the reverse complement orientation, 5' Mut Mir390BS in the reverse complement orientation, and 3' Mir390BS. The antisense is composed of 6 parts from 5' to 3': 3' Mir390BS in the reverse complement orientation, 5' Mut Mir390BS, a 234bp exogenous sequence in the sense orientation, 5' Mut Mir390BS in the reverse complement orientation, 3' Mir390BS and an Helicase BS as an overhang. For sequences, see Table 5 (Sense-Trigger #7, Antisense-Trigger #8).

FIG. 7 presents a schematic representation of dsRNA construct #7 having Sense dual Mir390BS coupled with Antisense Mir4376BS. This construct is composed of 5 parts from 5' to 3': 5' Mut Mir390BS, a 234bp exogenous sequence in the reverse complement orientation, Mir4376BS in the reverse complement orientation and 3' Mir390BS. For sequence, see Table 5 (Trigger #9).

FIG. 8 presents a schematic representation of dsRNA construct #8 an Endogenous Trigger Control. This construct is composed of one part: a 234bp of the endogenous TAS3 sequence. For sequence, see Table 5 (Trigger #10).

FIG. 9 presents a schematic representation of dsRNA construct #9 – Mir390BS + Endogenous insert. This construct is composed of 3 parts from 5' to 3': 5' Mut Mir390BS, a 234 bp of the endogenous TAS3 sequence and 3' Mir390BS. For sequence, see Table 5 (Trigger #11).

FIGs. 10A-E are schematic representations of dsRNA constructs of the present disclosure.

FIGs. 11A-B present graphs showing real-time PCR analyses of ARF3 and ARF4 mRNA expression in roots 14 days after seed treatment according to an

embodiment of the present disclosure.

FIGs. 11C-E present graphs showing the results of real-time PCR analyses of GFP in seedlings seven days after seed treatment according to an embodiment of the present disclosure.

5 FIG. 11F presents a graph showing the results of real-time PCR analyses of GFP in leaves 30 days after seed treatment according to an embodiment of the present disclosure.

10 FIGs. 12A-E present graphs showing the results of real-time PCR analyses of GFP in shoots seven days after seed treatment according to an embodiment of the present disclosure.

FIGs. 13A-B presents graphs showing the results of real-time PCR analyses of GFP in shoots 14 days after seed treatment according to an embodiment of the present disclosure.

15 FIGs. 14A-B presents graphs showing the results of real-time PCR analyses of GFP in shoots seven days (A) and 14 days (B), according to an embodiment of the present disclosure.

FIGs. 15A-B presents graphs showing the results of real-time PCR analyses of GUS in shoots seven days (A) and 14 days (B) after seed treatment according to an embodiment of the present disclosure.

20 **DETAILED DESCRIPTION**

The present disclosure, in some embodiments thereof, relates to and provides for isolated dsRNA molecules and methods of using same for silencing target molecules of interest.

25 The present disclosure further includes and provides for compositions and methods for silencing gene expression.

The present disclosure provides for, and includes tools for overcoming the delivery obstacle and amplifying the small interfering RNA (siRNA) levels within the plant cell to thereby efficiently down-regulate target genes of interest.

30 Before explaining at least one embodiment of the disclosure in detail, it is to be understood that the disclosure is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The disclosure is capable of other embodiments or of being practiced or carried out in various ways.

It is understood that any Sequence Identification Number (SEQ ID NO) disclosed in the instant application can refer to either a DNA sequence or a RNA sequence, depending on the context where that SEQ ID NO is mentioned, even if that SEQ ID NO is expressed only in a DNA sequence format or a RNA sequence format.

5 For example, SEQ ID NO: 1 is expressed in a DNA sequence format (*e.g.*, reciting T for thymine), but it can refer to either a DNA sequence that corresponds to a T7 DNA Dependent RNA Polymerase primer nucleic acid sequence, or the RNA sequence of an RNA molecule nucleic acid sequence. Similarly, though SEQ ID NO: 25 is expressed in a RNA sequence format (*e.g.*, reciting U for uracil), depending on the actual type of

10 molecule being described, SEQ ID NO: 25 can refer to either the sequence of a RNA molecule comprising a dsRNA, or the sequence of a DNA molecule that corresponds to the RNA sequence shown. In any event, both DNA and RNA molecules having the sequences disclosed with any substitutes are envisioned.

As used herein, the terms “homology” and “identity” when used in relation to

15 nucleic acids, describe the degree of similarity between two or more nucleotide sequences. The percentage of “sequence identity” between two sequences is determined by comparing two optimally aligned sequences over a comparison window, such that the portion of the sequence in the comparison window may comprise additions or deletions (gaps) as compared to the reference sequence (which does not comprise

20 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

25 sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be identical to the reference sequence and vice-versa. An alignment of two or more sequences may be performed using any suitable computer program. For example, a widely used and accepted computer program for performing sequence alignments is CLUSTALW v1.6 (Thompson, *et al.* Nucl. Acids Res., 22: 4673-4680, 1994).

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Homologous sequences include both orthologous and paralogous sequences. The term “paralogous” relates to gene-duplications within the genome of a species

leading to paralogous genes. The term “orthologous” relates to homologous genes in different organisms due to ancestral relationship. For instance in this case, other plant RNA viruses.

5 Identity (*e.g.*, percent homology) can be determined using any homology comparison software, including for example, the BlastN software of the National Center of Biotechnology Information (NCBI) such as by using default parameters.

According to some embodiments of the disclosure, the identity is a global identity, *i.e.*, an identity over the entire nucleic acid sequences of the disclosure and not over portions thereof.

10 The degree of homology or identity between two or more sequences can be determined using various known sequence comparison tools. Following is a non-limiting description of such tools which can be used along with some embodiments of the disclosure.

As used herein, the terms “exogenous polynucleotide” and “exogenous nucleic acid molecule” relative to an organisms refer to a heterologous nucleic acid sequence which is not naturally expressed within that organism, for example a plant. An exogenous nucleic acid molecule may be introduced into an organism in a stable or transient manner. An exogenous nucleic acid molecule may comprise a nucleic acid sequence which is identical or partially homologous to an endogenous nucleic acid sequence of the organism. In certain embodiments, an “exogenous polynucleotide” and “exogenous nucleic acid molecule” may refer to a nucleic acid sequence expressed or present in a plant, either transiently or stably. As used herein, the terms “endogenous polynucleotide” and “endogenous nucleic acid” refers to nucleic acid sequences that are found in an organism’s cell. In certain aspects, an endogenous nucleic acid may be part of the nuclear genome or the plastid genome. In other aspects, an endogenous nucleic acid may be found outside the nuclear or plastid genomes. As used herein, endogenous nucleic acids do not include viral, parasite or pathogen nucleic acids, for example an endovirus sequence. The present disclosure provides for, and includes, compositions comprising exogenous polynucleotides and exogenous nucleic acid molecules and methods for introducing them into a target organism. The present disclosure provides for, and includes, compositions comprising exogenous polynucleotides and exogenous nucleic acid molecules in combination with endogenous nucleic acids and

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polynucleotides and methods for introducing them into a target organism. The present disclosure provides for, and includes, compositions comprising recombinant endogenous nucleic acids and polynucleotides and methods for introducing them into a target organism.

5 The present disclosure provides for, and includes dsRNA molecules which are processed through the trans-acting siRNA (ta-siRNA) pathway. Transacting siRNAs are a subclass of siRNAs that function like miRNAs to repress expression of target genes. While not limited to any particular theory, trans-acting siRNAs form by transcription of ta-siRNA-generating genes found at trans-acting (TAS) loci. A ta-siRNA precursor is any nucleic acid molecule, including single-stranded or double-
10 stranded DNA or RNA, that can be transcribed and/or processed to release a ta-siRNA. Cleavage of the primary transcript occurs through a guided RISC mechanism, conversion of one of the cleavage products to dsRNA, and processing of the dsRNA by dicer or dicer-like (DCL) enzymes. While not limited by any particular theory, it is
15 thought that RNA-dependent RNA polymerase 6 (RDR6) (or related enzymes) function in posttranscriptional RNAi of sense transgenes, some viruses, and specific endogenous mRNAs that are targeted by trans-acting siRNAs (ta-siRNAs) (see Dalmay et al., *Cell* 101:543-553, 2000; Mourrain et al., *Cell* 101:533-542, 2000; Peragine et al., *Genes & Dev* 18:2369-2379, 2004; Vazquez et al., *Mol Cell* 16:69-79, 2004b; Yu et al., *Mol Plant Microbe Interact* 16:206-216, 2003). Again, while not being limited to any
20 particular theory, it is thought that ta-siRNAs arise from transcripts that are recognized by RDR6, in cooperation with SGS3, as a substrate to form dsRNA. The dsRNA is processed accurately in 21-nucleotide steps by DCL1 to yield a set of “phased” ta-siRNAs. These ta-siRNAs interact with target mRNAs to guide cleavage by the same
25 mechanism as do plant miRNAs (Peragine et al., *Genes & Dev* 18:2369-2379, 2004; Vazquez et al., *Mol Cell* 16:69-79, 2004; Allen et al., *Cell* 121:207-221, 2005). Trans-acting siRNAs are conserved among distantly related plant species and have been maintained over a long evolutionary period. The design and construction of ta-siRNA constructs and their use in the modulation of protein in transgenic plant cells is
30 disclosed by Allen and Carrington in US Patent Application Publication US 2006/0174380 A1 (now US Patent 8,030,473) which is incorporated herein by reference.

As used herein, the term “dsRNA sequence” refers to, and includes, a double-stranded sequences having a first strand and a second strand that is a reverse complement of the first strand. It will be understood that reference to an antisense RNA sequence for regulating a target gene of interest and a sense RNA sequence for regulating a target gene of interest, would necessarily include a dsRNA sequences when included in a dsRNA molecule. For clarity, the sequences for targeting a gene of interest for regulation will be generally referenced as the antisense RNA sequence and provides for a standard reference point for the 5’ and 3’ ends. As used herein, the ‘antisense strand’ refers to the strand having the antisense RNA sequence for regulating (e. g., suppressing or silencing) a target gene of interest. One of ordinary skill in the art would further understand that reference to a single strand, whether the sense or antisense strand, provides a definition and sequence for the reverse complement strand. Further, it is well understood that a single nucleic acid strand and its reverse complement provide for a double-stranded nucleic acid. One of ordinary skill in the art would understand that an RNA and DNA sequence may be readily substituted using the well known base pairing rules and as provided above. One of ordinary skill in the art would further understand that binding can occur between two polynucleotide sequences that are characterized by having sufficient sequence complementarity (which need not be 100% complementarity) to allow hybridization between the two polynucleotides (e. g., binding or hybridization under common physiological conditions). Thus, a “heterologous smRNA-binding sequence for binding a first small RNA” need not be 100% complementary to the sequence of the first small RNA (for example, where the heterologous smRNA-binding sequence is complementary to the sequence of the first small RNA except for one or more mutations or mismatches at the site where cleavage mediated by the small RNA would normally occur), although in some embodiments the complementarity is 100%. The present disclosure provides for, and includes, an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA sequence is flanked by two complementary sites to an smRNA expressed in the plant. In some embodiments, the dsRNA sequence may be flanked by two complementary sites from the same smRNA expressed in the plant. In other embodiments, the dsRNA sequence may be flanked by complementary sites from two different smRNAs. In yet other

embodiments, the dsRNA sequence may be flanked by four complementary sites corresponding to one or more smRNAs expressed in a plant (e.g., two heterologous sequences on one side and two heterologous sequences on the other side of the dsRNA sequence. In certain embodiments, the dsRNA molecule further comprises a helicase binding site positioned so as to allow unwinding of the strands of the isolated dsRNA molecule to single stranded RNA (ssRNA) and amplification by recruitment of an RNA-dependent RNA Polymerase (RDRP) when introduced into a host cell. In other embodiments, the helicase and other proteins may be provided *in vitro*, for example as part of a cell extract. Methods of *in vitro* analysis are known in the art. In certain embodiments, the host cell is a plant cell. In some embodiments, introduction of the dsRNA molecule into a plant cell results in the recruitment of a helicase and RDRP and the generation of secondary siRNA products corresponding to the dsRNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant. In certain embodiments, the target gene is silenced. In other embodiments, expression of the target gene is enhanced.

According to another embodiment of the disclosure there is provided, and included, an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA sequence is flanked by two complementary sites to an smRNA or smRNAs expressed in the plant. In certain embodiments, the target gene is silenced. In other embodiments, expression of the target gene is enhanced.

According to a further embodiment of the disclosure there is provided, and included, an isolated dsRNA molecule comprising an antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant, wherein the dsRNA molecule further comprises a complementary site to an smRNA expressed in the plant located upstream or downstream the dsRNA. In certain embodiments, the target gene is silenced. In other embodiments, expression of the target gene is enhanced.

Not to be limited by theory, a possible downstream mechanism for a dsRNA construct of the present disclosure having two flanking heterologous RNA sequences corresponding to an smRNA, with one sequence being a non-cleavable mutant (for example, mir390 BS and Mir390 Mut BS of Figure 2C) includes unwinding of the dsRNA in the cell. Following the opening of the double stranded RNA into two single

strands the sense strand may recruit an AGO7, or AGO7-like, protein to the flanking heterologous RNA sequences. This binding, in turn, may lead to cleavage at a non-mutated heterologous RNA sequence (for example Mir390 BS of Figure 2C) and localization of this single stranded RNA inside a cytoplasmic processing center (Evidence for such a processing center was reported in Kumakura et al. (2009). SGS3 and RDR6 interact and colocalize in cytoplasmic SGS3/RDR6-bodies. (2009). *FEBS Letters*, 583, 1261-1266 and Jouannet et al. (2012). Cytoplasmic *Arabidopsis* AGO7 accumulates in membrane-associated siRNA bodies and is required for ta-siRNA biogenesis. *EMBO Journal*, 31, 1704-1713.). The antisense strand may then either be

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diced or cleaved but is not expected to take part in additional amplification of the exogenous sequence (*e.g.*, the gene of interest of Figure 2C).

Not to be limited by theory, an alternative downstream mechanism for dsRNA construct of the present disclosure having two flanking heterologous RNA sequences corresponding to an smRNA (for example, as provided in Figure 2C) similarly starts with the unwinding of the dsRNA in a cell. In this non-limiting theoretical mechanism, the sense strand is translocated to a processing center that may have an accumulation of a RNA Dependent RNA Polymerase (RDRP) that is predicted to lead to the formation of antisense transcripts. Preferably, each template of sense RNA will serve for multiple rounds of antisense RNA production. Following antisense RNA accumulation, it may be that the mere localization of this transcript inside the processing center enables RDRP recruitment and creation of double-stranded RNAs (even though this strand may lack a recognizable element of the TAS system). Some of these double stranded RNAs may be translocated to the nucleus where to be diced into ta-siRNAs against an exogenous sequence and some of the double stranded RNA may remain in the processing center where it will unwind again and lead to further cycles of amplification. One possible mediator of the unwinding process inside the processing center is the SDE3 RNA helicase (*see* Garcia *et al.* (2012). Ago Hook and RNA Helicase Motifs Underpin Dual Roles for SDE3 in Antiviral Defense and Silencing of Nonconserved Intergenic Regions. *Mol Cell*, 48, 109-120.).

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Not to be limited by theory, a possible downstream mechanism for dsRNA construct #3 (Figure 3) also begins with unwinding of the dsRNA into two single strands.

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Focusing on the outcome of the sense strand, it may be recognized by Mir390-Ago7 at both Mir390 Binding sites. The binding of this complex may lead to cleavage at the 3' Mir390BS and to the translocation of this truncated transcript into a processing center. Inside the processing center it may serve as a template for the creation of multiple transcripts of antisense strands. The newly created antisense strands may contain recognizable Mir390 binding sites and therefore may be able to recruit Ago7 and Mir390 to the 5' Mut Mir390BS. This binding is may enable efficient recruitment of RDRP and creation of double stranded RNAs. Some of this double stranded RNA may be translocated to the nucleus and diced into ta-siRNAs whereas other dsRNA may be expected to continue to additional rounds of unwinding and amplification.

Not to be limited by theory, in the alternative, dsRNA construct #3 may be unwound in the cell to a ssRNA. Focusing on the outcome of the antisense strand, it may be recognized by Mir390-Ago7 at both Mir390 Binding sites. The binding of this complex may lead to cleavage at the 3' Mir390BS and to the translocation of this truncated transcript into the processing center. Inside the processing center it may serve as a template for the creation of multiple transcripts of sense strands. The newly created sense strands will contain recognizable Mir390 binding sites and therefore may be able to recruit Ago7 and Mir390 to the 5' Mut Mir390BS. This binding may enable efficient recruitment of RDRP and creation of double stranded RNAs. Some of this double stranded RNA may be translocated to the nucleus and diced into ta-siRNAs whereas other dsRNA may continue to additional rounds of unwinding and amplification.

Not to be limited by theory, in another alternative mechanism, dsRNA construct #3 is undergoes strand unwinding in a cell. Newly synthesized sense and antisense strands (possibly resulting from the mechanisms described above may serve as templates for multiple rounds of RDRP recruitment and dsRNA amplification. This construct may lead to an optimal amplification due to the presence of the 5' Mut Mir390BS on both strands enabling ongoing recruitment of Mir390-Ago7 complex.

As used herein, the term “upstream” refers to positions that are 5' end of the polynucleotide. In certain aspects, upstream refers to the 5' location of sequences relative to an antisense sequence for regulating a target gene.

As used herein the term “isolated” refers to separated from its natural environment. In the case of a dsRNA molecule, separated from the cytoplasm or the

nucleus, conversely, in the case of a plant part such as a seed, separated from the rest of the plant.

As used herein the term “isolated dsRNA molecule” refers to an isolated RNA molecule which is substantially in a double stranded form. As used herein, an isolated
5 dsRNA molecule may be in solution and may include buffers. An isolated dsRNA molecule is substantially separated from other nucleic acid molecules including DNA.

As used herein the term “dsRNA” refers to two strands of anti-parallel polyribonucleic acids held together by base pairing (*e.g.*, two sequences that are the reverse complement of each other in the region of base pairing). The two strands can be
10 of identical length or of different lengths provided there is enough sequence homology between the two strands that a double stranded structure is formed with at least 80%, 90%, 95 % or 100 % complementarity over the entire length. As used herein, the term “overhang” refers to non-double stranded regions of a dsRNA molecule (*i.e.*, single stranded RNA). According to an embodiment of the disclosure, there are no overhangs
15 for the dsRNA molecule. According to another embodiment of the disclosure, the dsRNA molecule comprises one overhang. According to other embodiments, a dsRNA molecule may comprise two overhangs.

In embodiments according to the present disclosure, an isolated dsRNA molecule comprises a second strand having an RNA sequence that is at least 80%, 90%,
20 95% or 100% complementary over its entire length to an antisense RNA sequence. In some embodiments, an isolated dsRNA molecule comprises a second strand that is 99% complementary over its entire length to an antisense RNA sequence. In other embodiments, the double stranded region is 98% complementary over the entire length of an antisense RNA sequence. In yet other embodiments, the double stranded region is
25 97% complementary over the entire length of an antisense RNA sequence. In further embodiments, the double stranded region may comprise 96% of the entire length of an antisense RNA sequences. In certain embodiments the double stranded region is between 90 and 100% complementary over the entire length of antisense RNA sequence. In certain embodiments the double stranded region is between 95 and 100%
30 complementary over the entire length of antisense RNA sequence.

The present disclosure provides for, and includes, embodiments of an isolated dsRNA molecule comprising a second strand having an RNA sequence that is nearly

100% complementary over its entire length to an antisense RNA sequence but having 1 mismatch. In some embodiments, the nearly 100% complementary dsRNA region may have 2 mismatches. In some embodiments, the nearly 100% complementary dsRNA region may have 3 mismatches. Some embodiments according to the present disclosure provide for 4, 5 or 6 mismatches in a dsRNA region. In some embodiments, the nearly 100% complementary dsRNA region may have 1 or more, 2 or more, or 3 or more mismatches.

According to an embodiment, an overhang may be 5' to a double stranded region comprising at least one antisense RNA sequence and its reverse complement (e.g., 5' to said antisense RNA sequence). According to an embodiment, an overhang may be 3' to a double stranded region comprising at least one antisense RNA sequence and its reverse complement (e.g., 3' to said antisense RNA sequence). In other embodiments according to the present disclosure, a dsRNA molecule may comprise two overhang regions flanking a double stranded region.

According to other embodiments, an overhang region comprises less than 10 bases. In certain embodiments, the strands are aligned such that there are at least 1, 2, or 3 bases at the end of the strands which do not align (*i.e.*, for which no complementary bases occur in the opposing strand) such that an overhang of 1, 2 or 3 residues occurs at one or both ends of the duplex when strands are annealed. In an embodiment, a less than 10 base overhang may be a 5' overhang (relative to the 5' and 3' positions on the end of a double stranded RNA region). In another embodiment, a less than 10 base overhang may be a 3' overhang. Relative to a dsRNA molecule having at least one antisense RNA sequence, the 5' overhang may be located at 5' of said antisense RNA sequences. In other embodiments, the 5' overhang may be located 3' of said antisense RNA sequence (e.g., the 5' overhang is on the complementary strand). Also provided by the present disclosure are embodiments wherein the 3' overhang is located 3' of said antisense RNA sequence or wherein the 3' overhang is located 5' of said antisense RNA sequence. According to embodiments of the present disclosure, a 5' overhanging sequence may be 9 bases. In an embodiment, a 3' overhanging sequence may be 9 bases. According to embodiments of the present disclosure, a 5' overhanging sequence may be 8 bases. In an embodiment, a 3' overhanging sequence may be 8 bases. According to embodiments of the present disclosure, a 5' overhanging sequence may be

7 bases. In an embodiment, a 3' overhanging sequence may be 7 bases. According to
embodiments of the present disclosure, a 5' overhanging sequence may be 6 bases. In
an embodiment, a 3' overhanging sequence may be 6 bases. In some embodiments, a
single stranded overhanging sequence may be less than 5 bases. According to
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embodiments of the present disclosure, a 5' overhanging sequence may be 5 bases. In
an embodiment, a 3' overhanging sequence may be 5 bases. According to embodiments
of the present disclosure, a 5' overhanging sequence may be 4 bases. In an
embodiment, a 3' overhanging sequence may be 4 bases. According to embodiments of
the present disclosure, a 5' overhanging sequence may be 3 bases. In an embodiment, a
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3' overhanging sequence may be 3 bases. According to embodiments of the present
disclosure, a 5' overhanging sequence may be 2 bases. In an embodiment, a 3'
overhanging sequence may be 2 bases.

As will be appreciated by one of ordinary skill in the art, a dsRNA molecule of
the present disclosure may refer to either strand of the anti-parallel nucleic acids. As
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will also be appreciated by one of ordinary skill in the art, a dsRNA molecule of the
present disclosure includes both a 'sense' and 'antisense' strand and that the sense and
antisense strands are reverse complements of each other in a region of base pairing. As
used herein the sequence of a dsRNA molecule for regulating a target gene of interest is
provided as the 'antisense' orientation with respect to the target gene of interest. Thus,
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one of ordinary skill in the art would appreciate that the 5' end of a dsRNA molecule for
regulating a target gene of interest corresponds to sequences towards the 3' end of the
target gene of interest. Similarly, the 3' end of a dsRNA molecule for regulating a
target gene of interest corresponds to sequences towards the 5' end of a target gene of
interest. As used herein, "the reverse complement of a dsRNA molecule for regulating
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a target gene of interest" refers to a nucleic acid sequence in the 'sense' orientation.

The term "corresponding to the target gene of interest" or "dsRNA for regulating
a target gene of interest" means that the dsRNA sequence contains an RNA silencing
agent to the target gene.

As used herein, the term "RNA silencing agent" refers to a nucleic acid which is
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capable of inhibiting or "silencing" the expression of a target gene. In certain aspects,
the RNA silencing agent is capable of preventing complete processing (*e.g.*, the full
translation and/or expression) of an mRNA molecule through a post-transcriptional

silencing mechanism. RNA silencing agents can be single- or double-stranded RNA or single- or double-stranded DNA or double-stranded DNA/RNA hybrids or modified analogues thereof. In some aspects, the RNA silencing agents are selected from the group consisting of (a) a single-stranded RNA molecule (ssRNA), (b) a ssRNA molecule that self-hybridizes to form a double-stranded RNA molecule, (c) a double-stranded RNA molecule (dsRNA), (d) a single-stranded DNA molecule (ssDNA), (e) a ssDNA molecule that self-hybridizes to form a double-stranded DNA molecule, and (f) a single-stranded DNA molecule including a modified Pol III gene that is transcribed to an RNA molecule, (g) a double-stranded DNA molecule (dsDNA), (h) a double-stranded DNA molecule including a modified Pol III promoter that is transcribed to an RNA molecule, (i) a double-stranded, hybridized RNA/DNA molecule, or combinations thereof. In some aspects these polynucleotides include chemically modified nucleotides or non-canonical nucleotides. In some aspects, the RNA silencing agents are noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. In some aspects, the RNA silencing agents are dsRNAs such as siRNAs, miRNAs and shRNAs. In one aspect, the RNA silencing agent is capable of inducing RNA interference. In another aspect, the RNA silencing agent is capable of mediating translational repression. As used herein, an RNA silencing agent is a type of agent for regulating a target gene.

In some embodiments, the dsRNA molecule is subject to amplification by RNA-Dependant RNA Polymerase (RDRP). According to some embodiments, a dsRNA molecule comprises a first strand having at least one antisense RNA sequence for regulating a target gene, one or two heterologous RNA sequences corresponding to a smRNA, a helicase binding site and a sequence encoding an RDRP, and a second complementary strand. According to some embodiments, a dsRNA molecule comprises a first strand having at least one antisense RNA sequence for regulating a target gene, one or two heterologous RNA sequences corresponding to a smRNA, a helicase binding site and a sequence encoding an RDRP and further including flanking 3' UTR and 5' UTR sequences from an endovirus and a second RNA strand that is the reverse complement.

As used herein, "small RNA" or "smRNA" refers to RNA molecules that

function to modulate (*e.g.*, inhibit), gene expression, and are present in diverse eukaryotic organisms, including plants. As known to those of skill in the art, smRNAs may be defined as low-molecular weight RNAs associated with gene silencing and in some embodiments may be further described as short (generally 21 to 26 nucleotides).
5 Small RNAs include siRNAs and miRNAs, which function in RNA silencing, also sometimes referred to as RNA interference (RNAi). RNA silencing encompasses a broad range of phenomena in which large, double-stranded RNA, fold-back structures, or stem-loop precursors are processed to about 21-26 nucleotide (nt) small RNAs (*e.g.*, siRNAs or miRNAs, which are described further below) that then guide the cleavage of
10 cognate RNAs, block productive translation thereof, or induce methylation of specific target DNAs (Meins, F., *et al.*, *Annu Rev. Cell Dev. Biol.*, 21:297-318, 2005).

As used herein, a small RNA is an RNA molecule that is at least 15 base pairs in length, generally 15-30 nucleotides long, preferably 20-24 nucleotides long. In some aspects, according to the present disclosure, a "small RNA" is greater than 30 base pairs
15 in length. In an aspect, the small RNA is greater than 30 base pairs in length but less than about 600 base pairs. In an aspect, the small RNA is greater than 100 base pairs in length but less than about 600 base pairs. In an aspect, the small RNA is greater than 200 base pairs in length but less than about 600 base pairs. A small RNA can be either double-stranded or single-stranded. Small RNA includes, without limitation, miRNA
20 (microRNA), ta-siRNA (trans activating siRNA), siRNA, activating RNA (RNAa), nat-siRNA (natural anti-sense siRNA), hc-siRNA (heterochromatic siRNA), cis-acting siRNA, lmiRNA (long miRNA), lsiRNA (long siRNA) and easiRNA (epigenetically activated siRNA) and their respective precursors. Preferred siRNA molecules of the disclosure are miRNA molecules, ta-siRNA molecules and RNAa molecules and their
25 respective precursors. A small RNA may be processed *in vivo* by an organism to an active form. According to aspects of the present disclosure, a selective insecticide may be a small RNA. In embodiments according to the present disclosure a small RNA is a dsRNA.

As provided for and included in the present disclosure, a dsRNA molecule may
30 comprise an antisense RNA sequence for regulating a target gene of interest. In some embodiments, a dsRNA molecule for regulating a target gene of interest may comprise an antisense RNA sequence that is greater than 30 base pairs in length to allow

processing of the dsRNA in a plant cell and generation of secondary siRNA molecules. In other embodiments, a dsRNA molecule for regulating a target gene of interest may comprise an antisense RNA sequence that is from 30 to 600 bp in length to allow processing of the dsRNA in a plant cell and generation of secondary siRNA molecules.

5 As used herein, “secondary siRNA”, “phase RNA” and “ta-siRNA” or refer to dsRNA molecules generated after processing a dsRNA molecule. In certain embodiments, the target gene regulation is silencing. In other embodiments, expression of the target gene is enhanced.

The present disclosure also includes and provides for embodiments having dsRNA molecules having various lengths of dsRNA sequences, whereby the shorter version *i.e.*, x is shorter or equals 50 bp (*e.g.*, 17-50), is referred to as siRNA or miRNA sequences. Longer dsRNA sequences of 51-600 nucleotides are referred to herein as dsRNA, which can be further processed for siRNA molecules.

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The term “siRNA” generally refers to small inhibitory RNA duplexes (generally between 17-30 base pairs, but also longer *e.g.*, 31-50 bp) that induce the RNA interference (RNAi) pathway. In certain embodiments, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. Without being limited by any theory, a role of siRNA is its involvement in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. Though not to be limiting, the observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into the RNA-induced silencing complex (RISC).

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It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose *et al.*, 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

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In some embodiments, the strands of a double-stranded interfering RNA (*e.g.*, an siRNA) may be connected to form a hairpin or stem-loop structure (*e.g.*, an shRNA). Thus, as mentioned the RNA silencing agent of some embodiments of the disclosure may also be a short hairpin RNA (shRNA).

5 The term “shRNA”, as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide
10 analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, 5 to 15, 7 to 13, 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (Brummelkamp, T. R. *et al.* (2002) *Science* 296: 550) and
15 5'-UUUGUGUAG-3' (Castanotto, D. *et al.* (2002) *RNA* 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

As used herein, the phrase “microRNA (also referred to herein interchangeably
20 as “miRNA” or “miR”) or a precursor thereof” refers to a microRNA (miRNA) molecule acting as a post-transcriptional regulator. Typically, the miRNA molecules are RNA molecules of about 20 to 22 nucleotides in length which can be loaded into a RISC complex and which direct the cleavage of another RNA molecule, wherein the other RNA molecule comprises a nucleotide sequence essentially complementary to the
25 nucleotide sequence of the miRNA molecule.

While not limited by a particular theory, a miRNA molecule is often processed from a “pre-miRNA” or as used herein a precursor of a miRNA molecule by proteins, such as DCL proteins. Pre-microRNA molecules are typically processed from pri-microRNA molecules (primary transcripts). The single-stranded RNA segments
30 flanking the pre-microRNA are important for processing of the pri-miRNA into the pre-miRNA. The cleavage site appears to be determined by the distance from the stem-loop junction (Han *et al.*, 2006, *Cell*, 125:887-901). In some embodiments, a miRNA

molecule is loaded onto a RISC complex where it can guide the cleavage of the target gene of interest.

Pre-microRNA molecules are typically processed from pri-microRNA molecules (primary transcripts). The single stranded RNA segments flanking the pre-microRNA are important for processing of the pri-miRNA into the pre-miRNA. The cleavage site appears to be determined by the distance from the stem-ssRNA junction (Han *et al.* 2006, Cell 125, 887-901, 887-901).

As used herein, a “pre-miRNA” molecule is an RNA molecule of about 100 to about 200 nucleotides, preferably about 100 to about 130 nucleotides which can adopt a secondary structure comprising an imperfect double stranded RNA stem and a single stranded RNA loop (also referred to as “hairpin”) and further comprising the nucleotide sequence of the miRNA (and its complement sequence) in the double stranded RNA stem. According to a specific embodiment, the miRNA and its complement are located about 10 to about 20 nucleotides from the free ends of the miRNA double stranded RNA stem. The length and sequence of the single stranded loop region are not critical and may vary considerably, *e.g.* between 30 and 50 nucleotides in length. The complementarity between the miRNA and its complement need not be perfect and about 1 to 3 bulges of unpaired nucleotides can be tolerated. The secondary structure adopted by an RNA molecule can be predicted by computer algorithms conventional in the art such as mFOLD. The particular strand of the double stranded RNA stem from the pre-miRNA which is released by DCL activity and loaded onto the RISC complex is determined by the degree of complementarity at the 5' end, whereby the strand which at its 5' end is the least involved in hydrogen bonding between the nucleotides of the different strands of the cleaved dsRNA stem is loaded onto the RISC complex and will determine the sequence specificity of the target RNA molecule degradation. However, if empirically the miRNA molecule from a particular synthetic pre-miRNA molecule is not functional (because the “wrong” strand is loaded on the RISC complex), it will be immediately evident that this problem can be solved by exchanging the position of the miRNA molecule and its complement on the respective strands of the dsRNA stem of the pre-miRNA molecule. As is known in the art, binding between A and U involving two hydrogen bounds, or G and U involving two hydrogen bounds is less strong than between G and C involving three hydrogen bounds.

In some embodiments according to the present disclosure, naturally occurring miRNA molecules may be comprised within their naturally occurring pre-miRNA molecules. In other embodiments, a miRNA can be introduced into a non-natural heterologous pre-miRNA molecule scaffold by exchanging the nucleotide sequence of the miRNA molecule. Thus, when processed the recombinant pre-miRNA produces an miRNA having a replaced sequence. In some embodiments, the scaffold of the pre-miRNA can also be completely synthetic. Likewise, synthetic miRNA molecules may be comprised within, and processed from, existing pre-miRNA molecule scaffolds or synthetic pre-miRNA scaffolds. Some pre-miRNA scaffolds may be preferred over others for their efficiency to be correctly processed into the designed microRNAs, particularly when expressed as a chimeric gene. In some aspects a chimeric pre-miRNA gene may include other DNA regions, such as untranslated leader sequences, transcription termination and polyadenylation regions that are incorporated in the primary transcript in addition to the pre-microRNA.

According to the present teachings, the dsRNA sequences may be naturally occurring or synthetic.

The dsRNA sequence for regulating a target gene of interest may contain multiple discrete portions or regions that correspond to the target gene, separated by portions that do not correspond to the target gene. In some embodiments, portions that do not correspond to the target gene may optionally correspond to a second, third, or fourth target gene. It will be appreciated that the portions that correspond to the target gene may have different lengths, different degrees of sequence identity to the target gene, and may correspond to regions located anywhere within the target gene.

In embodiments according to the present disclosure, an antisense RNA sequence may be flanked by two nucleic acid sequences that are complementary to an smRNA expressed in the plant. In some embodiments, the two flanking nucleic acid sequences may be complementary to two different smRNAs. In yet other embodiments, the two flanking nucleic acid sequences may comprise sequences that are complementary to more than two smRNAs. In further embodiments, the smRNAs may comprise two copies of one smRNA and a nucleic acid sequence complementary to a different smRNA.

The present disclosure provides for an includes dsRNA molecules comprising an

antisense RNA sequence and one or two nucleic acid sequences that are complementary to an smRNA expressed in the plant. The present disclosure provides for embodiments having the composition and orientation of one or two nucleic acid sequences that are complementary to an smRNA expressed in the plant of dsRNA molecules as shown in Table 1 below. In certain embodiments, the dsRNA molecules of Table 1 further comprise a helicase binding sequence. In some embodiments, the dsRNA molecules of Table 1, further comprise a helicase binding sequence and a RDRP polypeptide encoding sequence.

Table 1: Embodiments of first strands of dsRNA molecules having a first and second nucleic acid sequence complementary to an smRNA expressed in a plant

Construct	5' smRNA ₂	5' smRNA ₁	Antisense RNA sequence	3' smRNA ₁	3' smRNA ₂
A01 ¹	None	None	Present	None	None
A02	None	Direct	Present	Direct	None
A03	None	Direct	Present	Direct Mut	None
A04	None	Direct	Present	R/C	None
A05	None	Direct	Present	R/C Mut	None
A06	None	Direct Mut	Present	Direct	None
A07	None	Direct Mut	Present	Direct Mut	None
A08	None	Direct Mut	Present	R/C	None
A09	None	Direct Mut	Present	R/C Mut	None
A10	None	R/C	Present	Direct	None
A11	None	R/C	Present	Direct Mut	None
A12	None	R/C	Present	R/C	None
A13	None	R/C	Present	R/C Mut	None
A14	None	R/C Mut	Present	Direct	None
A15	None	R/C Mut	Present	Direct Mut	None
A16	None	R/C Mut	Present	R/C	None
A17	None	R/C Mut	Present	R/C Mut	None
B01	Direct	Direct	Present	Direct	Direct
B02	Direct	Direct	Present	Direct	Direct Mut
B03	Direct	Direct	Present	Direct	R/C
B04	Direct	Direct	Present	Direct	R/C Mut
B05	Direct	Direct	Present	Direct Mut	Direct
B06	Direct	Direct	Present	Direct Mut	Direct Mut

B07	Direct	Direct	Present	Direct Mut	R/C
B08	Direct	Direct	Present	Direct Mut	R/C Mut
B09	Direct	Direct	Present	R/C	Direct
B10	Direct	Direct	Present	R/C	Direct Mut
B11	Direct	Direct	Present	R/C	R/C
B12	Direct	Direct	Present	R/C	R/C Mut
B13	Direct	Direct	Present	R/C Mut	Direct
B14	Direct	Direct	Present	R/C Mut	Direct Mut
B15	Direct	Direct	Present	R/C Mut	R/C
B16	Direct	Direct	Present	R/C Mut	R/C Mut
B17	Direct	Direct Mut	Present	Direct	Direct
B18	Direct	Direct Mut	Present	Direct	Direct Mut
B19	Direct	Direct Mut	Present	Direct	R/C
B20	Direct	Direct Mut	Present	Direct	R/C Mut
B21	Direct	Direct Mut	Present	Direct Mut	Direct
B22	Direct	Direct Mut	Present	Direct Mut	Direct Mut
B23	Direct	Direct Mut	Present	Direct Mut	R/C
B24	Direct	Direct Mut	Present	Direct Mut	R/C Mut
B25	Direct	Direct Mut	Present	R/C	Direct
B26	Direct	Direct Mut	Present	R/C	Direct Mut
B27	Direct	Direct Mut	Present	R/C	R/C
B28	Direct	Direct Mut	Present	R/C	R/C Mut
B29	Direct	Direct Mut	Present	R/C Mut	Direct
B30	Direct	Direct Mut	Present	R/C Mut	Direct Mut
B31	Direct	Direct Mut	Present	R/C Mut	R/C
B32	Direct	Direct Mut	Present	R/C Mut	R/C Mut
B33	Direct	R/C	Present	Direct	Direct
B34	Direct	R/C	Present	Direct	Direct Mut
B35	Direct	R/C	Present	Direct	R/C
B36	Direct	R/C	Present	Direct	R/C Mut
B37	Direct	R/C	Present	R/C	Direct
B38	Direct	R/C	Present	R/C	Direct Mut
B39	Direct	R/C	Present	R/C	R/C
B40	Direct	R/C	Present	R/C	R/C Mut
B41	Direct	R/C	Present	R/C Mut	Direct
B42	Direct	R/C	Present	R/C Mut	Direct Mut
B43	Direct	R/C	Present	R/C Mut	R/C

B44	Direct	R/C	Present	R/C Mut	R/C Mut
B45	Direct	R/C Mut	Present	Direct	Direct
B46	Direct	R/C Mut	Present	Direct	Direct Mut
B47	Direct	R/C Mut	Present	Direct	R/C
B48	Direct	R/C Mut	Present	Direct	R/C Mut
B49	Direct	R/C Mut	Present	Direct Mut	Direct
B50	Direct	R/C Mut	Present	Direct Mut	Direct Mut
B51	Direct	R/C Mut	Present	Direct Mut	R/C
B52	Direct	R/C Mut	Present	Direct Mut	R/C Mut
B53	Direct	R/C Mut	Present	R/C	Direct
B54	Direct	R/C Mut	Present	R/C	Direct Mut
B55	Direct	R/C Mut	Present	R/C	R/C
B56	Direct	R/C Mut	Present	R/C	R/C Mut
B57	Direct	R/C Mut	Present	R/C Mut	Direct
B58	Direct	R/C Mut	Present	R/C Mut	Direct Mut
B59	Direct	R/C Mut	Present	R/C Mut	R/C
B60	Direct	R/C Mut	Present	R/C Mut	R/C Mut
C01	Direct Mut	Direct	Present	Direct	Direct
C02	Direct Mut	Direct	Present	Direct	Direct Mut
C03	Direct Mut	Direct	Present	Direct	R/C
C04	Direct Mut	Direct	Present	Direct	R/C Mut
C05	Direct Mut	Direct	Present	Direct Mut	Direct
C06	Direct Mut	Direct	Present	Direct Mut	Direct Mut
C07	Direct Mut	Direct	Present	Direct Mut	R/C
C08	Direct Mut	Direct	Present	Direct Mut	R/C Mut
C09	Direct Mut	Direct	Present	R/C	Direct
C10	Direct Mut	Direct	Present	R/C	Direct Mut
C11	Direct Mut	Direct	Present	R/C	R/C
C12	Direct Mut	Direct	Present	R/C	R/C Mut
C13	Direct Mut	Direct	Present	R/C Mut	Direct
C14	Direct Mut	Direct	Present	R/C Mut	Direct Mut
C15	Direct Mut	Direct	Present	R/C Mut	R/C
C16	Direct Mut	Direct	Present	R/C Mut	R/C Mut
C17	Direct Mut	Direct Mut	Present	Direct	Direct
C18	Direct Mut	Direct Mut	Present	Direct	Direct Mut
C19	Direct Mut	Direct Mut	Present	Direct	R/C
C20	Direct Mut	Direct Mut	Present	Direct	R/C Mut

C21	Direct Mut	Direct Mut	Present	Direct Mut	Direct
C22	Direct Mut	Direct Mut	Present	Direct Mut	Direct Mut
C23	Direct Mut	Direct Mut	Present	Direct Mut	R/C
C24	Direct Mut	Direct Mut	Present	Direct Mut	R/C Mut
C25	Direct Mut	Direct Mut	Present	R/C	Direct
C26	Direct Mut	Direct Mut	Present	R/C	Direct Mut
C27	Direct Mut	Direct Mut	Present	R/C	R/C
C28	Direct Mut	Direct Mut	Present	R/C	R/C Mut
C29	Direct Mut	Direct Mut	Present	R/C Mut	Direct
C30	Direct Mut	Direct Mut	Present	R/C Mut	Direct Mut
C31	Direct Mut	Direct Mut	Present	R/C Mut	R/C
C32	Direct Mut	Direct Mut	Present	R/C Mut	R/C Mut
C33	Direct Mut	R/C	Present	Direct	Direct
C34	Direct Mut	R/C	Present	Direct	Direct Mut
C35	Direct Mut	R/C	Present	Direct	R/C
C36	Direct Mut	R/C	Present	Direct	R/C Mut
C37	Direct Mut	R/C	Present	R/C	Direct
C38	Direct Mut	R/C	Present	R/C	Direct Mut
C39	Direct Mut	R/C	Present	R/C	R/C
C40	Direct Mut	R/C	Present	R/C	R/C Mut
C41	Direct Mut	R/C	Present	R/C Mut	Direct
C42	Direct Mut	R/C	Present	R/C Mut	Direct Mut
C43	Direct Mut	R/C	Present	R/C Mut	R/C
C44	Direct Mut	R/C	Present	R/C Mut	R/C Mut
C45	Direct Mut	R/C Mut	Present	Direct	Direct
C46	Direct Mut	R/C Mut	Present	Direct	Direct Mut
C47	Direct Mut	R/C Mut	Present	Direct	R/C
C48	Direct Mut	R/C Mut	Present	Direct	R/C Mut
C49	Direct Mut	R/C Mut	Present	Direct Mut	Direct
C50	Direct Mut	R/C Mut	Present	Direct Mut	Direct Mut
C51	Direct Mut	R/C Mut	Present	Direct Mut	R/C
C52	Direct Mut	R/C Mut	Present	Direct Mut	R/C Mut
C53	Direct Mut	R/C Mut	Present	R/C	Direct
C54	Direct Mut	R/C Mut	Present	R/C	Direct Mut
C55	Direct Mut	R/C Mut	Present	R/C	R/C
C56	Direct Mut	R/C Mut	Present	R/C	R/C Mut
C57	Direct Mut	R/C Mut	Present	R/C Mut	Direct

C58	Direct Mut	R/C Mut	Present	R/C Mut	Direct Mut
C59	Direct Mut	R/C Mut	Present	R/C Mut	R/C
C60	Direct Mut	R/C Mut	Present	R/C Mut	R/C Mut
D01	R/C	Direct	Present	Direct	Direct
D02	R/C	Direct	Present	Direct	Direct Mut
D03	R/C	Direct	Present	Direct	R/C
D04	R/C	Direct	Present	Direct	R/C Mut
D05	R/C	Direct	Present	Direct Mut	Direct
D07	R/C	Direct	Present	Direct Mut	R/C
D08	R/C	Direct	Present	Direct Mut	R/C Mut
D10	R/C	Direct	Present	R/C	Direct Mut
D11	R/C	Direct	Present	R/C	R/C
D12	R/C	Direct	Present	R/C	R/C Mut
D13	R/C	Direct	Present	R/C Mut	Direct
D14	R/C	Direct	Present	R/C Mut	Direct Mut
D15	R/C	Direct	Present	R/C Mut	R/C
D16	R/C	Direct	Present	R/C Mut	R/C Mut
D17	R/C	Direct Mut	Present	Direct	Direct
D18	R/C	Direct Mut	Present	Direct	Direct Mut
D19	R/C	Direct Mut	Present	Direct	R/C
D20	R/C	Direct Mut	Present	Direct	R/C Mut
D21	R/C	Direct Mut	Present	Direct Mut	Direct
D22	R/C	Direct Mut	Present	Direct Mut	Direct Mut
D23	R/C	Direct Mut	Present	Direct Mut	R/C
D24	R/C	Direct Mut	Present	Direct Mut	R/C Mut
D25	R/C	Direct Mut	Present	R/C	Direct
D26	R/C	Direct Mut	Present	R/C	Direct Mut
D27	R/C	Direct Mut	Present	R/C	R/C
D28	R/C	Direct Mut	Present	R/C	R/C Mut
D29 ³	R/C	Direct Mut	Present	R/C Mut	Direct
D30	R/C	Direct Mut	Present	R/C Mut	Direct Mut
D31	R/C	Direct Mut	Present	R/C Mut	R/C
D32	R/C	Direct Mut	Present	R/C Mut	R/C Mut
D33	R/C	R/C	Present	Direct	Direct
D34	R/C	R/C	Present	Direct	Direct Mut
D35	R/C	R/C	Present	Direct	R/C
D36	R/C	R/C	Present	Direct	R/C Mut

D37	R/C	R/C	Present	R/C	Direct
D38	R/C	R/C	Present	R/C	Direct Mut
D39	R/C	R/C	Present	R/C	R/C
D40	R/C	R/C	Present	R/C	R/C Mut
D41	R/C	R/C	Present	R/C Mut	Direct
D42	R/C	R/C	Present	R/C Mut	Direct Mut
D43	R/C	R/C	Present	R/C Mut	R/C
D44	R/C	R/C	Present	R/C Mut	R/C Mut
D45	R/C	R/C Mut	Present	Direct	Direct
D46	R/C	R/C Mut	Present	Direct	Direct Mut
D47	R/C	R/C Mut	Present	Direct	R/C
D48	R/C	R/C Mut	Present	Direct	R/C Mut
D49	R/C	R/C Mut	Present	Direct Mut	Direct
D50	R/C	R/C Mut	Present	Direct Mut	Direct Mut
D51	R/C	R/C Mut	Present	Direct Mut	R/C
D52	R/C	R/C Mut	Present	Direct Mut	R/C Mut
D53	R/C	R/C Mut	Present	R/C	Direct
D54	R/C	R/C Mut	Present	R/C	Direct Mut
D55	R/C	R/C Mut	Present	R/C	R/C
D56	R/C	R/C Mut	Present	R/C	R/C Mut
D57	R/C	R/C Mut	Present	R/C Mut	Direct
D58	R/C	R/C Mut	Present	R/C Mut	Direct Mut
D59	R/C	R/C Mut	Present	R/C Mut	R/C
D6	R/C	Direct	Present	Direct Mut	Direct Mut
D60	R/C	R/C Mut	Present	R/C Mut	R/C Mut
D9	R/C	Direct	Present	R/C	Direct
E01	R/C Mut	Direct	Present	Direct	Direct
E02	R/C Mut	Direct	Present	Direct	Direct Mut
E03	R/C Mut	Direct	Present	Direct	R/C
E04	R/C Mut	Direct	Present	Direct	R/C Mut
E05	R/C Mut	Direct	Present	Direct Mut	Direct
E06	R/C Mut	Direct	Present	Direct Mut	Direct Mut
E07	R/C Mut	Direct	Present	Direct Mut	R/C
E08	R/C Mut	Direct	Present	Direct Mut	R/C Mut
E09	R/C Mut	Direct	Present	R/C	Direct
E10	R/C Mut	Direct	Present	R/C	Direct Mut
E11	R/C Mut	Direct	Present	R/C	R/C

E12	R/C Mut	Direct	Present	R/C	R/C Mut
E13	R/C Mut	Direct	Present	R/C Mut	Direct
E14	R/C Mut	Direct	Present	R/C Mut	Direct Mut
E15	R/C Mut	Direct	Present	R/C Mut	R/C
E16	R/C Mut	Direct	Present	R/C Mut	R/C Mut
E17	R/C Mut	Direct Mut	Present	Direct	Direct
E18	R/C Mut	Direct Mut	Present	Direct	Direct Mut
E19	R/C Mut	Direct Mut	Present	Direct	R/C
E20	R/C Mut	Direct Mut	Present	Direct	R/C Mut
E21	R/C Mut	Direct Mut	Present	Direct Mut	Direct
E22	R/C Mut	Direct Mut	Present	Direct Mut	Direct Mut
E23	R/C Mut	Direct Mut	Present	Direct Mut	R/C
E24	R/C Mut	Direct Mut	Present	Direct Mut	R/C Mut
E25	R/C Mut	Direct Mut	Present	R/C	Direct
E26	R/C Mut	Direct Mut	Present	R/C	Direct Mut
E27	R/C Mut	Direct Mut	Present	R/C	R/C
E28	R/C Mut	Direct Mut	Present	R/C	R/C Mut
E29	R/C Mut	Direct Mut	Present	R/C Mut	Direct
E30	R/C Mut	Direct Mut	Present	R/C Mut	Direct Mut
E31	R/C Mut	Direct Mut	Present	R/C Mut	R/C
E32	R/C Mut	Direct Mut	Present	R/C Mut	R/C Mut
E33	R/C Mut	R/C	Present	Direct	Direct
E34	R/C Mut	R/C	Present	Direct	Direct Mut
E35	R/C Mut	R/C	Present	Direct	R/C
E36	R/C Mut	R/C	Present	Direct	R/C Mut
E37	R/C Mut	R/C	Present	R/C	Direct
E38	R/C Mut	R/C	Present	R/C	Direct Mut
E39	R/C Mut	R/C	Present	R/C	R/C
E40	R/C Mut	R/C	Present	R/C	R/C Mut
E41	R/C Mut	R/C	Present	R/C Mut	Direct
E42	R/C Mut	R/C	Present	R/C Mut	Direct Mut
E43	R/C Mut	R/C	Present	R/C Mut	R/C
E44	R/C Mut	R/C	Present	R/C Mut	R/C Mut
E45	R/C Mut	R/C Mut	Present	Direct	Direct
E46	R/C Mut	R/C Mut	Present	Direct	Direct Mut
E47	R/C Mut	R/C Mut	Present	Direct	R/C
E48	R/C Mut	R/C Mut	Present	Direct	R/C Mut

E49	R/C Mut	R/C Mut	Present	Direct Mut	Direct
E50	R/C Mut	R/C Mut	Present	Direct Mut	Direct Mut
E51	R/C Mut	R/C Mut	Present	Direct Mut	R/C
E52	R/C Mut	R/C Mut	Present	Direct Mut	R/C Mut
E53	R/C Mut	R/C Mut	Present	R/C	Direct
E54	R/C Mut	R/C Mut	Present	R/C	Direct Mut
E55	R/C Mut	R/C Mut	Present	R/C	R/C
E56	R/C Mut	R/C Mut	Present	R/C	R/C Mut
E57	R/C Mut	R/C Mut	Present	R/C Mut	Direct
E58	R/C Mut	R/C Mut	Present	R/C Mut	Direct Mut
E59	R/C Mut	R/C Mut	Present	R/C Mut	R/C
E60	R/C Mut	R/C Mut	Present	R/C Mut	R/C Mut

1: see construct #1, Fig 2B and construct #8, Fig 8;
 2: See construct #2, Fig 2A and 2C; construct #4 Fig 4; construct #9 Fig 9
 3: see construct, #3 and construct #6
 As used herein, "Direct" means the direct sequence (*i.e.*, a sequence having the same order of nucleotides and in the same orientation) of a smRNA; "Direct Mut" means the direct sequence of an smRNA having a mutation that renders it resistant to cleavage; "R/C" means the reverse complement of an smRNA; and "R/C Mut" means a reverse complement of an smRNA having a mutation that renders it resistant to cleavage.

In embodiments according to the present disclosure, the sequences of the embodiments of Table 1 include, but are not limited to combinations of SEQ ID NOs: 26 to 35, and 41 to 288, their complements, and non-cleavable mutants thereof. In some embodiments, RNA sequence for regulating a target gene of interest comprises a nucleic acid having 90 to 100% homology to a sequence selected from the group consisting of SEQ ID NOs:8, 11, 12, 36 to 38, and their complements thereof. It is understood that the present disclosure provides for, and includes, dsRNA constructs of Table 1 having a second reverse complementary strand at least to the antisense RNA sequence. The present disclosure further provides dsRNA constructs having a second reverse complimentary strand comprising an antisense RNA sequence and a 5' smRNA₁ sequence. The present disclosure further provides dsRNA constructs having a second reverse complimentary strand comprising an antisense RNA sequence, a 5' smRNA₁ sequence and a 3' smRNA₁ sequence. The present disclosure further provides dsRNA constructs having a second reverse complimentary strand comprising an antisense RNA sequence, a 5' smRNA₁ sequence, a 5' smRNA₂ sequence and a 3' smRNA₁ sequence.

The present disclosure further provides dsRNA constructs having a second reverse complimentary strand comprising an antisense RNA sequence, a 5' smRNA₁ sequence, a 5' smRNA₂ sequence, a 3' smRNA₁ sequence and a 4' smRNA₂ sequence.

5 The present disclosure provides for and includes second reverse complementary strand of the constructs of Table 1 having mismatches. In some embodiments, the second reverse complementary strand provides for a double stranded region comprising a smRNA and its non-cleavable mutant. Accordingly it is understood, the dsRNA comprises one or more mismatches corresponding to the mismatch between the smRNA and it's non-cleavable mutant. The present disclosure provides for combinations of the
10 first strands of Table 1 to produce dsRNA molecules. Accordingly, it is understood that construct A02 may be combined with, for example, the reverse complement of A02 to prepare a dsRNA of the present disclosure. In other embodiments, for example, construct A02 may be combined with the reverse complement of construct A06 to prepare a dsRNA of the present disclosure having a mismatch sequence at the non-cleavable site. One of ordinary skill in the art would recognize that additional
15 combinations of the constructs of Table 1 may be prepared in accordance with the present disclosure.

In embodiments according to the present disclosure, the sequence complementarity may be, but are not required to be, 100%. In certain embodiments of
20 the disclosure the degree of complementarity, *e.g.*, percent complementarity, need only be sufficient to provide for stable binding of a smRNA to the complementary site. In certain embodiments of the disclosure the degree of complementarity need only be sufficient such that the smRNA pairs to the complementary site and mediates cleavage of the target mRNA. For example, in certain embodiments of the disclosure the degree
25 of complementarity is at least 70%, at least 80%, or at least 90%. In certain embodiments of the disclosure the number of mismatched or unpaired nucleotides in the siRNA strand or miRNA, following binding to the complementary site, is between 0 and 5, *e.g.*, 1, 2, 3, 4, or 5.

In other embodiments according to the present disclosure, the sequence
30 complementarity of an smRNA may be greater than 90%. In some embodiments, the sequence complementarity of an smRNA may be greater than 91%. In some embodiments, the sequence complementarity of an smRNA may be greater than 92%.

In other embodiments, the sequence complementarity of an smRNA may be greater than 93%. In some embodiments, the sequence complementarity of an smRNA may be greater than 94%. In some embodiments, the sequence complementarity of an smRNA may be greater than 95%. In other embodiments, the sequence complementarity of an smRNA may be greater than 96%. In some embodiments, the sequence complementarity of an smRNA may be greater than 97%. In some embodiments, the sequence complementarity of an smRNA may be greater than 98%. In other embodiments, the sequence complementarity of an smRNA may be greater than 99%. In some embodiments, the sequence complementarity of an smRNA may be 100%. In embodiments according to the present disclosure, sequence complementarity may be between 90 and 100% or 95 and 100%. According to embodiments of the present disclosure the smRNAs may be selected from the group consisting of SEQ ID NOs 26 to 35, 41 to 288, and non-cleavable mutants thereof.

As used herein, the terms “complementarity” and “complementary” refer to a nucleic acid that can form one or more hydrogen bonds with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types of interactions. It will be recognized that complementarity and homology or identity are related terms. That is, a homology describes the degree of similarity between two or more nucleotide sequences when examined in the same 5' to 3' orientation. In contrast, complementarity describes the degree of similarity between two or more nucleotide sequences when comparing a sequence having a 5' to 3' orientation to a sequence having a 3' to 5' orientation. Thus, a first and second sequence having 90% homology will also have 90% complementarity when the first sequence is compared to the reverse complement of the second sequences. In reference to the nucleic molecules of the presently disclosed subject matter, the binding free energy of a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, in some embodiments, to form a duplex structure under physiological conditions in a plant cell, to mediate ribonuclease activity, *etc.* For example, the degree of complementarity between the sense and antisense strands of an miRNA precursor can be the same or different from the degree of complementarity between the miRNA-containing strand of an miRNA precursor and the target nucleic acid sequence. Determination of binding free energies for nucleic acid molecules is

well known in the art. See *e.g.*, Freier *et al.*, 1986; Turner *et al.*, 1987. One of ordinary skill in the art would be able to test for sufficiency of complementarity by random or site directed mutagenesis and screening of silencing activity and dsRNA molecule stability *in vivo*.

5 In certain embodiments, the phrase “percent complementarity” refers to the percentage of residues in a nucleic acid molecule that can form hydrogen bonds (*e.g.*, Watson-Crick base pairing) with a second nucleic acid sequence (*e.g.*, 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). The terms “100% complementary”, “fully complementary”, and “perfectly complementary”
10 indicate that all of the contiguous residues of a nucleic acid sequence can hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. It will be appreciated that the nucleic acids may have different lengths and/or that there may be bulges when the two nucleic acids are optimally aligned for maximum complementarity over a given portion of either sequence. Percent complementarity can,
15 in various embodiments of the disclosure, disregard such bulges in the computation or consider the percentage complementarity to be the number of paired (hydrogen bonded) residues divided by the total number of residues over a given length, which may be the length of the shorter or the longer nucleic acid in different embodiments.

 Any complementary sequence for a smRNA may be used in various
20 embodiments of the present disclosure. The sequence may be complementary to an miRNA or siRNA. The sequence may be perfectly (100%) complementary or may have imperfect complementarity as described herein and known in the art. The complementary sequence may be one that is naturally found in a trans-acting-siRNA-producing (TAS) locus or ta-siRNA precursor RNA, flanking the portion of the RNA
25 that is cleaved to produce ta-siRNAs. The complementary sequence may be any smRNA complementary sequence that is found on one side of a nucleic acid sequence that is cleaved to produce siRNA, wherein a second smRNA complementary sequence is found on the other side of the nucleic acid sequence. In various embodiments of the disclosure the complementary sequence is recognized by a smRNA selected from the
30 group consisting of:

miR390: (SEQ ID NO: 25) AAGCUCAGGAGGGGAUAGCGCC;

miR161.1: (SEQ ID NO: 26) UUGAAAGUGACUACAUCGGGG;

miR400: (SEQ ID NO: 27) UAUGAGAGUAUUAUAAGUCAC;
TAS2 3'D6(-): (SEQ ID NO: 28) AUAUCCCAUUUCUACCAUCUG;
TAS1b 3'D4(-): (SEQ ID NO: 29) UUCUUCUACCAUCCUAUCAAU;
TAS3 5'D7(+): (SEQ ID NO: 30) UUCUUGACCUUGUAAGACCCC;
5 TAS3 5'D8(+): (SEQ ID NO: 31) UUCUUGACCUUGUAAGGCCUU;
miR168: (SEQ ID NO: 32) UCGCUUGGUGCAGGUCGGGAA;
miR828 (SEQ ID NO: 33) UCUUGCUUAAAUGAGUAUCCA; and
miR393: (SEQ ID NO: 34) UCCAAAGGGAUCGCAUUGAUC.

10 In one embodiment, the miRNA is UUCGCUUGCAGAGAGAAAUCAC (SEQ
ID NO: 35). Note that these sequences may have been identified in one or more plants,
e.g., *Arabidopsis*, most land plants, moss, *etc.* It will be appreciated that in some cases
the sequences are conserved across multiple species while in other cases there could be
minor variations. Such variations are encompassed within the present disclosure. It
will be appreciated that homologous siRNAs or miRNAs from other plant species than
15 those listed could be used. Optionally, recognition of the complementary sequence by
the cognate miRNA or siRNA leads to cleavage. One of skill in the art could determine
whether binding and/or cleavage of a smRNA to a candidate complementary sequence
occurs *in vivo* (in living cells or organisms) or *in vitro*, *e.g.*, under conditions
approximating physiological intercellular conditions. In other embodiments, an
20 smRNA may be selected from the group consisting of SEQ ID NOs:41 to 288 (see,
Table 1 of U.S. Patent Number 8,143,480).

The length of the complementary sequence could vary. The length of a
complementary sequence may be defined as equal to the length of the smRNA that
binds to it, but it will be appreciated that a complementary sequence could differ in
25 length from that of the smRNA, *e.g.*, it may be shorter than the length of the smRNA.
Typically the complementary sequence is sufficiently long such that the smRNA can
bind (*e.g.*, hybridize) to the sequence with reasonable specificity and, optionally, direct
cleavage within a duplex structure formed upon binding. Such cleavage may occur at a
position within the duplex typical of cleavage directed by smRNAs, (*e.g.*, in certain
30 embodiments at position 10 or 11 of the smRNA). For example, a complementary
sequence could be between 15 and 24 nucleotides in length, or any intervening number,
wherein there are 1, 2, 3, 4, or 5 mismatches when the smRNA is paired with the

complementary sequence in the case of a 15 nucleotide sequence and up to 6, 7, or 8 mismatches in the case of a 24 nucleotide complementary sequence. Similar considerations would apply for other smRNA complementary sequence. It will be appreciated that there may be “bulges” in the duplex formed when an smRNA pairs with its complementary sequence. In such instances a bulge could be considered equivalent to a single mismatch or, in various embodiments of the disclosure a bulge of X nucleotides could be considered equivalent to X mismatches. It will also be appreciated that the specificity of binding of the smRNA to the complementary sequence need not be completely specific, *e.g.*, the smRNA may bind to different sequence having either a lesser or greater degree of complementarity.

In embodiments according to the present disclosure, a first or second complementary sequence may be between 15 and 30 nt, between 18 and 24 nt, between 20 and 22, or exactly 21 nt in length. In some embodiments a first or second complementary sequence may comprise any intervening range or specific value within the foregoing ranges in certain embodiments of the disclosure. In certain non-limiting embodiments of the disclosure the number of mismatched or unpaired nucleotides in the siRNA strand or miRNA, following binding to the complementary sequence, is between 0 and 5, *e.g.*, 1, 2, 3, 4, or 5. In certain non-limiting embodiments of the disclosure the number of mismatched or unpaired nucleotides (including those in both strands) in a duplex structure formed between the smRNA and its complementary sequence, is between 0 and 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. The mismatches or bulges may occur at any position within the duplex structure, in various embodiments of the disclosure. In certain embodiments the mismatches or bulges are located at positions known in the art not to typically inhibit or prevent smRNA-directed cleavage. In other embodiments the mismatches or bulges are located at such positions. One or more mismatches or bulges may occur, for example, at any position with respect to the 5' end of a smRNA depicted in the figures herein or contemplated when an smRNA described herein pairs with a sequence complementary to it. The mismatch may be any mismatch known in the art. . In certain embodiments a mismatch is said to occur when a nucleotide within an at least partly double-stranded structure is not paired in a conventional G-C, A-T, or A-U base pair. In certain embodiments a mismatch is said to occur when a nucleotide in an at least partly double-stranded structure is not paired in a

Watson-Crick base pair. It will be appreciated that the aforementioned mismatches may exclude “bulges”, wherein a nucleotide bulges outward from an otherwise duplex region by being located between two nucleotides that are base paired with adjacent nucleotides on the opposite strand of the duplex.

5 The present disclosure further includes and provides for embodiments wherein a first and a second complementary sequence are the same length. In other embodiments, a first complementary sequence may be a different length than a second complementary sequence.

10 The portion of the smRNA that is complementary to the complementary sequence could vary. For example, in certain embodiments the complementary site is at least 70%, at least 80%, or at least 90% complementary to the first 16 nucleotides of the smRNA. In certain embodiments the complementary sequence is at least 70%, at least 80%, or at least 90% complementary to the first 17, 18, or 19 nucleotides of the smRNA. In certain embodiments the complementary sequence is a subsequence of a
15 complementary sequence, wherein said subsequence is at least 16, 17, 18, 19, 20, or 21 nucleotides in length. In some embodiments the subsequence is the last 16, 17, 18, 19, 20, or 21 nucleotides of the listed sequence. In some embodiments the subsequence is at least 70%, at least 80%, at least 90%, or 100% complementary to the first 16, 17, 18, 19, 20, or 21 nucleotides of an smRNA.

20 The present disclosure also includes, and provides for, dsRNA molecules having a single smRNA complementary sequence and an antisense RNA sequence for regulating a target gene of interest. In embodiments according to the present disclosure, where the dsRNA molecule is presented as an antisense sequence, an smRNA sequence on the 5' end is an upstream sequences. In other embodiments, an smRNA sequence on
25 the 3' end is a downstream sequence. Not to be limited by theory, a smRNA complementary sequence binds (hybridizes) to a complementary smRNA and the duplex recruits an RDRP, such as RNA-dependent RNA polymerase 6 (RDR6). The dsRNA molecule can be further processed by Dicer-like enzymes such as dicer-like protein 4 (DCL4) to produce the siRNAs. It will be appreciated that the presence of a
30 second complementary sequence (e.g., flanking sequences) depends on the sequence of smRNA that the complementary sequence recognizes. In some instances (*i.e.*, miR390) it is required whereas in others it is not required (*i.e.*, miR173). In some aspects, for

smRNAs that do not require a second complementary sequence (flanking sequence), the inclusion of a second complementary sequence may increase the propensity, efficiency or rate of generating siRNAs.

5 When only one smRNA complementary sequence is present in a dsRNA molecule, then it can be upstream (5') or downstream (3') to the dsRNA sequence for silencing the target gene (where the dsRNA sequence is referenced in the antisense orientation). According to a specific embodiment, this smRNA complementary sequence is functional and is located 3' to the dsRNA sequence for silencing the target gene. According to another specific embodiment, this smRNA complementary
10 sequence is functional (*e.g.*, miR173) and is located 5' to the dsRNA sequence for silencing the target gene.

When two complementary sequences to one or more an smRNAs are included, one sequence (*e.g.*, the first site) is located 5' to the dsRNA sequence for silencing the target gene (where the dsRNA sequence is referenced in the antisense orientation), and
15 the second site is located 3' to the dsRNA sequence for silencing the target gene (*e.g.*, flanking smRNA sequences). Alternatively, the present disclosure also provides for dsRNA molecules having the second site located 5' to the dsRNA sequence for silencing the target gene, and the first site located 3' to dsRNA sequence for silencing the target gene.

20 It will be appreciated that the complementary sequences can be positioned on one strand (sense) and the other on the other strand (antisense). It will be further appreciated that in the presence of two complementary sequences to an smRNA or smRNAs, one of said sequences can mediate binding of the smRNA but not cleavage of the dsRNA sequence for silencing the target gene. Thus, one of the complementary
25 binding sequences is essentially an smRNA mimic sequence (*e.g.* sufficient for binding but not cleavage).

The smRNA mimic sequence is essentially complementary to the microRNA or siRNA provided that one or more mismatches are allowed: thus, a mismatch between the complementary nucleotides at position 10 or position 11 of the microRNA and the
30 corresponding nucleotide sequence in the micro-RNA resistant site. As used herein, the term "smRNA mimic," "smRNA mutant," and "miRNA Mut" are used interchangeably and refer to smRNAs that are not cleaved in a cell. Not to be limited by any particular

theory, mimic or mutant are thought mediate binding of the machinery, such as the Argonaute protein family, but are not processed, for example by a dicer-like protein. Accordingly, smRNA mimics or mutants interact with the RISC complex but cannot be cleaved. Thus a non-cleavable target mimic of a smRNA acts to sequester the corresponding target miRNA and arrest its activity. By incorporating a miRNA target mimic having a non-cleavable target site the accumulation of all MIR gene family members may be reduced. Methods for preparing smRNA mutants or mimics are known. See Todesco *et al.*, “A Collection of Target Mimics for Comprehensive Analysis of MicroRNA Function in *Arabidopsis thaliana*,” PLOS Genetics 6(7):e1001031 (2010); Wang Z., “The guideline of the design and validation of MiRNA mimics,” Methods Mol Biol. 676:211-23 (2011);

The complementary sequences may be identical or different and may be recognized by the same or different smRNAs, which may be miRNA or siRNA, or both, in any combination.

The complementary sequences(s) can be immediately adjacent to (*i.e.*, contiguous with) the dsRNA sequence for silencing the target gene. Alternatively, the complementary sequence (or at least one of same) may be separated from the dsRNA sequence for silencing the target gene by an intervening spacer or functional sequence (*e.g.*, Helicase binding site).

As used herein a “helicase binding site” refers to a binding site of an RNA helicase. RNA helicases are essential for most processes of RNA metabolism such as ribosome biogenesis, pri-mRNA splicing and translation initiation. Sequence information is available from the RNA Helicase Database, available on the internet at www.dotrnahelicase.org/. According to a specific embodiment, the helicase may be a DEAD RNA helicase (DEAD RH), such as described in Chi *et al.* (2012). “The Function of RH22, a DEAD RNA Helicase, in the Biogenesis of the 50S Ribosomal Subunits of *Arabidopsis* Chloroplasts,” *Plant Physiology*, 158, 693-707. In certain embodiments, a helicase binding site may be positioned so as to allow unwinding of the strands of a dsRNA molecule to single stranded RNA (ssRNA) and allow recruitment of an RNA-dependent RNA Polymerase such as RDR6. Unwinding and recruitment of an RDRP provides for amplification of a dsRNA molecule in the plant cell. Other proteins that are known to be cytosolic proteins and have helicase or helicase-like activity

include the Argonaute protein family, which are a key components of the RISC complex (RNA Induced Silencing Complex). Alternatively a helicase binding site may includes sequences recognized by the plant homolog of RNA helicase RIG-I (Yoneyama *et al.* 2004 Nat. Immun. 5:730-737). Alternatively or additionally, the present disclosure provides for helicase binding site sequences as described in Garcia *et al.* 2012 Mol. Cell 48(1):109-20, which is hereby incorporated by reference in its entirety.

According to a specific embodiment, the helicase binding site is positioned upstream or downstream of the dsRNA sequence for silencing expression of the target gene (where the dsRNA sequence is referenced in the antisense orientation).

According to a specific embodiment, the helicase binding site is positioned upstream or downstream of the dsRNA sequence (where the dsRNA sequence is referenced in the antisense orientation) and the two complementary sites to the smRNA or smRNAs flank the helicase binding site.

According to a further specific embodiment, the helicase binding site is located within the dsRNA sequence corresponding to the target site.

“Flanked by” as used herein, does not require that the smRNA complementary sequences are contiguous with the dsRNA sequence for silencing the target gene. All that is necessary is that there is an smRNA complementary sequences on at least one side or in case of two complementary sequences on each side of the dsRNA sequence for silencing the target gene. Either or both smRNA complementary sequences may, in various embodiments of the disclosure, be located contiguously with the dsRNA sequence for silencing the target gene. In certain embodiments either or both smRNA complementary sequences may, in various embodiments of the disclosure, be separated from the dsRNA sequence for silencing the target gene by between 1 nt and 2 kB, *e.g.*, between 1 nt and 1 kB, between 1 nt and 500 nt, between 1 nt and 250 nt, between 1 nt and 100 nt, *etc.* In certain embodiments either or both smRNA complementary sequences are separated from a portion of the nucleic acid sequence that corresponds to the target gene by between 10 and 20 nt, between 10 and 50 nt, or between 10 and 100 nt. Thus the spacer between either smRNA complementary sequences and the closest nucleotide that corresponds to a portion of a target gene may, in various embodiments of the disclosure, be between 1 nt and 2 kB, *e.g.*, between 1 nt and 1 kB, between 1 nt and 500 nt, between 1 nt and 250 nt, between 1 nt and 100 nt, between 10 and 20 nt,

between 10 and 50 nt, or between 10 and 100 nt in length.

Without being bound to theory, it is suggested that following introduction into the plant, the dsRNA molecule is unwound either by the binding of a helicase to a “helicase binding site” when present or by endogenous RNA helicases that recognize and unwind the dsRNA molecule in a manner similar to antiviral response in a case where it is absent.

Without being bound to a particular theory, once a single stranded molecule is formed is processed by miRNA-guided-cleavage. One product of the cleaved transcript may be stabilized possibly by Suppressor of Gene Silencing 3 (SGS3) and converted to dsRNA by RNA-Dependent RNA Polymerase 6 (RDR6). The resulting dsRNA may be processed through Dicer-Like 4 (DCL4) into 21-nt siRNA duplexes in register with the miRNA-cleavage site. One strand of each smRNA duplex may be selectively sorted to one or more Argonaute (AGO) proteins according to the 5' nucleotide sequence while the other is used as a template for RNA dependent RNA polymerase, thereby constantly generating more phase siRNA molecule.

Thus, the dsRNA molecule is designed for specifically targeting a target gene of interest. It will be appreciated that the dsRNA can be used to down-regulate one or more target genes. In some embodiments, a single isolated dsRNA molecule can target a number of different genes.

The present disclosure provides for and includes heterogenic compositions of dsRNA molecules. In certain embodiments wherein a dsRNA molecule targets a single target gene of interest, heterogenic compositions comprising two or more dsRNA molecules that target two or more target genes of interest may be prepared. A heterogenic composition comprises a plurality of dsRNA molecules for targeting a number of target genes may be prepared. In some embodiments, a plurality of dsRNA molecules may be separately applied to the seeds (but not as a single composition).

The present disclosure provides for an includes dsRNA molecules comprising a sequence, wherein said nucleic acid sequence shares between 100% and 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other

embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 91 % sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 92% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 93% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 94% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 95% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 96% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 97% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 98% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 99% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299. In other embodiments according to the present disclosure, a dsRNA molecule comprises a sequence that shares more than 100 % sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 289-299.

While conceiving the present disclosure, the present inventors realized that the long dsRNA identified by Fukuhara et al. is able to survive autonomously in rice cells, and as such can be used as a cassette (a building block) for introducing dsRNA sequenced for RNA silencing of a target gene of interest (endogenous to the plant or

exogenous thereto). Such a dsRNA molecule is expressed throughout the plant's life cycle, does not become integrated into the plant genome (plastid or nuclear), and does not get reverse-transcribed into DNA.

Thus, in some embodiments of the present disclosure provide for endovirus-derived sequences which have evolved to co-exist in plant cells in a dsRNA form, maintaining a near exact copy number in all cells.

As used herein the term "endovirus" refers to a dsRNA symbiotic virus which propagates in the plant cell and maintains a relatively stable copy number throughout the life cycle of the plant.

As used herein the term "5' UTR" refers to an untranslated region derived from the endovirus sequence (13,716 nucleotides, available from GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data bases with accession number D32136, according to Fukuhara 1995 *supra*), adjacent (in a 5' orientation) to its RDRP sequence.

As used herein the term "3' UTR" refers to an untranslated region derived from the endovirus sequence (13,716 nucleotides, available from GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data bases with accession number D32136, according to Fukuhara 1995 *supra*), adjacent (in a 3' orientation) to its RDRP sequence.

As used herein the term "RNA dependent RNA Polymerase" refers to the RDRP-like sequence derived from the 13,716 nucleotides described in Fukuhara *et al.* 1995 *supra*.

According to a specific embodiment said 5' UTR is as set forth in SEQ ID NO: 14. It will be appreciated that the sequences are provided in the form of DNA but will be made RNA upon subjecting to T7 activity. In some embodiments, the 5' UTR shares between 90% and 100% sequences identity to a nucleic acid sequence of SEQ ID NO:14.

According to a specific embodiment said 3' UTR is as set forth in SEQ ID NO: 22.

According to a specific embodiment said endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence is as set forth in SEQ ID NO: 23.

According to some embodiments of the disclosure, the nucleic acid sequence is at least about 80 %, at least about 81 %, at least about 82 %, at least about 83 %, at least about 84 %, at least about 85 %, at least about 86 %, at least about 87 %, at least about 88 %, at least about 89 %, at least about 90 %, at least about 91 %, at least about 92 %, at least about 93 %, at least about 94 %, at least about 95 %, at least about 96 %, at least about 97 %, at least about 98 %, at least about 99 %, and at least about 100 %.

at least about 93 %, at least about 94 %, at least about 95 %, at least about 96 %, at least about 97 %, at least about 98 %, at least about 99 %, or more say 100 % identical to the nucleic acid sequence of SEQ ID NO: 14, 22 or 23 (or a combination of same). It is understood that combinations of SEQ ID NOs: 14, 22, or 23 having differing percent
5 homology are envisioned. As a non-limiting example, a combination dsRNA molecule may comprise a sequence having 85% homology to SEQ ID NO:14, 90% homology to SEQ ID NO:22 and 99% homology to SEQ ID NO:23. Any number of such combinations are contemplated and provided for in the present disclosure.

One of ordinary skill in the art would understand that certain nucleotide
10 positions of a polynucleotide sequence play critical roles while other nucleotide positions may vary without significant effect. This understanding is well illustrated for polypeptide encoding sequences. For example, the first and second nucleotide of an amino acid encoding codon largely determine the identity of an amino acid in a polypeptide chain. In contrast, the third “wobble” position can vary, sometimes without
15 limitation. Accordingly, one of ordinary skill in the art can substitute approximately 30% of the nucleic acid sequence without affecting the amino acid sequence. A similar dependence for non-coding sequences, including 3' and 5' UTRs exists, though the positions of importance may not be predictable. However, one of ordinary skill in the art may mutagenize (randomly or through site directed) positions in a nucleic acid
20 sequence and confirm their activity using the functional assays disclosed in the present application (See, Examples below).

According to some embodiments, a nucleic acid sequence may encode a protein that shares between 70% and 100% homology with an RNA Dependent RNA Polymerase (RDRP) polypeptide sequence according to SEQ ID NO: 300. A nucleic
25 acid sequence according to the present disclosure may encode a protein having 70 to 75% homology to a polypeptide sequence according to SEQ ID NO: 300. In another embodiment, a nucleic acid sequence may encode a protein having 75 to 80% homology to a polypeptide sequence according to SEQ ID NO: 300. In another embodiment, a nucleic acid sequence may encode a protein having 80 to 85% homology to a
30 polypeptide sequence according to SEQ ID NO: 300. In an embodiment, a nucleic acid sequence may encode a protein having 85 to 90% homology to a polypeptide sequence according to SEQ ID NO: 300. In another embodiment, a nucleic acid sequence may

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encode a protein having 90 to 95% homology to a polypeptide sequence according to SEQ ID NO: 300. In an embodiment, a nucleic acid sequence may encode a protein having 95 to 100% homology to a polypeptide sequence according to SEQ ID NO: 300. In another embodiment, a nucleic acid sequence may encode a protein having 97 to 100% homology to a polypeptide sequence according to SEQ ID NO: 300. In an embodiment, a nucleic acid sequence may encode a protein having 98 to 100% homology to a polypeptide sequence according to SEQ ID NO: 300. In another embodiment, a nucleic acid sequence may encode a protein having 99 to 100% homology to a polypeptide sequence according to SEQ ID NO: 300.

10 Homologous sequences to the above can also be used according to the present teachings, as long as their main characteristics are maintained, *i.e.*, amplification by RDRP and maintenance of stable copy number in the cell throughout the plant life cycle.

15 The present disclosure further provides for, and includes, an isolated dsRNA molecule comprising a nucleic acid sequence in a sequential order from 5' to 3', an endovirus 5' UTR, an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence, an endovirus 3' UTR and a cloning site flanked by said RDRP and said 3' UTR.

20 The present disclosure further provides for, and includes, an isolated dsRNA molecule comprising a nucleic acid sequence in a sequential order from 5' to 3', an endovirus 5' UTR, an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence, an endovirus 3' UTR and a nucleic acid sequence for regulating a target gene flanked by said RDRP and said 3' UTR.

25 In some embodiments, a heterologous dsRNA sequence corresponding to a target gene is constructed such that it is flanked by the RDRP sequence and the 3' UTR. Alternatively, in other embodiments, a heterologous dsRNA sequence corresponding to the target gene is constructed such that it is flanked by the RDRP sequence and the 5' UTR. When introduced into the plant (*e.g.*, directly to the seed), and once germination has initiated, gene expression occurs including expression of endogenous plant
30 helicases, RDRPs and other components of the silencing machinery. Not to be limited by theory, at any given time, a portion of the dsRNA molecules is recognized and processed by the plant's dicer like (DCL) proteins into siRNA of different lengths. In

certain embodiments, this recognition and processing includes processing of the gene targeted for silencing, which is flanked between the 5' and the 3' UTR. In certain embodiments, the gene targeted for silencing and the RDRP sequence is flanked between the 5' and the 3' UTR. Not all of the heterologous dsRNA sequence is processed and a portion remains in double-stranded, full length form. Not to be limited by theory, it is thought that plant helicases recognize unique features in the 5' UTR and the 3' UTR of the dsRNA and unwind the dsRNA into two ssRNA molecules. Again, not limited by theory, the same or other feature in the 5' and 3' UTR are thought to also recruit and activate an RDRP. In some embodiments, the RDRP may be an RDRP that is encoded by the dsRNA. In other embodiments, the RDRP may be an endogenous RDRP, naturally occurring in the plant or introduced as a transgene.

Though not limited by theory, it is thought that the RDRP uses each of the ssRNA molecules thought to be produced by the activity of a helicase as templates to produce a dsRNA molecule identical to the original dsRNA molecule.

Accordingly, and not to be limited by theory, as long as a ratio between processed and un-processed dsRNA molecules is maintained, the cycle can go on and repeat throughout the plant's life cycle. Also not to be limited by theory, it is thought that some features in the 5' and 3' UTR can assist to maintain a stable copy number of the dsRNA in cells, in a similar manner to the endovirus which is maintained at a stable copy number.

As used herein the term "sequential" refers to multiple nucleic acid segments (*e.g.*, 5' UTR, RDRP and 3' UTR) arranged in sequence. In this case, from 5' to 3'. Each of the specified nucleic acid segments can be directly attached to each other and contiguous, however intervening nucleic acids can be implanted therebetween such that the segments are not directly attached to each other and are discontinuous.

According to an embodiment of the disclosure the target gene is endogenous to the plant. Downregulating such a gene is typically important for conferring the plant with an improved, agricultural, horticultural, nutritional trait ("improvement" or an "increase" is further defined hereinbelow).

As used herein, the terms "suppress," "repress," and "downregulate" when referring to the expression or activity of a nucleic acid molecule in a plant cell are used equivalently herein and mean that the level of expression or activity of the nucleic acid

molecule in a plant, a plant part, or plant cell after applying a method of the present disclosure is lower than its expression or activity in the plant, part of the plant, or plant cell before applying the method, or compared to a control plant lacking a dsRNA molecule of the disclosure.

5 The terms “suppressed,” “repressed” and “downregulated” as used herein are synonymous and mean herein lower, preferably significantly lower, expression or activity of the nucleic acid molecule to be expressed.

10 As used herein, a “suppression,” “repression,” or “downregulation” of the level or activity of an agent such as a protein, mRNA, or RNA means that the level or activity is reduced relative to a substantially identical plant, part of a plant, or plant cell grown under substantially identical conditions, lacking a dsRNA molecule of the disclosure, for example, lacking an RNA sequence for regulating a target gene of interest. As used herein, “suppression,” “repression,” or “downregulation” of the level or activity of an agent, such as, for example, a preRNA, mRNA, rRNA, tRNA, snoRNA, snRNA
15 expressed by the target gene, and/or of the protein product encoded by it, means that the amount is reduced by 10% or more, for example, 20% or more, preferably 30% or more, more preferably 50% or more, even more preferably 70% or more, most preferably 80% or more, for example, 90%, relative to a cell or organism lacking a dsRNA molecule of the disclosure.

20 As used herein “a suppressive amount” refers to an amount of dsRNA molecule which is sufficient to down regulate the target gene by at least 20 %. In an aspect, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by 30 % or more. In an aspect, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene
25 by 40 % or more. In an aspect, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by at least 50 %. In other aspects, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by 60 % or more. In an aspect, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by at least 70 %.
30 In an aspect, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by 80 % or more. In an aspect, a suppressive amount according to the present disclosure is an

amount sufficient to downregulate a target gene by greater than 90 %. In certain aspects, a suppressive amount according to the present disclosure is an amount sufficient to downregulate a target gene by 100 % (e.g., wherein the remaining amount of the target gene is not detectable). The suppressive amount can be a result of the formation of amplification in the plant or the phytopathogen.

As used herein “endogenous” refers to a gene which expression (mRNA or protein) takes place in the plant. Typically, the endogenous gene is naturally expressed in the plant or originates from the plant. Thus, the plant may be a wild-type plant. However, the plant may also be a genetically modified plant (transgenic).

Downregulation of the target gene may be important for conferring improved one of-, or at least one of (e.g., two of- or more), biomass, vigor, yield, abiotic stress tolerance, biotic stress tolerance or improved nitrogen use efficiency.

Examples of target genes include, but are not limited to, an enzyme, a structural protein, a plant regulatory protein, an miRNA target gene, or a non-coding RNA such as a miRNA of the plant. WO2011067745, WO 2009125401 and WO 2012056401 provide examples of miRNA sequences or targets of miRNAs (e.g., miRNA167, miRNA169, miRNA 156, miR164 and targets thereof ARF, NFY, SPL17 and NAC, respectively) which expression can be silenced to improve a plant trait.

The target gene may comprise a nucleic acid sequence which is transcribed to an mRNA which codes for a polypeptide.

Alternatively, the target gene can be a non-coding gene such as an miRNA or a siRNA.

For example, in order to silence the expression of an mRNA of interest, synthesis of the dsRNA molecule suitable for use with some embodiments of the disclosure can be selected as follows. First, the mRNA sequence may be scanned including the 3' UTR and the 5' UTR.

Second, the mRNA sequence may be compared to an appropriate genomic database using any sequence alignment software, such as the BLAST software available from the NCBI server (available on the internet at www.ncbi.nlm.nih.gov/BLAST/). Putative regions in the mRNA sequence which exhibit significant homology to other coding sequences may be filtered out.

Qualifying target sequences may be selected as for the preparation of dsRNA

templates for dsRNA molecule synthesis. Preferred sequences are those that have as little homology to other genes in the genome to reduce an “off-target” effect.

It will be appreciated that the RNA regulating or silencing agent of some embodiments of the disclosure need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

The dsRNA molecules of the present disclosure may be synthesized using any method known in the art, including either enzymatic syntheses or solid-phase syntheses. These are especially useful in the case of short polynucleotide sequences with or without modifications as explained above. Equipment and reagents for executing solid-phase synthesis are commercially available from, for example, Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the capabilities of one skilled in the art and can be accomplished via established methodologies as detailed in, for example: Sambrook, J. and Russell, D. W. (2001), “Molecular Cloning: A Laboratory Manual”; Ausubel, R. M. *et al.*, eds. (1994, 1989), “Current Protocols in Molecular Biology,” Volumes I-III, John Wiley & Sons, Baltimore, Maryland; Perbal, B. (1988), “A Practical Guide to Molecular Cloning”, John Wiley & Sons, New York; and Gait, M. J., ed. (1984), “Oligonucleotide Synthesis”; utilizing solid-phase chemistry, *e.g.*, cyanoethyl phosphoramidite followed by deprotection, desalting, and purification by, for example, an automated trityl-on method or HPLC.

The nucleic acids of the present disclosure may comprise heterocyclic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3' to 5' 5phosphodiester linkage. Preferably used nucleic acids are those modified in either backbone, internucleoside linkages or bases, as is broadly described hereinunder.

Specific examples of preferred nucleic acids useful according to this aspect of the present disclosure include nucleic acids containing modified backbones or non-natural internucleoside linkages. Nucleic acids having modified backbones include those that retain a phosphorus atom in the backbone, as disclosed in U.S. Patent NOs: 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified polynucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms can also be used.

Alternatively, modified polynucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts, as disclosed in U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,214,134; 5,466,677; 5,610,289; 5,633,360; 5,677,437; and 5,677,439.

Other nucleic acids which can be used according to the present disclosure, are those modified in both sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for complementation with the appropriate polynucleotide target. An example for such an polynucleotide mimetic, includes peptide nucleic acid (PNA). A PNA polynucleotide refers to a polynucleotide where the sugar-backbone is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion

of the backbone. United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Other backbone modifications, which can be used in the present disclosure are disclosed in U.S. Pat. No: 6,303,374.

5 Polynucleotide agents of the present disclosure may also include base modifications or substitutions. As used herein, “unmodified” or “natural” bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include but are not limited to other synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, 10 xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted 15 adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further bases include those disclosed in U.S. Pat. No: 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 20 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*, 1991, , 613, and those disclosed by Sanghvi, Y. S., Chapter 15, *Antisense Research and Applications*, pages 289-2, Crooke, S. T. and Lebleu, B. , ed., CRC Press, 1993. Such bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the disclosure. These 25 include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi YS *et al.* (1993) *Antisense Research and Applications*, CRC Press, Boca Raton 276-278) and are presently preferred base substitutions, even more 30 particularly when combined with 2'-O-methoxyethyl sugar modifications.

Following synthesis, the polynucleotide agents of the present disclosure may optionally be purified. For example, polynucleotides can be purified from a mixture by

extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, polynucleotides may be used with no, or a minimum of, purification to avoid losses due to sample processing. The polynucleotides may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers
5 or salts to promote annealing, and/or stabilization of the duplex strands.

It will be appreciated that a polynucleotide agent of the present disclosure may be provided *per se*, or as a nucleic acid construct comprising a nucleic acid sequence encoding the polynucleotide agent. Typically, the nucleic acid construct comprises a promoter sequence which is functional in the host cell, as detailed herein below.

10 The polynucleotide sequences of the present disclosure, under the control of an operably linked promoter sequence, may further be flanked by additional sequences that advantageously affect its transcription and/or the stability of a resulting transcript. Such sequences are generally located upstream of the promoter and/or downstream of the 3' end of the expression construct.

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

25 The present disclosure provides for and includes DNA templates for the preparation of dsRNA molecules. As used herein, “dsRNA template” refers to a DNA sequence having the same sequence as the corresponding dsRNA molecule. In certain embodiments, a dsRNA template may further include additional sequences, such as promoters, sufficient for the expression of one or more RNA molecules via
30 transcription. In some embodiments the promoters are bacteriophage promoters, for example but not limited to, SP6, T3 and T7. In some embodiments a promoter may be a bacterial or a eukaryotic promoters.

In certain embodiments, a dsRNA template according to the present teachings may be used as a cassette for the cloning of a nucleic acid sequence corresponding to a target gene of interest (exogenous to the plant or endogenous thereto) for silencing expression of same when expressed as a dsRNA molecule.

5 Thus according to an embodiment of the disclosure, a dsRNA template may comprise a cloning site (multiple cloning site for instance) to which a nucleic sequence for silencing a target gene of interest is ligated while being flanked by the RDRP encoding and the 3'UTR encoding nucleic acid sequences. In other aspects according to present disclosure, a dsRNA molecule may be prepared by chemical synthesis using
10 methods known in the art. In other embodiments, a dsRNA template may comprise a plasmid vector having the dsRNA molecule coding sequences. In certain embodiments, the dsRNA template may be a linear polynucleotide having a RNA polymerase promoter at one end. It will be appreciated that such a template produces a single strand RNA.

15 In certain embodiments, a dsRNA template may comprise a mixture of two linear polynucleotides having the same dsRNA coding sequence but promoters at opposite ends. It will be appreciated that transcription results in the production of two complementary RNA strands from the separate template that may be annealed and recovered, or recovered and annealed. By providing separate transcription templates,
20 and recovering annealed double stranded dsRNA molecules according to the present disclosure, asymmetric dsRNA molecules may be produced. As provided below, dsRNA construct #4 provides for a dsRNA region corresponding to a region of a target gene of interest and having non-double stranded Mir390 Mut BS and Mir390BS sequences. As used herein, the term "overhang" refers to non-double stranded regions
25 of a dsRNA molecule (*i.e.*, single stranded RNA). Accordingly dsRNA construct #4 has two overhang regions comprising Mir390 Mut BS and Mir390 BS sequences respectively. Similarly, dsRNA construct #6 provides for an asymmetric dsRNA having a non-double stranded helicase binding sequence or overhang.

In other aspects, a dsRNA template may have two promoters flanking the
30 dsRNA coding sequences. It will be appreciated that, like the separate templates, two complementary strands are produced that may be annealed and recovered, or recovered and annealed. The promoters of the dsRNA templates of some embodiments may be

the same or different.

As mentioned, in certain embodiments, the dsRNA molecule may be directly contacted with the seed.

5 The seed may be of any plant, such as of the *Viridiplantae* super family including monocotyledon and dicotyledon plants. Other plants are listed below. According to an embodiment of the disclosure, the cells of the plant comprise RNA dependent RNA polymerase activity and the target RNA molecule of the dsRNA to ensure amplification of the dsRNA.

10 The term “plant” as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and isolated plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. It will be appreciated, that the plant or seed thereof may be transgenic plants.

15 As used herein the phrase “plant cell” refers to plant cells which are derived and isolated from disintegrated plant cell tissue or plant cell cultures.

20 As used herein the phrase “plant cell culture” refers to any type of native (naturally occurring) plant cells, plant cell lines and genetically modified plant cells, which are not assembled to form a complete plant, such that at least one biological structure of a plant is not present. Optionally, the plant cell culture of this embodiment of the present disclosure may comprise a particular type of a plant cell or a plurality of different types of plant cells. It should be noted that optionally plant cultures featuring a particular type of plant cell may be originally derived from a plurality of different types of such plant cells. In certain embodiments according to the present disclosure, the plant cell is a non-sexually producing plant cell. In other aspects, a plant cell of the present disclosure is a non-photosynthetic plant cell.

25 Any commercially or scientifically valuable plant is envisaged in accordance with some embodiments of the disclosure. Plants that are particularly useful in the methods of the disclosure include all plants which belong to the super family *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp.*, *Acer spp.*, *Actinidia spp.*, *Aesculus spp.*, *Agathis australis*,

30

Albizia amara, *Alsophila tricolor*, *Andropogon* spp., *Arachis* spp., *Areca catechu*,
Astelia fragrans, *Astragalus cicer*, *Baikiaea plurijuga*, *Betula* spp., *Brassica* spp.,
Bruguiera gymnorrhiza, *Burkea africana*, *Butea frondosa*, *Cadaba farinosa*, *Calliandra*
5 *spp.*, *Camellia sinensis*, *Canna indica*, *Capsicum* spp., *Cassia* spp., *Centroema*
pubescens, *Chacoomeles* spp., *Cinnamomum cassia*, *Coffea arabica*, *Colophospermum*
mopane, *Coronillia varia*, *Cotoneaster serotina*, *Crataegus* spp., *Cucumis* spp.,
Cupressus spp., *Cyathea dealbata*, *Cydonia oblonga*, *Cryptomeria japonica*,
Cymbopogon spp., *Cynthea dealbata*, *Cydonia oblonga*, *Dalbergia monetaria*, *Davallia*
divaricata, *Desmodium* spp., *Dicksonia squarosa*, *Dibeteropogon amplectens*, *Dioclea*
10 *spp.*, *Dolichos* spp., *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehaffia* spp.,
Eleusine coracana, *Eragrestis* spp., *Erythrina* spp., *Eucalyptus* spp., *Euclea schimperi*,
Eulalia villosa, *Pagopyrum* spp., *Feijoa sellowiana*, *Fragaria* spp., *Flemingia* spp.,
Freycinetia banksii, *Geranium thunbergii*, *GinAgo biloba*, *Glycine javanica*, *Gliricidia*
spp., *Gossypium hirsutum*, *Grevillea* spp., *Guibourtia coleosperma*, *Hedysarum* spp.,
15 *Hemaffhia altissima*, *Heteropogon contoffus*, *Hordeum vulgare*, *Hyparrhenia rufa*,
Hypericum erectum, *Hypeffhelia dissolute*, *Indigo incamata*, *Iris* spp., *Leptarrhena*
pyrolifolia, *Lespediza* spp., *Lettuca* spp., *Leucaena leucocephala*, *Loudetia simplex*,
Lotonus bainesii, *Lotus* spp., *Macrotyloma axillare*, *Malus* spp., *Manihot esculenta*,
Medicago saliva, *Metasequoia glyptostroboides*, *Musa sapientum*, *Nicotianum* spp.,
20 *Onobrychis* spp., *Ornithopus* spp., *Oryza* spp., *Peltophorum africanum*, *Pennisetum*
spp., *Persea gratissima*, *Petunia* spp., *Phaseolus* spp., *Phoenix canariensis*, *Phormium*
cookianum, *Photinia* spp., *Picea glauca*, *Pinus* spp., *Pisum sativum*, *Podocarpus totara*,
Pogonarthria fleckii, *Pogonaffhria squarrosa*, *Populus* spp., *Prosopis cineraria*,
Pseudotsuga menziesii, *Pterolobium stellatum*, *Pyrus communis*, *Quercus* spp.,
25 *Rhaphiolepis umbellata*, *Rhopalostylis sapida*, *Rhus natalensis*, *Ribes grossularia*,
Ribes spp., *Robinia pseudoacacia*, *Rosa* spp., *Rubus* spp., *Salix* spp., *Schyzachyrium*
sanguineum, *Sciadopitys vefficillata*, *Sequoia sempervirens*, *Sequoiadendron*
giganteum, *Sorghum bicolor*, *Spinacia* spp., *Sporobolus fimbriatus*, *Stiburus*
alopecuroides, *Stylosanthos humilis*, *Tadehagi* spp., *Taxodium distichum*, *Themeda*
30 *triandra*, *Trifolium* spp., *Triticum* spp., *Tsuga heterophylla*, *Vaccinium* spp., *Vicia* spp.,
Vitis vinifera, *Watsonia pyramidata*, *Zantedeschia aethiopica*, *Zea mays*, amaranth,
artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower,

celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash tea, maize, wheat, barley, rye, oat, peanut, pea, lentil and alfalfa, cotton, rapeseed, canola, pepper, sunflower, tobacco, eggplant, eucalyptus, a tree, an ornamental plant, a perennial grass and a forage crop.

5 Alternatively algae and other non-Viridiplantae can be used for the methods of the present disclosure.

According to some embodiments of the disclosure, the plant used by the method of the disclosure is a crop plant including, but not limited to, cotton, *Brassica* vegetables, oilseed rape, sesame, olive tree, palm oil, banana, wheat, corn or maize, 10 barley, alfalfa, peanuts, sunflowers, rice, oats, sugarcane, soybean, turf grasses, barley, rye, sorghum, sugar cane, chicory, lettuce, tomato, zucchini, bell pepper, eggplant, cucumber, melon, watermelon, beans, hibiscus, okra, apple, rose, strawberry, chili, garlic, pea, lentil, canola, mums, *Arabidopsis*, broccoli, cabbage, beet, quinoa, spinach, squash, onion, leek, tobacco, potato, sugar beet, papaya, pineapple, mango, *Arabidopsis* 15 *thaliana*, and also plants used in horticulture, floriculture or forestry, such as, but not limited to, poplar, fir, eucalyptus, pine, an ornamental plant, a perennial grass and a forage crop, coniferous plants, moss, algae, as well as other plants available on the internet at, for example, www.dotnationmasterdotcom/encyclopedia/Plantae.

According to a specific embodiment, the plant is selected from the group 20 consisting of corn, rice, wheat, tomato, cotton and sorghum. In certain embodiments, the plant is a corn plant. In certain embodiments, the plant is a rice plant. In certain embodiments, the plant is a wheat plant. In certain embodiments, the plant is a cotton plant. In certain embodiments, the plant is a sorghum plant.

Introduction of the compositions of the present disclosure can be performed to 25 any organs/cells of the plant (as opposed to seeds) using conventional delivery methods such as particle bombardment, grafting, soaking and the like.

According to a specific embodiment, the composition is introduced directly to the seed.

According to a specific embodiment, the seed is an uncoated or fresh seed that 30 hasn't been subjected to chemical/physical treatments. In certain embodiments, the seed is a corn seed. In certain embodiments, the seed is a rice seed. In certain embodiments, the seed is a wheat seed. In certain embodiments, the seed is a cotton

seed. In certain embodiments, the seed is a sorghum seed.

The seed may be subjected to priming or washing prior to contacting with the dsRNA.

5 In embodiments according to the present disclosure, washing of the seeds is effected for 30 min to 4 hours. In other embodiments, the seeds may be washed up to 5 hours. In an embodiment a seed may be washed up 6, 7 or 8 hours. In another embodiment, seeds are washed for less than 4 or less than 3 hours. In some embodiments, a seed may be washed less than two hours or less than one hour.

10 The present disclosure provides for and includes washing of a seed between 1 minute and 1 hours. Also included are brief washes comprising less than 1 minute wherein the seed is completely wet and then removed from the wash. In some embodiments, a seed may be washed from 1 minute to 10 minutes. In another embodiment, a seed may be washed from 1 minute to 10 minutes. In an embodiment, a seed may be washed from 10 to 30 minutes. In yet another embodiment, a seed may be washed from 1 to 30 minutes. In certain embodiments, a seed may be washed from 5 to 15 minutes or 5 to 15 minutes. In some embodiments, a seed may be washed from 15 to 30 minutes or 10 to 25 minutes.

20 In some embodiments according to the present disclosure, the wash solution may include a weak detergent such as Tween-20, or its equivalents. In some embodiments, the detergent may be less than 1% by volume. In other embodiments, the detergent may be less than 0.5% by volume. In some embodiments, the detergent may be less than 0.25% or 0.2% by volume. In other embodiments, the detergent may be less than 0.1% or 0.05% by volume. In embodiments according to the present disclosure, the wash solution may contain a detergent at between 0.01 to 0.2 % or 0.2 to 1 %. In other 25 embodiments, the wash solution may contain a detergent at between 0.05 to 0.5 % or 0.5 to 1.5 %.

30 As used herein the term "priming" refers to controlling the hydration level within seeds so that the metabolic activity necessary for germination can occur but radicle emergence is prevented. Different physiological activities within the seed occur at different moisture levels (Leopold and Vertucci, 1989; Taylor, 1997). The last physiological activity in the germination process is radicle emergence. The initiation of radicle emergence requires a high seed water content. By limiting seed water content,

all the metabolic steps necessary for germination can occur without the irreversible act of radicle emergence. Prior to radicle emergence, the seed is considered desiccation tolerant, thus the primed seed moisture content can be decreased by drying. After drying, primed seeds can be stored until time of sowing.

5 Several different priming methods are used commercially. Among them, liquid or osmotic priming and solid matrix priming appear to have the greatest following (Khan *et al.*, 1991).

10 According to an embodiment of the disclosure, priming is effected in the presence of salt, a chelating agent, polyethylene glycol or a combination of same (*e.g.*, chelating agent and salt).

 Alternatively priming is effected in the presence of water such as deionized water or double deionized water (ddW). According to a specific embodiment, the priming is effected in the presence of 100 % ddW.

 Several types of seed priming are commonly used:

15 Osmopriming (osmoconditioning) is a standard priming technique. Seeds are incubated in well aerated solutions with a low water potential, and afterwards washes and dried. The low water potential of the solutions can be achieved by adding osmotica like mannitol, polyethyleneglycol (PEG) or salts like KCl. In embodiments according to the present disclosure, the seeds are osmoprimed. In certain embodiments, the
20 osmoprimed seed is a corn seed. In certain embodiments, the osmoprimed seed is a rice seed. In certain embodiments, the osmoprimed seed is a wheat seed. In certain embodiments, the osmoprimed seed is a cotton seed. In certain embodiments, the osmoprimed seed is a sorghum seed.

25 Hydropriming (drum priming) is achieved by continuous or successive addition of a limited amount of water to the seeds. A drum is used for this purpose and the water can also be applied by humid air. 'On-farm steeping' is a cheap and useful technique that is practiced by incubating seeds (cereals, legumes) for a limited time in warm water. In embodiments according to the present disclosure, the seeds are hydroprimed. In certain embodiments, the hydroprimed seed is a corn seed. In certain embodiments,
30 the hydroprimed seed is a rice seed. In certain embodiments, the hydroprimed seed is a wheat seed. In certain embodiments, the hydroprimed seed is a cotton seed. In certain embodiments, the hydroprimed seed is a sorghum seed.

Matrixpriming (matricconditioning) is the incubation of seeds in a solid, insoluble matrix (vermiculite, diatomaceous earth, cross-linked highly water-absorbent polymers) with a limited amount of water. This method confers a slow imbibition. In
embodiments according to the present disclosure, the seeds are matricconditioned. In
5 certain embodiments, the matricconditioned seed is a corn seed. In certain embodiments,
the matricconditioned seed is a rice seed. In certain embodiments, the matricconditioned
seed is a wheat seed. In certain embodiments, the matricconditioned seed is a cotton
seed. In certain embodiments, the matricconditioned seed is a sorghum seed.

Pregerminated seeds may be used in certain embodiments however not all
10 species can be primed using this method. In contrast to normal priming, seeds are
allowed to perform radicle protrusion. This is followed by sorting for specific stages, a
treatment that reinduces desiccation tolerance, and drying. The use of pregerminated
seeds causes rapid and uniform seedling development.

Thus, according to one embodiment, the seeds are primed seeds.

15 Of note, it may be possible that the seeds are treated with water (double-distilled
water, ddW), prior to contacting with the dsRNA without effecting any priming on the
seeds. For instance, treatment for a short while with water (*e.g.*, 30 seconds to 1 hours,
30 seconds to 0.5 hour, 30 seconds to 10 min, 30 seconds to 5 min or 45 seconds to 5
min).

20 Thus, according to one embodiment, the seeds are non-primed seeds.

A non-limiting example of a method of introducing the dsRNA into the seed is
provided in Example 1, which is considered as an integral part of the specification.

The temperature at the washing/priming and drying steps may be the same or
differ.

25 According to one embodiment, the temperature for washing/priming is between
4 and 28 °C. In some embodiments, the washing/priming temperature is less than 28 °C.
In some embodiments, the washing/priming temperature is less than 25 °C. In some
embodiments, the washing/priming temperature is less than 20 °C. In some
embodiments, the washing/priming temperature is less than 15 °C. In some
30 embodiments, the washing/priming temperature is less than 10 °C. In some
embodiments, the washing/priming temperature is between 4 and 10 °C. In an
embodiment the washing/priming temperature is between 10 and 15 °C. In an another

embodiment the washing/priming temperature is between 15 and 20 °C or 15 and 25 °C. In another embodiment the washing/priming temperature is between 10 and 20 °C or 10 and 25 °C.

5 According to one embodiment, the priming/washing solution or the dsRNA containing solution is devoid of a solid carrier.

According to one embodiment, the priming/washing solution or the dsRNA containing solution is devoid of a transferring agent such as a surfactant or a salt.

10 According to a further embodiment of the disclosure, the seeds subject to contacting with the dsRNA molecule are washed in order to remove agents, to which the seeds have been subjected, such as a pesticide, a fungicide, an insecticide, a fertilizer, a coating agent and a coloring agent.

Thus, according to one embodiment, the seeds (prior to treatment with dsRNA) are substantially free (*i.e.*, do not comprise effective amounts) of pesticide, a fungicide, an insecticide, a fertilizer, a coating agent and a coloring agent.

15 The seeds are then subjected to drying.

According to one embodiment, the drying temperature is between 20 to 37 °C. In another embodiment, the drying temperature is between 20 to 30 °C. In another embodiment, the drying temperature is between 22 and 37 °C. In another embodiment, the drying temperature is between 15 to 22 °C or 20 to 25 °C. In embodiments according to the present disclosure, the drying time at a temperature of the present disclosure may be for 10 to 20 hours. In other embodiments the drying time may be from 10 to 16 hours. In other embodiments, the drying time may be 2 to 5 hours.

20 Various considerations are to be taken when calculating the concentration of the dsRNA in the contacting solution. Considerations include, but are not limited to, at least one of the group consisting of seed size, seed weight, seed volume, seed surface area, seed density and seed permeability.

For example, related to seed size, weight, volume and surface area, it is estimated that corn seeds will require longer treatment than *Arabidopsis* and tomato seeds. Regarding permeability and density, it is estimated that wheat seeds will require longer treatments at higher concentrations than tomato seeds.

30 Examples of concentrations of dsRNA in the treating solution include, but are not limited to 0.1 to 100 micrograms (1×10^{-6} grams) per microliter (1×10^{-6} liter)

($\mu\text{g}/\mu\text{l}$). In an embodiment the dsRNA concentration in the treating solution may be 0.04 to 0.15 $\mu\text{g}/\mu\text{l}$. In an another embodiment the dsRNA concentration in the treating solution may be 0.1 to 50 $\mu\text{g}/\mu\text{l}$. In certain embodiments, the dsRNA concentration in the treating solution may be 0.1 to 10 $\mu\text{g}/\mu\text{l}$. In yet other embodiments the dsRNA concentration in the treating solution may be 0.1 to 5 $\mu\text{g}/\mu\text{l}$. In some embodiments the dsRNA concentration in the treating solution may be 0.1 to 1 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.1 to 0.5 $\mu\text{g}/\mu\text{l}$. Also included and provided for in the present disclosure are embodiments having a dsRNA concentration in the treating solution of between 0.15 and 0.5 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.1 to 0.3 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.01 to 0.1 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.01 to 0.05 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.02 to 0.04 $\mu\text{g}/\mu\text{l}$. In an embodiment the dsRNA concentration in the treating solution may be 0.001 to 0.02 $\mu\text{g}/\mu\text{l}$. According to a specific embodiment, the concentration of the dsRNA in the treating solution is 0.04 to 0.15 $\mu\text{g}/\mu\text{l}$.

According to a specific embodiment, the contacting with the dsRNA is effected in the presence of a chelating agent such as EDTA or another chelating agent such as DTPA (0.01 to 0.1 mM).

The contacting solution may comprise a transferring agent such as a surfactant or a salt.

Examples of such transferring agents include but are not limited salts such as sodium or lithium salts of fatty acids (such as tallow or tallowamines or phospholipids lipofectamine or lipofectin (1 to 20 nM, or 0.1 to 1 nM)) and organosilicone surfactants. Other useful surfactants include organosilicone surfactants including nonionic organosilicone surfactants, *e.g.*, trisiloxane ethoxylate surfactants or a silicone polyether copolymer such as a copolymer of polyalkylene oxide modified heptamethyl trisiloxane and allyloxypolypropylene glycol methylether (commercially available as Silwet™ L-77 surfactant having CAS Number 27306-78-1 and EPA Number: CAL.REG.NO. 5905-50073-AA, currently available from Momentive Performance Materials, Albany, N.Y.).

Useful physical agents can include (a) abrasives such as carborundum,

corundum, sand, calcite, pumice, garnet, and the like, (b) nanoparticles such as carbon nanotubes or (c) a physical force. Carbon nanotubes are disclosed by Kam *et al.* (2004) J. Am. Chem. Soc., 126 (22):6850-6851, Liu *et al.* (2009) Nano Lett., 9(3):1007-1010, and Khodakovskaya *et al.* (2009) ACS Nano, 3(10):3221-3227. Physical force agents
5 can include heating, chilling, the application of positive pressure, or ultrasound treatment. Agents for laboratory conditioning of a plant to permeation by polynucleotides include, *e.g.*, application of a chemical agent, enzymatic treatment, heating or chilling, treatment with positive or negative pressure, or ultrasound treatment. Agents for conditioning plants in a field include chemical agents such as surfactants and
10 salts.

Contacting of the seeds with the dsRNA molecule can be effected using any method known in the art as long as a suppressive amount of the dsRNA molecule enters the seeds. These examples include, but are not limited to, soaking, spraying or coating with powder, emulsion, suspension, or solution; similarly, the polynucleotide molecules
15 are applied to the plant by any convenient method, *e.g.*, spraying or wiping a solution, emulsion, or suspension.

According to a specific embodiment contacting may be effected by soaking (*i.e.*, inoculation) so that shaking the seeds with the treating solution may improve penetration and soaking and therefore reduce treatment time. Shaking is typically
20 performed at 50 to 150 RPM and depends on the volume of the treating solution. Shaking may be effected for 4 to 24 hours (1 to 4 hours, 10 minutes to 1 hour or 30 seconds to 10 minutes). The incubation takes place in the dark at 4 to 28 °C or 15 to 22 °C (*e.g.*, 8 to 15 °C, 4 to 8 °C, 22 to 28 °C).

According to a specific embodiment, contacting occurs prior to breaking of seed
25 dormancy and embryo emergence.

Following contacting, preferably prior to breaking of seed dormancy and embryo emergence, the seeds may be subjected to treatments (*e.g.*, coating) with the above agents (*e.g.*, pesticide, fungicide etc.).

Contacting is effected such that the dsRNA molecule enters the embryo,
30 endosperm, the coat, or a combination of the three.

After contacting with the treatment solution, the seeds may be subjected to drying for up to 30 hours at 25 to 37 °C.

According to a specific embodiment, the seed (*e.g.*, isolated seed) comprises the exogenous dsRNA and wherein at least 10 or 20 molecules of the dsRNA are in the endosperm of the isolated seed.

5 As used herein the term “isolated” refers to separation from the natural physiological environment. In the case of seed, the isolated seed is separated from other parts of the plant. In the case of a nucleic acid molecule (*e.g.*, dsRNA) separated from the cytoplasm.

According to a specific embodiment, the dsRNA molecule is not expressed from the plant genome, thereby not being an integral part of the genome.

10 Methods of qualifying successful introduction of the dsRNA molecule include but are not limited to, RT-PCR (*e.g.*, quantifying the level of the target gene or the dsRNA), phenotypic analysis such as biomass, vigor, yield and stress tolerance, root architecture, leaf dimensions, grain size and weight, oil content, cellulose, as well as cell biology techniques.

15 Seeds may be stored for 1 day to several months prior to planting (*e.g.*, at 4 to 10 °C).

The resultant seed can be germinated in the dark so as to produce a plant.

20 Thus there is provided a plant or plant part comprising an exogenous dsRNA molecule and devoid of a heterologous promoter for driving expression of the dsRNA molecule in the plant.

As used herein “devoid of a heterologous promoter for driving expression of the dsRNA” means that the plant or plant cell doesn't include a cis-acting regulatory sequence (*e.g.*, heterologous) transcribing the dsRNA in the plant.

25 As used herein, the term “heterologous” means not naturally occurring together. In some embodiments, the term “heterologous” refers to exogenous, not-naturally occurring within the native plant cell (such as by position of integration, or being non-naturally found within the plant cell). Thus the isolated seed in the absence of a heterologous promoter sequence for driving expression of the dsRNA in the plant, comprises a homogenic (prior to amplification) or heterogenic (secondary siRNAs, following amplification) population of plant non-transcribable dsRNA. In embodiments
30 according to the present disclosure, an antisense RNA sequence may be a heterologous sequence.

The present methodology can be used for modulating gene expression such as in a plant, the method comprising: (a) contacting a seed of the plant with a dsRNA, under conditions which allow penetration of the dsRNA into the seed, thereby introducing the dsRNA into the seed; and optionally (b) generating a plant of the seed.

5 When used for down-regulating a plant gene, the dsRNA is designed of the desired specificity using bioinformatic tools which are well known in the art (*e.g.*, BLAST).

10 This methodology can be used in various applications starting from basic research such as in order to assess gene function and lasting in generating plants with altered traits which have valuable commercial use.

Such plants can exhibit agricultural beneficial traits including altered morphology, altered flowering, altered tolerance to stress (*i.e.*, biotic and/or abiotic), altered biomass vigor and/or yield and the like.

15 The phrase “abiotic stress” as used herein refers to any adverse effect on metabolism, growth, viability and/or reproduction of a plant. Abiotic stress can be induced by any of suboptimal environmental growth conditions such as, for example, water deficit or drought, flooding, freezing, low or high temperature, strong winds, heavy metal toxicity, anaerobiosis, high or low nutrient levels (*e.g.* nutrient deficiency), high or low salt levels (*e.g.* salinity), atmospheric pollution, high or low light intensities
20 (*e.g.* insufficient light) or UV irradiation. Abiotic stress may be a short term effect (*e.g.* acute effect, *e.g.* lasting for about a week) or alternatively may be persistent (*e.g.* chronic effect, *e.g.* lasting for example 10 days or more). The present disclosure contemplates situations in which there is a single abiotic stress condition or alternatively situations in which two or more abiotic stresses occur.

25 According to one embodiment the abiotic stress refers to salinity.

 According to another embodiment the abiotic stress refers to drought.

 According to another embodiment the abiotic stress refers to a temperature stress.

30 As used herein the phrase “abiotic stress tolerance” refers to the ability of a plant to endure an abiotic stress without exhibiting substantial physiological or physical damage (*e.g.* alteration in metabolism, growth, viability and/or reproducibility of the plant).

As used herein the phrase “nitrogen use efficiency (NUE)” refers to a measure of crop production per unit of nitrogen fertilizer input. Fertilizer use efficiency (FUE) is a measure of NUE. Crop production can be measured by biomass, vigor or yield. The plant's nitrogen use efficiency is typically a result of an alteration in at least one of the uptake, spread, absorbance, accumulation, relocation (within the plant) and use of nitrogen absorbed by the plant. Improved NUE is with respect to that of a non-transgenic plant (*i.e.*, lacking the transgene of the transgenic plant) of the same species and of the same developmental stage and grown under the same conditions.

As used herein the phrase “nitrogen-limiting conditions” refers to growth conditions which include a level (*e.g.*, concentration) of nitrogen (*e.g.*, ammonium or nitrate) applied which is below the level needed for optimal plant metabolism, growth, reproduction and/or viability.

As used herein the term/phrase “biomass”, “biomass of a plant” or “plant biomass” refers to the amount (*e.g.*, measured in grams of air-dry tissue) of a tissue produced from the plant in a growing season. An increase in plant biomass can be in the whole plant or in parts thereof such as aboveground (*e.g.* harvestable) parts, vegetative biomass, roots and/or seeds or contents thereof (*e.g.*, oil, starch etc.).

As used herein the term/phrase “vigor”, “vigor of a plant” or “plant vigor” refers to the amount (*e.g.*, measured by weight) of tissue produced by the plant in a given time. Increased vigor could determine or affect the plant yield or the yield per growing time or growing area. In addition, early vigor (*e.g.* seed and/or seedling) results in improved field stand.

As used herein the term/phrase “yield”, “yield of a plant” or “plant yield” refers to the amount (*e.g.*, as determined by weight or size) or quantity (*e.g.*, numbers) of tissues or organs produced per plant or per growing season. Increased yield of a plant can affect the economic benefit one can obtain from the plant in a certain growing area and/or growing time.

According to one embodiment the yield is measured by cellulose content, oil content, starch content and the like.

According to another embodiment the yield is measured by oil content.

According to another embodiment the yield is measured by protein content.

According to another embodiment, the yield is measured by seed number, seed

weight, fruit number or fruit weight per plant or part thereof (*e.g.*, kernel, bean).

A plant yield can be affected by various parameters including, but not limited to, plant biomass; plant vigor; plant growth rate; seed yield; seed or grain quantity; seed or grain quality; oil yield; content of oil, starch and/or protein in harvested organs (*e.g.*,
5 seeds or vegetative parts of the plant); number of flowers (*e.g.* florets) per panicle (*e.g.* expressed as a ratio of number of filled seeds over number of primary panicles); harvest index; number of plants grown per area; number and size of harvested organs per plant and per area; number of plants per growing area (*e.g.* density); number of harvested organs in field; total leaf area; carbon assimilation and carbon partitioning (*e.g.* the
10 distribution/allocation of carbon within the plant); resistance to shade; number of harvestable organs (*e.g.* seeds), seeds per pod, weight per seed; and modified architecture [such as increase stalk diameter, thickness or improvement of physical properties (*e.g.* elasticity)].

Improved plant NUE is translated in the field into either harvesting similar
15 quantities of yield, while implementing less fertilizers, or increased yields gained by implementing the same levels of fertilizers. Thus, improved NUE or FUE has a direct effect on plant yield in the field.

As used herein “biotic stress” refers stress that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, beneficial
20 and harmful insects, weeds, and cultivated or native plants. Example 7 of the Examples section which follows, implements the present teachings towards conferring resistance to *Spodoptera littoralis*.

As used herein the term “improving” or “increasing” refers to at least about 2 %, at least about 3 %, at least about 4 %, at least about 5 %, at least about 10 %, at least
25 about 15 %, at least about 20 %, at least about 25 %, at least about 30 %, at least about 35 %, at least about 40 %, at least about 45 %, at least about 50 %, at least about 60 %, at least about 70 %, at least about 80 %, at least about 90 % or greater increase in NUE, in tolerance to stress, in yield, in biomass or in vigor of a plant, as compared to a native or wild-type plants (*i.e.*, isogenic plants (not modified to comprise the dsRNA)) of the
30 disclosure.

As mentioned the target gene of the dsRNA may not be an endogenous plant gene but rather a gene exogenous to the plant such as of a phytopathogenic organism

which feeds on the plant or depends thereon for growth/replication (*e.g.*, bacteria or viruses) and survival.

As used herein. The term “phytopathogen” refers to an organism that benefits from an interaction with a plant, and has a negative effect on that plant.

5 Thus, according to an embodiment of the disclosure there is provided a method of inhibiting expression of a target gene in a phytopathogenic organism, the method comprising providing (*e.g.*, feeding or contacting under infecting conditions) to the phytopathogenic organism the plant as described herein (at least part thereof includes the dsRNA), thereby inhibiting expression of a target gene in the phytopathogenic
10 organism.

The phytopathogenic organism refers to a multicellular organism *e.g.*, insects, fungi, animals or a microorganism that can cause plant disease, including viruses, bacteria, fungi as well as oomycetes, chytrids, algae, and nematodes.

Reference herein to a “nematode” refers to a member of the phylum *Nematoda*.
15 Members of the family *Heteroderidae* are sedentary parasites that form elaborate permanent associations with the target host organism. They deprive nutrients from cells of an infected organism through a specialized stylet. The cyst nematodes (genera *Heterodera* and *Globodera*) and root-knot nematodes (genus *Meloidogyne*), in particular, cause significant economic loss in plants, especially crop plants. Examples of
20 cyst nematodes include, *inter alia*, *H. avenae* (cereal cyst nematodes), *H. glycines* (beet cyst nematode) and *G. pallida* (potato cyst nematode). Root-knot nematodes include, for example, *M. javanica*, *M. incognita* and *M. arenaria*. These pathogens establish “feeding sites” in the plant, by causing the morphological transformation of root cells into giant cells. Hence, nematode “infestation” or “infection” refers to invasion of and
25 feeding upon the tissues of the host plant. Other nematodes that cause significant damage include the lesion nematodes such as *Pratylenchus*, particularly *P. penetrans*, which infects maize, rice and vegetables, *P. brachyurus* which infects pineapple and *P. thornei* which infects *inter alia*, wheat.

Insects that may cause damage and disease in plants belong to three categories,
30 according to their method of feeding: chewing, sucking and boring. Major damage is caused by chewing insects that eat plant tissue, such as leaves, flowers, buds and twigs. Examples from this large insect category include beetles and their larvae (grubs), web-

worms, bagworms and larvae of moths and sawflies (caterpillars). By comparison, sucking insects insert their mouth parts into the tissues of leaves, twigs, branches, flowers or fruit and suck out the plant's juices. Typical examples of sucking insects include but are not limited to aphids, mealy bugs, thrips and leaf-hoppers. Damage caused by these pests is often indicated by discoloration, drooping, wilting and general lack of vigor in the affected plant.

According to a specific embodiment, the phytopathogen is *prodentia* of the family *Noctuidae* e.g., *Spodoptera littoralis*.

Examples of significant bacterial plant pathogens include, but are not limited to, Burkholderia, *Proteobacteria* (*Xanthomonas spp.* and *Pseudomonas spp.*, *Pseudomonas syringae pv. tomato*).

A number of virus genera are transmitted, both persistently and non-persistently, by soil borne zoosporic protozoa. These protozoa are not phytopathogenic themselves, but parasitic. Transmission of the virus takes place when they become associated with the plant roots. Examples include *Polymyxa graminis*, which has been shown to transmit plant viral diseases in cereal crops and *Polymyxa betae* which transmits Beet necrotic yellow vein virus. *Plasmodiophorids* also create wounds in the plant's root through which other viruses can enter.

Specific examples of viruses which can be targeted according to the present teachings includes, but are not limited to:

(1) Tobacco mosaic virus (TMV, RNA virus) which infects plants, especially tobacco and other members of the family *Solanaceae*.

(2) Tomato spotted wilt virus (TSWV, RNA virus) which causes serious diseases of many economically important plants representing 35 plant families, including dicots and monocots. This wide host range of ornamentals, vegetables, and field crops is unique among plant-infecting viruses. Belongs to *tospoviruses* in the Mediterranean area, affect vegetable crops, especially tomato, pepper and lettuce (Turina *et al.*, 2012, Adv Virus Res 84:403-437).

(3) Tomato yellow leaf curl virus (TYLCV) which is transmitted by whitefly, mostly affects tomato plants. *Geminiviruses* (DNA viruses) in the genus *Begomovirus* (including *sweepoviruses* and *legumoviruses*) - most devastating pathogens affecting a variety of cultivated crops, including cassava, sweet potato, beans, tomato, cotton and

grain legumes (Rey *et al.* 2012, Viruses 4;1753-1791). Members include TYLCV above and tomato leaf curl virus (ToLCV).

(4) Cucumber mosaic virus (CMV) - CMV has a wide range of hosts and attacks a great variety of vegetables, ornamentals, and other plants (as many as 191 host species in 40 families). Among the most important vegetables affected by cucumber mosaic are peppers (*Capsicum annuum L.*), cucurbits, tomatoes (*Lycopersicon esculentum Mill.*), and bananas (*Musa L. spp.*).

Other vegetable hosts include: cucumber, muskmelon, squash, tomato, spinach, celery, peppers, water cress, beet, sweet potato, turnip, chayote, gherkin, watermelon, pumpkin, citron, gourd, lima bean, broad bean, onion, ground-cherry, eggplant, potato, rhubarb, carrot, dill, fennel, parsnip, parsley, luffa, and artichoke (Chabbouh and Cherif, 1990, FAO Plant Prot. Bull. 38:52-53.).

Ornamental hosts include: China aster, chrysanthemum, delphinium, salvia, geranium, gilia, gladiolus, heliotrope, hyacinth, larkspur, lily, marigold, morning glory, nasturtium, periwinkle, petunia, phlox, snapdragon, tulip, and zinnia (Chupp and Sherf, 1960; Agrios, 1978).

(5) Potato virus Y (PVY) - one of the most important plant viruses affecting potato production.

(6) Cauliflower mosaic virus (CaMV, DNA virus (Rothnie *et al.*, 1994)).

(7) African cassava mosaic virus (ACMV).

(8) Plum pox virus (PPV) is the most devastating viral disease of stone fruit from the genus *Prunus*.

(9) Brome mosaic virus (BMV) - commonly infects *Bromus inermis* and other grasses, can be found almost anywhere wheat is grown.

(10) Potato virus X (PVX) There are no insect or fungal vectors for this virus. This virus causes mild or no symptoms in most potato varieties, but when Potato virus Y is present, synergy between these two viruses causes severe symptoms in potato.

Additional viruses:

Citrus tristeza virus (CTV) - causes the most economically damaging disease to Citrus, including sour orange (*Citrus aurantium*), and any *Citrus* species grafted onto sour orange root stock, sweet orange (*C. sinensis*), grapefruit (*C. paradisi*), lime and Seville orange (*C. aurantifolia*), and mandarin (*C. reticulata*). CTV is also known to

infect *Aeglopsis chevalieri*, *Afraegle paniculata*, *Pamburus missionis*, and *Passiflora gracilis*. CTV is distributed worldwide and can be found wherever citrus trees grow.

Barley yellow dwarf virus (BYDV)- most widely distributed viral disease of cereals. It affects the economically important crop species barley, oats, wheat, maize, triticale and rice.

Potato leafroll virus (PLRV) infects potatoes and other members of the family *Solanaceae*.

Tomato bushy stunt virus (TBSV), RNA virus, a member of the genus *Tombusvirus* and mostly affects tomatoes and eggplant.

Additional reviews:

Hamilton *et al.*, 1981, J Gen Virol 54;223-241 – mentions TMV, PVX, PVY, CMV, CaMV

Additional scientific papers:

Makkouk *et al.*, 2012, Adv Virus Res 84;367-402 -Viruses affecting peas and beans with narrow (Faba bean necrotic yellow virus (FBNYN)) and wide (alfalfa mosaic virus (AMV) and CMV) host range.

Insect pests causing plant disease include those from the families of, for example, *Apidae*, *Curculionidae*, *Scarabaeidae*, *Tephritidae*, *Tortricidae*, amongst others.

The target gene of the phytopathogenic organism encodes a product essential to the viability and/or infectivity of the pathogen, therefore its down-regulation (by the dsRNA) results in a reduced capability of the pathogen to survive and infect host cells. Hence, such down-regulation results in a “deleterious effect” on the maintenance viability and/or infectivity of the phytopathogen, in that it prevents or reduces the pathogen's ability to feed off and survive on nutrients derived from host cells. By virtue of this reduction in the phytopathogen's viability and/or infectivity, resistance and/or enhanced tolerance to infection by a pathogen is facilitated in the cells of the plant. Genes in the pathogen may be targeted at the mature (adult), immature (juvenile) or embryo stages.

Examples of genes essential to the viability and/or infectivity of the pathogen are provided herein. Such genes may include genes involved in development and reproduction, *e.g.* transcription factors (see, *e.g.* Xue *et al.*, 1993; Finney *et al.*, 1988),

cell cycle regulators such as *wee-1* and *ncc-1* proteins (see, *e.g.* Wilson *et al.*, 1999; Boxem *et al.*, 1999) and embryo-lethal mutants (see, *e.g.* Schnabel *et al.*, 1991); proteins required for modeling such as collagen, ChR3 and LRP-1 (see, *e.g.* Yochem *et al.*, 1999; Kostrouchova *et al.*, 1998; Ray *et al.*, 1989); genes encoding proteins involved in the motility/nervous system, *e.g.* acetylcholinesterase (see, *e.g.* Piotee *et al.*, 1999; Talesa *et al.*, 1995; Arpagaus *et al.*, 1998), ryanodine receptor such as *unc-68* (see, *e.g.* Maryon *et al.*, 1998; Maryon *et al.*, 1996) and glutamate-gated chloride channels or the avermeetin receptor (see, *e.g.*, Cully *et al.*, 1994; Vassilatis *et al.*, 1997; Dent *et al.*, 1997); hydrolytic enzymes required for deriving nutrition from the host, *e.g.* serine proteinases such as HGSP-1 and HGSP-III (see, *e.g.* Lilley *et al.*, 1997); parasitic genes encoding proteins required for invasion and establishment of the feeding site, *e.g.* cellulases (see, *e.g.* de Boer *et al.*, 1999; Rosso *et al.*, 1999) and genes encoding proteins that direct production of stylar or amphidial secretions such as *sec-1* protein (see, *e.g.* Ray *et al.*, 1994; Ding *et al.*, 1998); genes encoding proteins required for sex or female determination, *e.g.* *tra-1*, *tra-2* and *egl-1*, a suppressor of *ced9* (see, *e.g.* Hodgkin, 1980; Hodgkin, 1977; Hodgkin, 1999; Gumienny *et al.*, 1999; Zarkower *et al.*, 1992); and genes encoding proteins required for maintenance of normal metabolic function and homeostasis, *e.g.* sterol metabolism, embryo lethal mutants (see, *e.g.* Schnabel *et al.*, 1991) and trans-spliced leader sequences (see, *e.g.* Ferguson *et al.*, 1996), *pos-1*, cytoplasmic Zn finger protein; *pie-1*, cytoplasmic Zn finger protein; *mei-1*, ATPase; *dif-1*, mitochondrial energy transfer protein; *rba-2*, chromatin assembly factor; *skn-1*, transcription factor; *plk-1*, kinase; *gpb-1*, G-protein B subunit; *par-1*, kinase; *bir-1*, inhibitor of apoptosis; *mex-3*, RNA-binding protein, *unc-37*, G-protein B subunit; *hlh-2*, transcription factor; *par-2*, *dnc-1*, dynactin; *par-6*, *dhc-1*, dynein heavy chain; and *pal-1*, homeobox. Such genes have been cloned from parasitic nematodes such as *Melioidogyne* and *Heterodera* species or can be identified by one of skill in the art using sequence information from cloned *C. elegans* orthologs (the genome of *C. elegans* has been sequenced and is available, see The *C. elegans* Sequencing Consortium (1998)).

As used herein, a “pathogen resistance” trait is a characteristic of a plant that causes the plant host to be resistant to attack from a pathogen that typically is capable of inflicting damage or loss to the plant. Once the phytopathogen is provided with the

plant material comprising the dsRNA, expression of the gene within the target pathogen is suppressed by the dsRNA, and the suppression of expression of the gene in the target pathogen results in the plant being resistant to the pathogen.

5 In this case, the target gene can encode an essential protein or transcribe a non-coding RNA which, the predicted function is for example selected from the group consisting of ion regulation and transport, enzyme synthesis, maintenance of cell membrane potential, amino acid biosynthesis, amino acid degradation, development and differentiation, infection, penetration, development of appressoria or haustoria, mycelial growth, melanin synthesis, toxin synthesis, siderophore synthesis, sporulation, fruiting
10 body synthesis, cell division, energy metabolism, respiration, and apoptosis, among others.

According to a specific embodiment, the phytopathogenic organism is selected from the group consisting of a fungus, a nematode, a virus, a bacteria and an insect.

To substantiate the anti-pest activity, the present teachings also contemplate
15 observing death or growth inhibition and the degree of host symptomatology following said providing.

To improve the anti-phytopathogen activity, embodiments of the present disclosure further provide a composition that contains two or more different agents each toxic to the same plant pathogenic microorganism, at least one of which comprises a
20 dsRNA described herein. In certain embodiments, the second agent can be an agent selected from the group consisting of inhibitors of metabolic enzymes involved in amino acid or carbohydrate synthesis; inhibitors of cell division; cell wall synthesis inhibitors; inhibitors of DNA or RNA synthesis, gyrase inhibitors, tubulin assembly inhibitors, inhibitors of ATP synthesis; oxidative phosphorylation uncouplers; inhibitors
25 of protein synthesis; MAP kinase inhibitors; lipid synthesis or oxidation inhibitors; sterol synthesis inhibitors; and melanin synthesis inhibitors.

In addition, plants generated according to the teachings of the present disclosure or parts thereof can exhibit altered nutritional or therapeutic efficacy and as such can be employed in the food or feed and drug industries. Likewise, the plants generated
30 according to the teachings of the present disclosure or parts thereof can exhibit altered oil or cellulose content and as such can be implemented in the construction or oil industry.

The seeds of the present disclosure can be packed in a seed containing device which comprises a plurality of seeds at least some of which (*e.g.*, 5 %, 10 % or more) containing an exogenous dsRNA, wherein the seed is devoid of a heterologous promoter for driving expression of the dsRNA.

5 The seed containing device can be a bag, a plastic bag, a paper bag, a soft shell container or a hard shell container.

Reagents of the present disclosure can be packed in a kit including the dsRNA, instructions for introducing the dsRNA into the seeds and optionally a priming solution. According to one embodiment, the dsRNA and priming solution are comprised in
10 separate containers.

Compositions of some embodiments of the disclosure may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied
15 by instructions for introduction to the seed.

As used herein the term “about” refers to $\pm 10\%$.

The terms “comprises”, “comprising”, “includes”, “including”, “having” and their conjugates mean “including but not limited to”.

The term “consisting of” means “including and limited to”.

20 The term “consisting essentially of” means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form “a”, “an” and “the” include plural references
25 unless the context clearly dictates otherwise. For example, the term “a compound” or “at least one compound” may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this disclosure may be presented in a range format. It should be understood that the description in range format
30 is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as

individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies
5 regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein
10 interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term “method” refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known
15 manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, may also be provided in combination
20 in a single embodiment. Conversely, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the disclosure. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

25 Various embodiments and embodiments of the present disclosure as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

30 Reference is now made to the following examples, which together with the above descriptions, illustrate some embodiments of the disclosure in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present disclosure include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein

by reference.

EXAMPLE 1: PROTOCOLS FOR dsRNA PRODUCTION AND SEED TREATMENT

Generating dsRNA/siRNA Sequences

5 The dsRNA sequences were custom-created for each gene using *in vitro* transcription of PCR products. Part of the mRNA, including either the ORF, 3' UTR or 5' UTR for which dsRNA to be produced was PCR-amplified using gene-specific primers, which contain the sequence of the T7 promoter on either side. This product was used as a template for dsRNA production using commercial kits such as the MaxiScript dsRNA kit (Life Technologies) or T7 High Yield RNA Synthesis kit (NEB). Next, the sample is treated with DNase Turbo at 37 °C for 15-30 min followed by phenol treatment and nucleic acid precipitation. Next, one of two different reactions is carried out: (1) dsRNA is ready to use, (2) processing of the dsRNA with Dicer (Shortcut RNase III (NEB)) to create small interfering RNAs (siRNA).

10 Either dsRNA or a combination of dsRNA and siRNA were used for seed treatments as described below.

General Seed Treatment Protocol for Gene Silencing using a dsRNA/siRNA Mixture

15 Uncoated organic corn seeds were from variety "popcorn", uncoated organic whole grain rice seeds, organic soybean and wheat seeds were purchased from Nitsat Haduvdevan (Israel), Fresh tomato seeds were retrieved from M82 tomato fruits, which are propagated in-house. Uncoated or fresh plant seeds were washed with double distilled water (DDW) prior to treatment for four hours. Next, seeds were dried at 30 °C for 10-16 hours. Following the drying step, seeds were treated with a solution containing the dsRNA formulation, which is made of dsRNA at a final concentration of 40-150 µg/ml in 0.1mM EDTA. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C. Finally, seeds were washed twice briefly and planted on soil or dried for 0-30 hours and germinated at 25 °C in a dark growth chamber and planted in soil or planted directly in soil. Control seeds were treated in a similar way, with a formulation that lacked the dsRNA or with non-specific dsRNA.

EXAMPLE 2: STABILITY OF THE DSRNA IN SEEDLINGS OF RICE, TOMATO AND SORGHUM

As an example for an exogenous gene that is not present/expressed in plants, the ORFs encoding the replicase and coat protein of CGMMV (accession number AF417242) were used to as targets for dsRNA treatment of plant seeds using the protocol described in Example 1. Rice, tomato and sorghum seeds were washed for 4 hours at 20 °C, tomato and sorghum were dried at 30 °C and rice at 20 °C for overnight. Seeds were immediately treated at 15 °C with 132.7 µg/ml dsRNA (final concentration) for 39 hours for rice, 93.8 µg/ml dsRNA (final concentration) for 48 hours for tomato, and 75 µg/ml dsRNA (final concentration) for 40 hours for sorghum.

Briefly, the virus-derived ORFs were amplified by PCR with specifically designed forward and reverse primers that contain the T7 sequence (5'-TAATACGACTCACTATAGGG-3', SEQ ID NO: 1) at their 5' (see Table 2, below). PCR products were purified from agarose gel and since they carry T7 promoters at both ends they were used as templates for T7-dependent in-vitro transcription, resulting in dsRNA product of the CGMMV genes. PCR on a housekeeping gene, tubulin, was used as a positive control (forward primer 5'-GGTGCTCTGAACGTGGATG-3' (SEQ ID NO: 2), and reverse primer 5'-CATCATCGCCATCCTCATTCTC-3' (SEQ ID NO: 3)).

Table 2: PCR primers served as Templates for *in vitro* Transcription and detection of CGMMV, and CGMMV dsRNA products .

Virus Name	Product Name	Product Sequence/SEQ ID NO:	Forward primer/SEQ ID NO:	Reverse primer/SEQ ID NO:
1) CGMMV (NCBI Accession number AF417242)	CGMVV dsRNA product 1	TAATACGACTCACTATAGGGGGTAAGCG GCATTCTAAACCTCCAAATCGGAGGTTGG ACTCTGCTTCTGAAGAGTCCAGTTCTGTT TCTTTTGAAGATGGCTTACAATCCGATCA CACCTAGCAAACCTATTGCGTTTAGTGCT TCTTATGTTCCCGTCAGGACTTACTTAAT TTTCTAGTTGCTTCACAAGGTACCGCTTTC CAGACTCAAGCGGAAGAGATTCTTTCCG	TAATACGACT CACTATAGGG GGTAAGCGGC ATTCTAAACC/ (SEQ ID NO:5)	Set 1: TAATACGA CTCACTATA GGGGAAGA CCCTCGAA ACTAAGC/(S EQ ID NO:4)
		CGAGTCCCTGTCTGCGTTACCCCTCGTCTG TCGTAGATATTAATTCTAGATTCCCAGAT		CTTCTTATGT TCCCGTCAGG /(SEQ ID NO:7)

		<p>GCGGGTTTTTACGCTTTCCTCAACGGTCC TGTGTTGAGGCCTATCTTCGTTTCGCTTCT CAGCTCCACGGATACGCGTAATAGGGTC ATTGAGGTTGTAGATCCTAGCAATCCTAC GACTGCTGAGTCGCTTAACGCCGTAAGC GTACTGATGACGCGTCTACGGCCGCTAGG GCTGAGATAGATAATTTAATAGAGTCTAT TTCTAAGGGTTTTGATGTTTACGATAGGG CTTCATTTGAAGCCGCGTTTTTCGGTAGTC TGGTCAGAGGCTACCACCTCGAAAGCTTA GTTTCGAGGGTCTTCCCCTATAGTGAGTC GTATTA/(SEQ ID NO:8)</p>		<p>ATTG/(SEQ ID NO:6)</p>
	<p>CGMVV dsRNA product 2</p>	<p>TAATACGACTCACTATAGGGGCTTTACCG CCACTAAGAACTCTGTACACTCCCTTGCG GGTGGTCTGAGGCTTCTTGAATTGGAATA TATGATGATGCAAGTGCCCTACGGCTCAC CTTGTTATGACATCGGCGGTAACTATACG CAGCACTTGTCAAAGGTAGATCATATGT GCATTGCTGCAATCCGTGCCTAGATCTTA AAGATGTTGCGAGGAATGTGATGTACAA CGATATGATCACGCAACATGTACAGAGG CACAAGGGATCTGGCGGGTGCAGACCTC TTCCAACCTTCCAGATAGATGCATTCAGG AGGTACGATAGTTCTCCCCTGTGCGGTCAC CTGTTTCAGACGTTTTCCAAGAGTGTTCCCT ATGATTTTGGGAGTGGTAGGGATAATCAT GCAGTCTCGTTGCATTCAATCTACGATAT CCCTTATTCTTCGATCGGACCTGCTCTTCA TAGGAAAAATGTGCGAGTTTGTATGCAG CCTTTCATTTCTCGGAGGCATTGCTTTTAG GTTTCGCTGTAGGTAATTTAAATAGTATT GGGGCTCAGTTTAGGGTCGATGGTGATGC CCTATAGTGAGTCGTATTA/(SEQ ID NO:11)</p>	<p>TAATACGACT CACTATAGGG GCTTTACCGC CACTAAGAAC /(SEQ ID NO:10)</p>	<p>Set 3: TAATACGA CTCACTATA GGGCATCA CCATCGAC CCTAAAC /(SEQ ID NO:9)</p>

dsRNA homologous to green mottle mosaic virus was observed to be stable in rice seedlings. Rice seeds were treated at 15 °C with 132.7 µg/ml dsRNA (final concentration) for 39 hours and dsRNA was detected by real time polymerase chain

reaction (RT-PCR) 1 week post germination. Detection of tubulin cDNA serves as a positive control for the cDNA quality. At two weeks post germination, dsRNA was detectable in 10 out of 10 seedlings. At 3 weeks post germination, dsRNA homologous to green mottle mosaic virus was detected in 5 out of 5 samples of rice seedlings.

5 Tomato seeds were treated at 15 °C with 93.8 µg/ml dsRNA (final concentration) for 48 hours and sorghum seeds treated at 5 µg/ml dsRNA (final concentration) for 40 hours. CGMMV dsRNA was detected by RT-PCR in 5 out of 13 tomato seedlings tested at 10 day post-germination and 3 out of four sorghum seedlings 4 weeks after germination.

10 The exogenous dsRNA was found to be stable for at least three weeks in rice seedlings and at least 10 days in tomato seedlings and four weeks in Sorghum plants.

EXAMPLE 3: THE dsRNA IS NOT INTEGRATED INTO THE GENOME OF RICE

15 Rice seeds were treated with an exogenous dsRNA as in Example 2. Plants were germinated and grown for five weeks, DNA was extracted and PCR reactions were performed to demonstrate that the dsRNA did not integrate into the Rice's genome. Two sets of primers that gave a positive reaction when checked on the RNA level were used, set 1 (see Table 2) of primers were the set of primers used to amplify the template (all the dsRNA sequence). Set 2 (see Table 3) are the primers that were used in the PCR
20 above. A Rice endogenous housekeeping gene (tubulin) was used as a positive control for the PCR reaction (see Table 3).

Three different DNA PCR reactions were carried out on dsRNA treated and untreated plants. No amplified DNA corresponding to CGMMV was detected in any treated or untreated plant.

25 Table 3: Tubulin Primers Used for PCR Amplification.

Primer Name and Direction	Primer Sequence/(SEQ ID NO:)	Primer Length
osa_TubA1_736F	GGTGCTCTGAACGTGGATG (SEQ ID NO: 12)	19
osa_TubA1_1342R	CATCATCGCCATCCTCATTCTC (SEQ ID NO: 13)	22

EXAMPLE 4: EXOGENOUS dsRNA MOLECULES ARE HIGHLY STABLE IN SOLUTION AND DO NOT GET INCORPORATED INTO THE GENOME OF TREATED PLANTS

Corn seeds were treated using the protocol described in Example 1, seeds were washed for 4 h at 20 °C, dried at 30 °C overnight and immediately treated with 40 µg/ml dsRNA (final concentration) directed against the β-glucuronidase (GUS) reporter gene for 60 hours at 15 °C, dried and were germinated. Leaves and roots were harvested from control and dsGUS-treated plants 7 and 15 days following germination. RNA was extracted from the harvested tissues and RT-PCR with specific GUS primers was run (Table 4). In addition, a corn endogenous housekeeping gene (ubiquitin) was used as a positive control for the PCR reaction. The GUS dsRNA molecules were found to be extremely stable in the treated seeds, and can be detected in corn plants 7 and 15 days post germination of the seeds.

GUS dsRNA was detected in corn seedlings by RT-PCR at 7 and 15 days after germination according to an aspect of the present disclosure. At one week, GUS dsRNA was detected in shoots of nine of eleven corn seedlings tested. GUS dsRNA was not detected in untreated plants. At 1 week post-germination, GUS dsRNA was detected in five of five corn seedlings' roots. At 15 days post germination, GUS dsRNA was detected in corn seedlings' roots. GUS dsRNA molecules do not get incorporated in the genome of treated corn plants one week after germination as determined by agarose gel electrophoresis of DNA PCR reactions using GUS primers on DNA isolated from treated corn plants.

Table 4: Primers for PCR Amplification of GUS and Ubiquitin Genes and GUS dsRNA product.

Primer Length	Primer Sequence/SEQ ID NO:	Primer Name
GUS_T7_For	TAATACGACTCACTATAGGGAGATCGACGGCCTGTGGGCATTC /(SEQ ID NO:15)	
GUS_T7_Rev	TAATACGACTCACTATAGGGAGCATTCCCGCGGGATAGTCTG /(SEQ ID NO:16)	43
GUS208For	CAGCGCGAAGTCTTTATACC/(SEQ ID NO:17)	43
GUS289Rev	CTTTGCCGTAATGAGTGACC/(SEQ ID NO:18)	20

zmaUBQ-947F	CCATAACCCTGGAGGTTGAG/(SEQ ID NO:19)	20
zmaUBQ1043R	ATCAGACGCTGCTGGTCTGG/(SEQ ID NO:20)	20
GUS dsRNA product	TAATACGACTCACTATAGGGAGATCGACGGCCTGTGGGCATTC AGTCTGGATCGCGAAAAGTGTGGAATTGATCAGCGTTGGTGG GAAAGCGCGTTACAAGAAAGCCGGGCTATTGCTGTGCCAGGC AGTTTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATG CGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCGAAAAG GTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTAC TCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGA GCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCC GTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTG AACAACGAACTGAACTGGCAGACTATCCCGCCGGGAATGCTC CCTATAGTGAGTCGTATTA/(SEQ ID NO:21)	

EXAMPLE 5 : FLUORESCENCE MICROSCOPY OF siRNA SEQUENCES IN VARIOUS PLANT SEEDS

Plant seeds as per the protocol described in example 1. Seeds were washed for 4 h at 20 °C, dried at 25 °C and were immediately treated with a fluorescent siRNA (siGLO, 2µM final concentration, Thermo Scientific) at 15 °C for 24 h. The quality of the siGLO before application to a plant seed was verified by gel electrophoresis analysis. Bands corresponding to the expected size of 20-24 bp of the fluorescent siRNA molecules was detected.

Fluorescent pictures of the seeds were taken 24-48 hours post treatment using an Olympus microscope at the lowest objective magnification (5X for bigger seeds such as rice and tomato seeds, and 10X for smaller seeds such as *Arabidopsis* seeds). To eliminate the possibility of non-specific auto-fluorescence, dsRNA-treated seeds were compared to control untreated seeds. Penetration of fluorescent siRNA molecules into plant seeds was observed at 24 hours after seed treatment with siRNA at 2 µM final concentration in rice seeds and tomato seeds.

Penetration of fluorescent siRNA molecules into rice seeds was observed at 24 hours following treatment with siGLO dsRNA.

In order to evaluate the distribution efficiency of the fluorescent siRNA inside the seeds, different plant seeds were cut into slices and imaged with a fluorescent microscope 48 hours after treatment. Each treated seed was imaged alongside a control untreated seed. Light and fluorescent images were taken where applicable for rice,

tomato, cucumber, bean, sorghum and wheat seed samples.

Penetration of fluorescent siRNA molecules into rice seeds was observed at 48 hours following treatment with siGLO dsRNA. siGLO-treated and control rice seeds were sliced to view the interior distribution of the fluorescent dsRNA using a fluorescent microscope and fluorescent siRNA molecules detected in the treated seed. Fluorescent siGLO RNA is detected throughout the seed.

Penetration of fluorescent siRNA molecules into tomato seeds was observed at 48 hours following treatment with siGLO dsRNA. siGLO-treated and control tomato seeds were sliced to view the interior distribution of the fluorescent dsRNA using a fluorescent microscope. Fluorescent siGLO RNA is detected in the endosperm and the embryo.

Penetration of fluorescent siRNA molecules into cucumber seeds was observed at 48 hours following treatment with siGLO dsRNA. siGLO-treated and control cucumber seeds were sliced to view the interior distribution of the fluorescent dsRNA using a fluorescent microscope. Penetration of fluorescent siRNA molecules into cucumber seeds was observed at Fluorescent siGLO RNA is detected in the endosperm and the embryo.

Penetration of fluorescent siRNA molecules is detected in sliced seeds of various plant species, including bean, tomato, sorghum and wheat, 48 hours following treatment with siGLO dsRNA. siGLO-treated and control seeds were sliced to view the interior distribution of the fluorescent dsRNA using a fluorescent microscope. Light images were also taken for each seed and are shown alongside the fluorescent image of the seed for reference.

Figure 1 presents fluorescent images of siGLO-treatment rice seeds over a 24 hour period. The effect of incubation time with siGLO dsRNA on fluorescence intensity, indicating quantity and quality of dsRNA penetration, was tested. Control seeds that were left untreated (1), were imaged along with seeds treated with siGLO dsRNA for four different incubation times; 10 min (2), 3.5 hours (3), 5.5 hours (4), and 24 hours (5).

It is clear that the siRNA is distributed at various levels between the embryo and the endosperm. Accordingly, dsRNA molecules enter the embryo directly. Though not to be limited by any particular theory, the dsRNA molecules are carried by the water-

based solution used for the seed treatment. The dsRNA molecules enter the endosperm as part of the endosperm's water-absorption process. These molecules then are transferred to the embryo as it develops as part of the endosperm to embryo nutrient flow during germination and seed development.

5 These present findings suggest the RNA molecules used to treat the seeds both penetrate the embryo and function in the embryo as it develops and also penetrate the endosperm and feed the embryo following germination.

EXAMPLE 6: TIME COURSE EXPERIMENT WITH siGLO TREATMENT

10 A time course experiment was performed on rice seeds to monitor the kinetics of siGLO penetration into the seeds following the seed treatment (Figure 1). The results indicate that the siRNA efficiently penetrates the plant seeds using the protocol described in Example 1.

EXAMPLE 7: EXAMPLE EMBODIMENTS OF dsRNA MOLECULES

15 Example 7A provides A backbone sequence with two smRNA complementary sites and a helicase binding site:
 5' GCATCCTCATCTTAATCTCGGTGCTATCCTACCTGAGCTTGATATCTAG
GCGAAGCAGCCCGAATGCTGCACCCTAGATGGCGAAAGTCCAGTAGCG
 ATATCGAATTCCTCGAGGGATCCAAGCTTCCTTGTCTATCCCTCCTGAGCTG
 TTGATTTTATTCCATGT 3' (SEQ ID NO: 14). This example contains a sequence for
 20 mutated microRNA 390 binding (bold), followed by a helicase binding site (bold and underlined) and a microRNA 390 binding sequence (underlined). DNA sequences for restriction enzyme recognition are added for cloning of the sequence to be silenced.

Example 7B is the same as example 7A, without the helicase binding site:

25 GCATCCTCATCTTAATCTCGGTGCTATCCTACCTGAGCTTGATATCG
 ATATCGAATTCCTCGAGGGATCCAAGCTTCCTTGTCTATCCCTCCTGAGCTG
 TTGATTTTATTCCATGT (SEQ ID NO: 22).

Example 7C provides a backbone sequence with two smRNA complementary sites and a helicase binding site:

30 GCATCCTCATCTTAATCTCGTGATTTTCTCTACAAGCGAAGATATC
TAGGCGAAGCAGCCCGAATGCTGCACCCTAGATGGCGAAAGTCCAGTA
GCGATATCGAATTCCTCGAGGGATCCAAGCTTCTTGCTCAAATGAGTATTC
CAGTTGATTTTATTCCATGT (SEQ ID NO: 23). Example 7C contains a sequence

for microRNA 173 binding (bold), followed by a helicase binding site (bold and underlined) and the reverse-complement sequence of microRNA 828 binding sequence (underlined). DNA sequences for restriction enzyme recognition are added for cloning of the sequence to be silenced. In this case a single complementary site is sufficient
5 (*i.e.*, miR173BS) yet a second complementary site is placed on the complementary strand (*i.e.*, miR828BS) so as to enhance amplification from both strands.

Example 7D is the same as Example 7C, without the helicase binding site:

GCATCCTCATCTTAATCTCGTGATTTTTCTCTACAAGCGAAGATATC
GATATCGAATTCCTCGAGGGATCCAAGCTTTCTTGCTCAAATGAGTATTCCA
10 GTTGATTTTATTCCATGT (SEQ ID NO: 24).

EXAMPLE 8 SCHEMATIC REPRESENTATION OF THE *SOLANUM LYCOPERSICUM* TAS3 GENE

Figure 2A presents a schematic representation of the *Solanum Lycopersicum* (*Lycopersicon esculentum*) TAS3 gene. This gene contains two Mir390 binding sites
15 (BS). The 5' Mir390BS has mutations in critical positions for Mir390 dependent cleavage and therefore it is bound by Mir390 but not cleaved (will be referred hereafter as 5' Mut Mir390BS). The 3' Mir390 does lead to Mir390 binding and cleavage. In between these two sequences there is a 234bp sequence that contains all the different ta-siRNAs that will be created following RDRP recruitment, RNA dependent RNA
20 polymerization and dicing (Allen et al. (2005). MicroRNA-Directed Phasing during Trans-Acting siRNA Biogenesis in Plants. *Cell*, 121, 207-221., Axtell et al. (2006). A Two-Hit Trigger for siRNA Biogenesis in Plants. *Cell*, 127,565-577., Montgomery et al. (2008). Specificity of ARGONAUTE7-miR390 Interaction and Dual Functionality in TAS3 Trans-Acting siRNA Formation. *Cell*, 133, 128-141).

EXAMPLE 9: ADDITIONAL dsRNA CONSTRUCTS ACCORDING TO THE PRESENT DISCLOSURE

Example 9A provides dsRNA Construct #1 that is an exogenous trigger control. Figure 2B presents a schematic representation of dsRNA Construct #1 that will serve as a control for the other experiments since it contains only the exogenous sequence with
30 no additional features that should lead to its amplification. The length of the exogenous sequence is 234bp, the same size of the original insert between the two Mir390BS in TAS3.

Example 9B provides dsRNA Construct #2 having a dual Mir390BS on sense strand and an exogenous sequence. Figure 2C presents schematic representation of dsRNA Construct #2 having a dual Mir390BS on sense strand and an exogenous sequence. Double-stranded RNA Construct #2 is based on the dual Mir390BS from the TAS3 gene with an exogenous sequence replacing the original insert between the two Mir390BS.

Example 9C provides dsRNA construct #3 having a Dual Mir390BS both on the sense and antisense strands. Figure 3 presents a schematic of dsRNA construct #3. This construct contains dual Mir390BS on both the sense and antisense strands and therefore we hypothesize that it will continuously recruit Mir390-Ago7 and RDRP to both strands. As a result, it is predicted to lead to long lasting amplification of the exogenous sequence and to ongoing production of its ta-siRNAs.

Example 9D provides dsRNA construct #4 having miR390BS as overhangs. Figure 4 presents dsRNA construct #4 composed of two different strands. Having the Mir390BS present as overhangs will ease the initial requirement for the unwinding of the dsRNA since the Mir390BS will already be accessible for mir390 and Ago7 binding. The Mir390BS sequences will facilitate the unwinding and as a result the translocation into the processing center and the initiation of the entire process as explained in example #3 above.

Example 9E provides dsRNA construct #5 having Dual miR390BS sequences and helicase binding sequences. Figure 5 presents dsRNA construct #5. The presence of the helicase binding sequences will enable more efficient unwinding of the dsRNA through active recruitment of a helicase and therefore leading to a strong and efficient amplification.

Example 9F provides dsRNA construct #6 having Mir390BS on both strands and a helicase binding sequence (helicase BS) overhang. Figure 6 presents dsRNA construct #6 having Mir390BS on both strands and a helicase overhang. This dsRNA construct #6 is composed of two different strands. The sense strand is the same as in construct #3 and the antisense strand is the same as construct #3 with an addition of an overhang of a helicase BS at the 3' end. The helicase BS leads to recruitment of a helicase that will unwind the dsRNA and enable efficient initiation of the entire process. Each of the strands will contains Mir390-Ago7 sequences for binding and localization

into the processing center enabling long lasting amplification.

Example 9G provides dsRNA construct #7 having a sense dual Mir390BS sequence coupled with an antisense Mir4376BS. Figure 7 presents dsRNA construct #7 containing a dualMir390BS on its sense strand and a single Mir4376BS on its antisense strand. The presence of ta-siRNA inducing miRNAs on both strands will lead to ongoing amplification.

Example 9H provides dsRNA construct #8 having an Endogenous Trigger Control. Figure 8 presents dsRNA construct #8 that is based on an exact endogenous insert sequence (the insert is the region between the two Mir390BS) in order to serve as an endogenous trigger control for the dsRNA construct #9 of Example 9I.

Example 9I presents dsRNA construct #9 having a Mir390BS sequence and an the Endogenous insert of Example 9H. Figure 9 presents a schematic of dsRNA construct #9. Construct #9 maintains the endogenous sequence of the Mir390BS including the original insert region. This construct result in production of ta-siRNAs targeting ARF3 and ARF4.

EXAMPLE 10: TREATMENT OF SEEDS WITH ta-siRNA CONSTRUCTS AND ANALYSIS OF RNA LEVELS.

Tomato seeds were treated with dsRNA molecules corresponding to the constructs and sequences of Table 5.

Trigger #	Trigger alias	Sequence (5'-3')	Length	DS/SS	S/AS
1	GFP234	CTAATACGACTCACTATAGGGAGATTTCG TCCTCCTTGAAATCAATCCCTTAAGCTCG ATCCTGTTGACGAGGGTGTCTCCCTCAAAC TTGACTTCAGCACGTGTCTTGTAGTTCCTG TCGTCCTTGAAAGAGATGGTCCTCTCCTGC ACGTATCCCTCAGGCATGGCGCTCTTGAAG AAGTCGTGCCGCTTCATATGATCTGGGTAT CTTGAAAAGCATTGAACACCATAAGAGAA AGTAGTGACAAGTGTGGCTCTCCCTATAG TGAGTCGTATTAG	282	DS	Sense
2	GFP234 Mir390	CTAATACGACTCACTATAGGGAGAGGTGC TATCCTACCTGAGCTTTTTCCTCCTCTTG AAATCAATTCCTTAAGCTCGATCCTGTTG ACGAGGGTGTCTCCCTCAAACCTGACTTCA	325	DS	Sense

		GCACGTGTCCTTGTAGTTCCCGTCGTCCTTG AAAGAGATGGTCCTCTCCTGCACGTATCCC TCAGGCATGGCGCTCTTGAAGAAGTCGTGC CGCTTCATATGATCTGGGTATCTTGAAAAG CATTGAACACCATAAGAGAAAAGTAGTGAC AAGTGTGGCCCTTGTCTATCCCTCCTGAG CTTCTCCCTATAGTGAGTCGTATTAG			
3	GFP234 Mir390 X2	CTAATACGACTCACTATAGGGAGAAGCTC AGGAGGGATAGACAAGGGGTGCTATCCTA CCTGAGCTTTTTCCGTCCCTTGAAATCA ATCCCTTAAGCTCGATCCTGTTGACGAGG GTGTCTCCCTCAAACCTGACTTCAGCACGT GTCTTGTAGTTCCCGTCGTCCTTGAAAGAG ATGGTCCTCTCCTGCACGTATCCCTCAGGC ATGGCGCTCTTGAAGAAGTCGTGCCGCTTC ATATGATCTGGGTATCTTGAAAAGCATTGA ACACCATAAGAGAAAAGTAGTGACAAGTGT TGGCAAGCTCAGGTAGGATAGCACCCCTT GTCTATCCCTCCTGAGCTTCTCCCTATAGT GAGTCGTATTAG	368	DS	Sense
4	GFP234 Mir390 _Sense	CTAATACGACTCACTATAGGGAGAGGTGC TATCCTACCTGAGCTTTTTCCGTCCCTTG AAATCAATTCCTTAAGCTCGATCCTGTTG ACGAGGGTGTCTCCCTCAAACCTGACTTCA GCACGTGTCCTTGTAGTTCCCGTCGTCCTTG AAAGAGATGGTCCTCTCCTGCACGTATCCC TCAGGCATGGCGCTCTTGAAGAAGTCGTGC CGCTTCATATGATCTGGGTATCTTGAAAAG CATTGAACACCATAAGAGAAAAGTAGTGAC AAGTGTGGCCCTTGTCTATCCCTCCTGAG CT	301	SS	Sense
5	GFP234 Mir390 _Antise nse	CTAATACGACTCACTATAGGGAGAGCCAA CACTTGTCACTACTTTCTCTTATGGTGTTC ATGCTTTTCAAGATACCCAGATCATATGAA GCGGCACGACTTCTTCAAGAGCGCCATGCC TGAGGGATACGTGCAGGAGAGGACCATCT CTTTCAAGGACGACGGGAACAACAAGACA CGTGCTGAAGTCAAGTTTGAGGGAGACAC	258	SS	Antisense

		CCTCGTCAACAGGATCGAGCTTAAGGGAA TTGATTTCAAGGAGGACGGAAA			
6	GFP234 Mir390 _Helica se	CTAATACGACTCACTATAGGGAGAGGTGC TATCCTACCTGAGCTTTTTCCGTCCTCCTTG AAATCAATTCCCTTAAGCTCGATCCTGTTG ACGAGGGTGTCTCCCTCAAACCTGACTTCA GCACGTGTCTTGTAGTTCCCGTCGTCCTTG AAAGAGATGGTCCTCCTGCACGTATCCC TCAGGCATGGCGCTCTTGAAGAAGTCGTGC CGCTTCATATGATCTGGGTATCTTGAAAAG CATTGAACACCATAAGAGAAAGTAGTGAC AAGTGTGGCGCTACTGGACTTTCGCCATC TAGGGTGCAGCATTCGGGCTGCTTCGCCTA CCTTGTCTATCCCTCCTGAGCTTCTCCCTAT AGTGAGTCGTATTAG	375	DS	Sense
7	GFP234 Mir390 _Helica se_Sens e	CTAATACGACTCACTATAGGGAGAAGCTC AGGAGGGATAGACAAGGGGTGCTATCCTA CCTGAGCTTTTTCCGTCCTCCTTGAAATCA ATCCCTTAAGCTCGATCCTGTTGACGAGG GTGTCTCCCTCAAACCTGACTTCAGCACGT GTCTTGTAGTCCCGTCGTCCTTGAAAGAG ATGGTCCCTCCTGCACGTATCCCTCAGGC ATGGCGCTCTTGAAGAAGTCGTGCCGCTTC ATATGATCTGGGTATCTTGAAAAGCATTGA ACACCATAAGAGAAAGTAGTGACAAGTGT TGGCAAGCTCAGGTAGGATAGCACCCCTT GTCTATCCCTCCTGAGCT	344	SS	Sense
8	GFP234 Mir390 _Helica se_Anti Sense	CTAATACGACTCACTATAGGGAGAAGCTC AGGAGGGATAGACAAGGGGTGCTATCCTA CCTGAGCTTGCCAACACTTGCTACTACTTT CTCTTATGGIGTTCAATGCTTTTCAAGATA CCCAGATCATATGAAGCGGCACGACTTCTT CAAGAGCGCCATGCCTGAGGGATACGTGC AGGAGAGGACCATCTCTTTCAAGGACGAC GGGAACAACAAGACACGTGCTGAAGTCAA GTTTGAGGGAGACACCCTCGTCAACAGGA TCGAGCTTAAGGGAATTGATTTCAAGGAG GACGGAAAAGCTCAGGTAGGATAGCACC	394	SS	Antisense

		CCTTGTCTATCCCTCCTGAGCTTAGGCGAA GCAGCCCGAATGCTGCACCCTAGATGGCG AAAGTCCAGTAGC			
9	GFP234 Mir390 _Mir43 76	CTAATACGACTCACTATAGGGAGAGGTGC TATCCTACCTGAGCTTTTTCCGTCCTCCTTG AAATCAATTCCCTTAAGCTCGATCCTGTTG ACGAGGGTGTCTCCCTCAAACCTGACTTCA GCACGTGTCTTGTAGTTCCTCGTCGTCCTTG AAAGAGATGGTCCTCTCCTGCACGTATCCC TCAGGCATGGCGCTCTTGAAGAAGTCGTGC CGCTTCATATGATCTGGGTATCTTGAAAAG CATTGAACACCATAAGAGAAAGTAGTGAC AAGTGTGGCTCGCAGGAGAGATGACACC AGACCTTGTCTATCCCTCCTGAGCTTCTCC CTATAGTGAGTCGTATTAG	347	DS	Sense
10	TAS3	CTAATACGACTCACTATAGGGAGATTTCTC ACCGCTTTTTTTTTTCTGTTGTGTATTCTCT TTTTTGACTTGTTCCTTTCGTTCCCTCTACC TACCCCATCTTCTTGACCTTGTAAGACCTT TTCTTGACCTTGTAAGACCCCGTGTTATCT CTTACGTCTTTATGTTTTGTTTTTTGCAA TCTTACGTCATGACTTCTTCATGTAAGCTTT GTTTGGTCTCCTTCTTCTTCTACTCAACT CTCGTTCTCCTTCTCCCTATAGTGAGTCGT ATTAG	282	DS	Sense
11	TAS3M ir390	CTAATACGACTCACTATAGGGAGAGGTGC TATCCTACCTGAGCTTTTTCTCACCCTTTT TTTTTCTGTTGTGTATTCTCTTTTTTGACTT GTTGCCTTTCGTTCCCTCTACCTACCCCATTC TTCTTGACCTTGTAAGACCTTTTCTTGACCT TGTAAGACCCCGTGTTATCTCTTACGTCTTT ATGTTTTGTTTTTTGCAAATCTTACGTCAT GACTTCTTCATGTAAGCTTTGTTTGGTCTCC TTCTTCTTCTACTCAACTCTCGTTCTCCT TCCTTGTCTATCCCTCCTGAGCTTCTCCCTA TAGTGAGTCGTATTAG	325	DS	Sense
12	GUS23 4	GCCACTTGCAAAGTCCCCTAGTGCCT TGTCCAGTTGCAACCACCTGTTGATCC	236	DS	Sense

		GCATCACGCAGTTCAACGCTGACATCA CCATTGGCCACCACCTGCCAGTCAACA GACGCGTGGTTACAGTCTTGCGCGACA TGCGTCACCACGGTGATATCGTCCACC CAGGTGTTCCGGCGTGGTGTAGAGCATT ACGCTGCGATGGATTCCGGCATAGTTA AAGAAATCATGGAAGTAAGC			
13	GUS23 4Mir39 0	GGTGCTATCCTACCTGAGCTTCCACTT GCAAAGTCCCGCTAGTGCCTTGTCCAG TTGCAACCACCTGTTGATCCGCATCAC GCAGTTCAACGCTGACATCACCATTGG CCACCACCTGCCAGTCAACAGACGCGT GGTTACAGTCTTGCGCGACATGCGTCA CCACGGTGATATCGTCCACCCAGGTGT TCGGCGTGGTGTAGAGCATTACGCTGC GATGGATTCCGGCATAGTTAAAGAAAT CATGGAAGTAAGCCTTGTCTATCCCTC CTGAGCTC	278	DS	Sense

GFP234 (Figure 10A; Trigger #1), GFP234Mir390 (Trigger #2), TAS3 (Trigger #10) and TAS3Mir390 (Figure 10B, Trigger #11) were prepared as provided in Example 1. A final concentration of 50 µg/ml dsRNA diluted with 0.1mM EDTA was used. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C followed by washing with water three times for one minute. After treatment, seeds were germinated either on wet paper or in soil and grown at about 25 °C with a 16 hour photoperiod. The plants germinated in soil were watered with tap water as necessary. Seeds that were treated with a similar solution not containing dsRNA (*e.g.*, 0.1mM EDTA “EDTA”) were germinated and grown alongside the treated plants as a control.

Total RNA was extracted from whole seedlings, leaves or roots of germinated seeds seven, 14 and 30 days post treatment. For seeds that were germinated on paper, the entire seedling was harvested after seven days. For seeds that were germinated in soil, the leaves and roots were harvested and analyzed separately 14 and 30 days after treatment.

cDNA was prepared using oligo-dT primers and the expression levels of TAS3,

ARF3 and ARF4 was determined by real-time PCR with SYBR Green (Quanta BioSciences). The results are presented in Figure 11A-E. The house-keeping genes *Expressed* and *Tip41* were used as endogenous control genes to normalize for input RNA amounts. ARF3 and ARF4 genes are regulated by the TAS3 system and their expression is predicted to decrease following TAS3Mir390 treatment. No significant difference in TAS3, ARF3 or ARF4 expression was detected in seedlings when comparing the TAS3 construct with the TAS3Mir390 construct seven days after treatment (t-test, p-value>0.05). Similarly, no significant difference in TAS3, ARF3 or ARF4 expression was detected in leaves when comparing the two constructs 14 days after treatment. When comparing expression levels in roots 14 days after treatment, a down-regulation trend was observed for ARF3 and ARF4 genes following treatment with the TAS3Mir390 construct (Figures 11A-B). Figure 11A shows relative quantification of ARF3 mRNA following treatment with either TAS3 Insert or Mir390BS TAS3 dsRNA constructs. Each point represents the expression value per individual plant. Expression values were normalized to the average expression values of all plants treated with TAS3_Insert, which was set to 100%. The red line represents the normalized average expression values for each treatment. Figure 11B shows the same analysis for ARF4 mRNA levels.

The RNA extracted from seven-day old seedlings, as well as RNA extracted from 30-day old roots and 30-day old leaves was used in a second cDNA reaction with random primers and then subjected to real-time PCR with SYBR Green (Quanta BioSciences). The primers used for real-time were derived from the GFP sequence that appears in the dsRNA constructs. Therefore, this analysis provides an indication for the presence of the dsRNA that was used for seed treatment and/or for RNA that was synthesized from this dsRNA in the plant tissue. *Expressed* and *Tip41* were used as endogenous control genes. For seven day old seedlings, a 2.6 fold difference in GFP level was observed in plants treated with GFP234 compared to plants treated with TAS3 (t-test, p-value=0.08). However, no significant difference in GFP level was observed when comparing between GFP234 and GFP234Mir390 treatments (Figures 11C-E). Figure 11C shows normalized Ct values for all treatments analyzed; for each RNA sample tested, the Ct value obtained from the real-time amplification plot was normalized to the average Ct value of the two endogenous control genes. This value

was then subtracted from the number 50 to assign larger values for higher expression levels. Each dot represents one plant and the red line represents the average value per treatment. RNA samples that gave no Ct values were assigned a value of 40. Figure 11D shows a comparison between GFP234 and TAS3 treatments. Figure 11E shows a comparison between GFP234 and GFP234Mir390 treatments. For 30 day old roots, no significant difference in GFP level was observed when comparing between GFP234 and GFP234Mir390 treatments. For 30 day old leaves, a significant (t-test, p-value=0.005), 7.1-fold difference in GFP level was observed in plants treated with GFP234Mir390 compared to plants treated with GFP234 (Figure 11F).

EXAMPLE 11: DETECTION OF GFP SEQUENCE IN PLANTS FOLLOWING SEED TREATMENT WITH TA-SI dsRNA CONSTRUCTS

In a second experiment, tomato seeds were treated with dsRNA molecules corresponding to GFP234 (Trigger#1), GFP234Mir390 (Trigger#2), GFP234Mir390X2 (Trigger#3), GFP234Mir390_Helicase (Trigger#6), GFP234Mir390_Mir4376 (Trigger#9), TAS3 (Trigger#10) and TAS3Mir390 (Trigger#11) as provided above in Example 10 and Table 5, according to the protocol described in Example 10. A final concentration of 50 µg/ml dsRNA diluted with 0.1mM EDTA was used. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C followed by washing with water three times for one minute. After treatment, seeds were germinated on wet paper and grown at about 25 °C with 16 hours photoperiod. Seeds that were treated with EDTA solution alone were germinated and grown alongside the treated plants as a control.

Total RNA was extracted from shoots (including hypocotyl, cotyledon and shoot apical meristem) seven and fourteen days after treatment. cDNA was prepared using random primers and the presence of the GFP sequence was determined and quantified by real-time PCR as described in Example 10. A significant difference in GFP level was observed in plants seven days after treatment with GFP234Mir390, GFP234Mir390_Helicase or GFP234Mir390_Mir4376 dsRNAs compared to treatment with GFP234, TAS3Mir390 or EDTA. The GFP level detected was between 5.9 fold (following treatment with GFP234Mir390_Mir4376) to 25 fold (following treatment with GFP234Mir390_Helicase) higher compared to plants treated with GFP234, with a p-value<0.05 (t-test). No significant difference was detected when comparing

GFP234Mir390X2 to GFP234 treatment (Figures 12A-E). The analyses were performed as described for Figures 11C-F of Example 10. Figure 12A shows normalized Ct values for all treatments. Figure 12B shows a comparison between GFP234 and GFP234Mir390 treatments. Figure 12C shows a comparison between GFP234 and GFP234Mir390_Helicase treatments. Figure 12D shows a comparison between GFP234 and GFP234Mir390_Mir4376 treatments. Figure 12E shows a comparison between GFP234 and GFP234Mir390X2 treatments.

A significant, 33 fold difference in GFP level was observed in plants 14 days after treatment with GFP234Mir390X2 dsRNA compared to treatment with GFP234 (t-test, p-value<0.05). Higher levels of GFP were also detected for GFP234Mir390, GFP234Mir390_Helicase and GFP234Mir390_Mir4376 treatments compared to GFP234 treatment, but with no significant difference (Figures 13A-B). The analyses were performed as described for Figures 11C-F. Figure 13A shows normalized Ct values for all treatments. Figure 13B shows a comparison between GFP234 and GFP234Mir390X2 treatments.

The same cDNA prepared from RNA extracted from seven-day old seedlings, was used in a second real-time PCR, where the expression levels of TAS3, ARF3 and ARF4 was determined as described in Example 10 (except that random primers, and not oligo-dT primers were used in the cDNA reaction). No significant difference in TAS3, ARF3 or ARF4 expression was detected in seedlings when comparing between TAS3 and TAS3Mir390 treatments (t-test, p-value>0.05).

EXAMPLE 12: DETECTION OF GFP SEQUENCE IN PLANTS FOLLOWING SEED TREATMENT WITH TA-SI dsRNA CONSTRUCTS

Tomato seeds were treated with dsRNA molecules corresponding to GFP234 (Trigger#1), GFP234Mir390_Helicase (Trigger#6) and GFP234Mir390_Mir4376 (Trigger#9), as provided above in Example 10 and Table 4, according to the protocol described in Example 1. A final concentration of 50 µg/ml dsRNA diluted with 0.1mM EDTA was used. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C followed by washing with water three times for one minute. After treatment, seeds were germinated on wet paper and grown at about 25 °C with 16 hours photoperiod. Seeds that were treated with EDTA solution alone were germinated and grown alongside the treated plants as a control.

Total RNA was extracted from shoots (including hypocotyl, cotyledon and shoot apical meristem) seven days after treatment. cDNA was prepared using random primers and the presence of the GFP sequence was determined and quantified by real-time PCR as described in Example 10. No significant difference in GFP levels was observed when comparing the GFP234 treated plants to the GFP234Mir390_Helicase or GFP234Mir390_Mir4376 treated plants (Dunnett's test).

EXAMPLE 13: DETECTION OF GFP SEQUENCE IN PLANTS FOLLOWING SEED TREATMENT WITH TA-SI dsRNA CONSTRUCTS

Tomato seeds were treated with dsRNA molecules corresponding to GFP234 (Trigger#1), GFP234Mir390 (Trigger#2), GFP234Mir390X2 (Trigger#3), GFP234Mir390_Helicase (Trigger#6), GFP234Mir390_Mir4376 (Trigger#9), TAS3 (Trigger#10) and TAS3Mir390 (Trigger#11) as provided above in Example 10 and Table 5, according to the protocol described in Example 1. A final concentration of 50 µg/ml dsRNA diluted with 0.1mM EDTA was used. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C followed by washing with water three times for one minute. After treatment, seeds were germinated on wet paper and grown at about 25 °C with 16 hours photoperiod. Seeds that were treated with EDTA solution alone were germinated and grown alongside the treated plants as a control.

Total RNA was extracted from shoots (including hypocotyl, cotyledon and shoot apical meristem) seven and 14 days after treatment. cDNA was prepared using random primers and the presence of the GFP sequence was determined and quantified by real-time PCR as described in Example 10. Seven days after treatment, a significant, 10-fold difference in GFP level was observed in plants treated with GFP234Mir390 compared to plants treated with GFP234 (Dunnett's test, p-value=0.035). A 6.6-fold difference in GFP level was observed in plants following treatment with GFP234Mir390_Helicase compared to treatment with GFP234 (Dunnett's test, p-value=0.11). For 14 days old plants, a significant, 34-fold difference in GFP level was observed in plants treated with GFP234Mir390_Helicase compared to plants treated with GFP234 (Dunnett's test, p-value=0.0004). An 8.5-fold difference in GFP level was observed in plants following treatment with GFP234Mir390 compared to treatment with GFP234 (Dunnett's test, p-value=0.058). Figure 14A shows normalized Ct values seven days after treatment.

Figure 14B shows normalized Ct values 14 days after treatment. The analysis was performed essentially as described for Figure 11C-F, except that instead of subtracting the normalized Ct values from the number 50 to assign larger values for higher GFP levels, an inverse y-axis is presented.

5 RNA extracted from seven-day old shoots treated with TAS3 (Trigger#10) and TAS3Mir390 (Trigger#11) dsRNAs was used in a second real-time PCR to determine the expression levels of TAS3, ARF3 and ARF4 as described in Example 11. A significant, 1.3-fold up-regulation in ARF4 expression was detected in plants following treatment with TAS3Mir390 compared to treatment with TAS3 (Dunnett's test, p-
10 value=0.05). No significant difference in TAS3 or ARF3 expression was detected in those plants.

EXAMPLE 14: DETECTION OF GUS SEQUENCE IN PLANTS FOLLOWING SEED TREATMENT WITH TA-SI dsRNA CONSTRUCTS

Tomato seeds were treated with dsRNA molecules corresponding to GUS234
15 (Trigger#12) and GUS234Mir390 (Trigger#13), as provided above in Example 10 and Table 5, according to the protocol described in Example 1. A final concentration of 50 µg/ml dsRNA diluted with 0.1mM EDTA was used. Treatment was performed by gently shaking the seeds in the solution for 24 hours in a dark growth chamber at 15 °C followed by washing with water three times for one minute. After treatment, seeds were
20 germinated on wet paper and grown at about 25 °C with 16 hours photoperiod. Seeds that were treated with EDTA solution alone were germinated and grown alongside the treated plants as a control.

Total RNA was extracted from shoots (including hypocotyl, cotyledon and shoot
25 apical meristem) seven and 14 days after treatment. cDNA was prepared using random primers and the presence of the GUS sequence was determined and quantified by real-time PCR as described in Example 10. A significant increase in GUS levels was observed in plants seven days after treatment with GUS234Mir390 compared to treatment with GUS234 (Dunnett's test, p-value=0.0005). Most of the RNA samples extracted from GUS234-treated plants gave no Ct value, meaning GUS was not detected
30 in those samples. Accordingly, these samples were assigned a value of 40. The resulting difference in the average Ct value between the GUS234 and the GUS234Mir390 treatments was calculated to be about 10, translating into a 994-fold difference in GUS

levels. For 14 days old plants, a 12.2-fold difference in GUS levels was observed (Dunnett's test, p-value=0.03865). Figure 15A shows normalized Ct values seven days after treatment with the two dsRNA constructs. Figure 15B shows normalized Ct values 14 days after treatment. The analysis was performed as described for Figures 14A-B.

5 **EXAMPLE 15: SMALL RNA DEEP SEQUENCING OF PLANTS FOLLOWING SEED TREATMENT WITH TA-SI dsRNA CONSTRUCTS**

RNA samples from the seven day old shoots described in Examples 11 and 14 were further analyzed by small RNA deep sequencing. cDNA libraries were prepared with an Illumina TruSeq™ Small RNA kit according to the manufacturer's protocol, and
10 sequenced by Illumina MiSeq® instrument. Each cDNA library was prepared from RNA pooled from three plants originating from the same treatment. For GFP-based dsRNAs, a total of ten libraries were prepared. Two libraries (representing a total of six plants) were prepared from GFP234 treated plants, three libraries (nine plants) were prepared from GFP234Mir390 treated plants, one library (three plants) was prepared
15 from GFP234Mir390X2 treated plants, two libraries (six plants) were prepared from GFP234Mir390_Helicase treated plants and two libraries (six plants) were prepared from TAS3Mir390 treated plants. Table 6 shows the average Ct value of each of the pooled RNA samples, according to the real-time PCR analysis shown in Figure 12A. The values presented in the table were normalized by subtracting the average Ct value
20 of RNA pooled from the TAS3Mir390 treatment. Low quality reads and reads that contain adaptor sequences were filtered out from the raw sequencing data. Table 6 summarizes the number of reads from each library that were mapped to GFP. In accordance with the real-time PCR results, more reads were mapped to the GFP sequence in the GFP234Mir390 and GFP234Mir390_Helicase treatments compared to
25 the GFP234 treatment.

Table 6: Small RNA MiSeq analysis of RNA extracted from plants following seed treatment with ta-si-GFP dsRNA constructs

Treatment	GFP234		GFP234Mir390			GFP234Mir390_Helicase		GFP234Mir390X2	TAS3Mir390	
RT-PCR normalized Ct value	1.3	2.8	3.2	6.6	9.5	7.9	5.9	2.7	0.0	4.0
Libraries size	1.23	1.24	2.09	1.46	1.4	1.59	1.23	1	1.61	1.07

ratio										
Total # of reads mapped to GFP234 sequence	36	12	74	130	273	122	117	20	0	2
Normalized # of reads mapped to GFP234 sequence	29	10	35	89	195	77	95	20	0	2

For GUS-based dsRNAs, two libraries were prepared, one library (representing a total of three plants) was prepared from GUS234 treated plants and one library (three plants) was prepared from GUS234Mir390 treated plants. Table 7 shows the average Ct value of each of the pooled RNA samples, according to the real-time PCR analysis shown in Figure 15A. The values presented in the Table were normalized by subtracting the average Ct value of RNA pooled from the GUS234 treatment. Table 7 summarizes the number of reads from each library that were mapped to GUS. Data was analyzed as described for Table 6. In accordance with the real-time PCR results, no reads were mapped to the GUS sequence in the GUS234 treatment while some reads were mapped to GUS in the GUS234Mir390 treatment.

Table 7: Small RNA MiSeq analysis of RNA extracted from plants following seed treatment with ta-si-GUS dsRNA constructs.

Treatment	GUS234	GUS234Mir390
RT-PCR normalized Ct value	0	7.6
Libraries size ratio	1	1.77
Total # of reads mapped to GUS234 sequence	0	27
Normalized # of reads mapped to GUS234 sequence	0	15

EXAMPLE 16: ADDITIONAL dsRNA CONSTRUCTS

Additional dsRNA constructs based on the constructs and sequences provided in Examples 9 and 10 are provided. Deep-Sequencing analysis described in Example 15 indicated that the first nucleotide in the small RNA reads mapped to the endogenous TAS3 transcript is predominantly “T”. The most abundant reads mapped to the TAS3 transcript were located at positions 37, 38, 79, 100, 101, 103, 184 and 185 of the 234nt sequence and with the exception of the reads mapped to position 185, where the first nucleotide was A, the first nucleotide was T. Therefore, the inclusion of a “T” or “A” in the sequence of the target gene of interest that is flanked by the two miR390 binding sites is expected to improve the efficiency of cleavage and direct it to specific sites within the sequence or alternatively improves the interaction of the resulting small RNAs with downstream effectors.

Trigger #14 (SEQ ID No. 314), is designed as a modified GFP234Mir390 sequence (based on Example 9B and trigger #2), where the nucleotides at positions 37, 38, 79, 100, 101, 103 and 184 are “T”, and the nucleotide at position 185 is A (only positions 37, 79 and 101 were changed. Sequence appears in 5'-3' orientation, the mentioned positions are in lowercase, bold).

SEQ ID No. 314:

GGTGCTATCCTACCTGAGCTTTTTCCGTCCTCCTTGAAATCAATTCCTTAAG
 CTCG**tt**CCTGTTGACGAGGGTGTCTCCCTCAAACCTTGACTTCAGCA**tt**GTGTCTT
 GTAGTTCCCGTCG**tt**C**tt**TGAAAGAGATGGTCCTCTCCTGCACGTATCCCTCAG
 GCATGGCGCTCTTGAAGAAGTCGTGCCGCTTCATATGATCTGGG**ta**TCTTGAA
 AAGCATTGAACACCATAAGAGAAAGTAGTGACAAGTGTTGGCCCTTGTCTA
 TCCCTCCTGAGCT

Trigger #15 (SEQ ID No. 315) is designed as a modified TAS3Mir390 sequence (based on Example 9I and trigger #11). In this sequence, four 21 nucleotides segments that begin with “TT” are selected from the GFP234 sequence and used to replace the original 21 nucleotide segments from the TAS3Mir390 sequence at positions 37, 79, 100 and 184. These positions are in phase with the miR390-guided cleavage site (sequence appears in 5'-3' orientation, the mentioned four segments are in lowercase, bold).

SEQ ID No. 315:

GGTGCTATCCTACCTGAGCTTTTTCTCACCGCTTTTTTTTTTCTGTTGTGTATT
 CTC**tttccgctccttgaatca**GTTCCCTCTACCTACCCCATTC**ttcccttaagctegatcctgtttgac**
ttcagcacgtgtcttgGTGTTATCTCTTACGTCTTTATGTTTTGTTTTTTTGCAAATCTT
 5 ACGTCATGACTTCTTCATG**ttcccgctccttgaagag**CTTCTTTCCTACTCAACTCT
 CGTTCTCCTTCCTTGTCTATCCCTCCTGAGCT

Trigger #16 (SEQ ID No.316) is designed as a modified GFP234Mir390
 sequence (based on Example 9B and trigger #2), where “T” appears every 21
 nucleotides. The most 3’ “T” is located at position 226 of the GFP234 sequence, 21
 10 nucleotides upstream to the miR390-guided cleavage site and all other “T” positions are
 in phase with this site (sequence appears in 5’-3’ orientation, only mutated nucleotides
 are in lowercase, bold).

SEQ ID No. 316:

GGTGCTATCCTACCTGAGCTTTTTCCGTCCTCCTG**t**AATCAATCCCTTAAG
 15 CTCG**t**TCCTGTTGACGAGGGTGTCT**t**CCTCAA**ACTT**GACTTCAGCA**t**GTGTCTT
 GTAGTTCCCGTCGTCCTTGAAAGAGATGGTCCTCCTGCACGTATCCCTCA
 GGC**t**TGGCGCTCTTGAAGAAGTCGTGCCGCTTCATATGATCTGGGTATCTTG
 AAAAGCATTGAACA**t**CATAAGAGAAAGTAGTGACA**t**GTGTTGGCCCTTGTCT
 ATCCCTCCTGAGCT

20 Trigger #17 (SEQ ID No. 317) is designed as a modified GFP234Mir390
 sequence (based on Example 9B and trigger #2), where ten 21 nucleotide segments that
 originate from the full-length GFP sequence and begins with a “T” are placed in tandem
 to produce the 234 nucleotide sequence. The first 15 and the last 9 nucleotides are from
 the endogenous TAS3 sequence, in order to position the ten segments in-phase with the
 25 miR390-guided cleavage site (sequence appears in 5’-3’ orientation, the first “T” in
 each segment is in lowercase, bold, and the TAS3 sequence is underlined).

SEQ ID No. 317:

GGTGCTATCCTACCTGAGCTTTTTCTCACCGCTTTT**t**AATGGTTGTCTGGTAAA
 AGG**t**CGCCAATTGGAGTATTTTGT**t**GATAATGATCAGCGAGTTGC**t**CTTCGAT
 30 GTTGTGGCGGGT**Ct**TGAAGTTGGCTTTGATGCCG**t**TCTTTTGCTTGTGGCCA
 TG**t**GTATACGTTGTGGGAGTTGT**t**TGTATTCCA**ACTT**GTGGCCG**t**GTTTCCGTC
 CTCCTTGA**AA**T**t**TCCCTTAAGCTCGATCCTGTGTTCTCCTTCCTTGTCTATCCC

TCCTGAGCT

Tomato seeds are treated with dsRNA triggers 14, 15, 16 and 17 as described in Example 10. The seeds are germinated on paper and the seedlings is harvested at 7, 14 and 30 days post treatment and total RNA is extracted from whole seedlings, leaves or roots of the germinated seeds. cDNA is prepared using oligo-dT primers and the expression levels of TAS3, ARF3 and ARF4 are determined by real-time PCR with SYBR Green (Quanta BioSciences). ARF3 and ARF4 genes are regulated by the TAS3 system and their expression is predicted to decrease following trigger treatment.

Although the disclosure has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present disclosure. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An isolated double-stranded RNA (dsRNA) molecule comprising
 - (a) a first RNA strand having
 - at least one antisense RNA sequence for suppressing expression of a target gene of interest in a plant or a phytopathogen of a plant, and
 - a first heterologous smRNA-binding sequence for binding to a first small RNA (smRNA) expressed in said plant or phytopathogen, and
 - a helicase-binding sequence having a helicase binding site; and
 - (b) a second RNA strand that is a reverse complement of said first RNA strand.
2. The isolated dsRNA molecule of claim 1, wherein said first smRNA comprises a nucleic acid sequence wherein said nucleic acid sequence shares between 100% and 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 to 288, and complements thereof.
3. The isolated dsRNA molecule of claim 1, wherein said first heterologous smRNA-binding sequence is 5' to said antisense RNA sequence.
4. The isolated dsRNA molecule of claim 1, wherein said first heterologous smRNA-binding sequence is 3' to said antisense RNA sequence.
5. The isolated dsRNA molecule of claim 1, wherein said first heterologous smRNA-binding sequence is the complement of said first smRNA.
6. The isolated dsRNA molecule of claim 1, wherein said first RNA strand further comprises a second heterologous smRNA-binding sequence for binding a second smRNA expressed in said plant or phytopathogen, and said first heterologous smRNA-binding sequence and said second heterologous smRNA-binding sequence flank said at least one antisense RNA sequence.
7. The isolated dsRNA molecule of claim 6, wherein said first smRNA and said second smRNA comprise a nucleic acid sequence having at least 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 to 288, and complements thereof.
8. The isolated dsRNA molecule of claim 6, wherein said second heterologous smRNA-binding sequence is the complement of said second smRNA.

9. The isolated dsRNA molecule of claim 6, wherein said second smRNA is identical to said first smRNA.
10. The isolated dsRNA molecule of claim 6, wherein said second smRNA is non-identical to said first smRNA.
11. The isolated dsRNA molecule of claim 6, wherein said first heterologous smRNA-binding sequence and said second heterologous smRNA-binding sequence comprise a nucleotide sequence selected from the group consisting of:
 - a direct sequence of said first smRNA and a direct sequence of said second smRNA;
 - a reverse complement of said first smRNA and a direct sequence of said second smRNA;
 - a reverse complement of said first smRNA and a direct sequence of said second smRNA;
 - a direct sequence of said first smRNA and a reverse complement of said second smRNA;
 - a direct sequence of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA;
 - a reverse complement of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA;
 - a reverse complement of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA;
 - a direct sequence of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a reverse complement of said second smRNA;
 - a direct sequence of said first smRNA and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage;
 - a reverse complement of said first smRNA and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage;

a reverse complement of said first smRNA and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage;

a direct sequence of said first smRNA and a reverse complement of said second smRNA and further comprising a mutation rendering it resistant to cleavage;

a direct sequence of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage;

a reverse complement of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage;

a reverse complement of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a direct sequence of said second smRNA and further comprising a mutation rendering it resistant to cleavage; and

a direct sequence of said first smRNA and further comprising a mutation rendering it resistant to cleavage and a reverse complement of said second smRNA and further comprising a mutation rendering it resistant to cleavage.

12. The isolated dsRNA molecule of claim 1, wherein said helicase binding sequence is positioned within said at least one antisense RNA sequence.
13. The isolated dsRNA molecule of claim 1, wherein said helicase binding sequence is positioned 5' or 3' of said at least one antisense RNA sequence.
14. The isolated dsRNA molecule of claim 13, wherein said first RNA strand further comprises a second heterologous smRNA-binding sequence for binding to a second smRNA expressed in said plant or phytopathogen, and wherein said first heterologous smRNA and said second heterologous smRNA-binding sequence flank said at least one antisense RNA sequence and said helicase binding sequence.
15. The isolated dsRNA molecule of claim 1, wherein said first smRNA has a nucleotide sequence selected from the group consisting of an RNA sequence of a microRNA (miRNA) and an RNA sequence of an siRNA.
16. The isolated dsRNA molecule of claim 6, wherein said first smRNA has a nucleotide sequence selected from the group consisting of an RNA sequence of a

miRNA and an RNA sequence of an siRNA and said second smRNA has a nucleotide sequence selected from the group consisting of an RNA sequence of a miRNA and an RNA sequence of an siRNA.

17. The isolated dsRNA molecule of claim 14, wherein said first smRNA is selected from the group consisting of an RNA sequence of a miRNA and an RNA sequence of an siRNA and said second smRNA is selected from the group consisting of an RNA sequence of a miRNA and an RNA sequence of an siRNA.
18. The isolated dsRNA molecule of claim 1, wherein said first smRNA is a miRNA.
19. The isolated dsRNA molecule of claim 6, wherein said first smRNA is a miRNA and said second smRNA is a miRNA.
20. The isolated dsRNA molecule of claim 14, wherein said first smRNA is a miRNA and said second smRNA is a miRNA.
21. The isolated dsRNA molecule of claim 1, wherein said first smRNA is a miRNA selected from the group consisting of miR390, miR161.1, miR168, miR393, miR828, and miR173.
22. The isolated dsRNA molecule of claim 6, wherein said first smRNA is a miRNA selected from the group consisting of miR390, miR161.1, miR168, miR393, miR828, and miR173.
23. The isolated dsRNA molecule of claim 14, wherein said first smRNA is a miRNA selected from the group consisting of miR390, miR161.1, miR168, miR393, miR828, and miR173.
24. The isolated dsRNA molecule of claim 6, wherein said plant or phytopathogen comprises a trans-acting-siRNA-producing (TAS) locus that comprises a binding site for binding said second smRNA.
25. The isolated dsRNA molecule of claim 6, wherein said plant or phytopathogen comprises a TAS locus, wherein said TAS locus naturally comprises a binding site for binding said first smRNA and a binding site for binding said second smRNA, and wherein said binding sites flank said TAS locus in said plant or phytopathogen.
26. The isolated dsRNA molecule of claim 6, wherein at least one of said first and second smRNA is an smRNA for which binding sites are naturally found flanking a TAS locus in a plant.
27. The isolated dsRNA molecule of claim 1, wherein said plant is a crop plant.

28. The isolated dsRNA molecule of claim 1, wherein said helicase binding site provides for the separation of the two strands of said dsRNA molecule to produce a region of single stranded RNA (ssRNA) when introduced into a plant cell or provided with a helicase and RNA Dependent RNA Polymerase (RDRP) *in vitro*.
29. The isolated dsRNA molecule of claim 1, wherein said helicase binding site provides for the recruitment of an RNA-dependent RNA Polymerase and transcription of a secondary small interfering RNA (siRNA) complementary to at least one strand of said dsRNA molecule when introduced into a plant cell or provided with a helicase *in vitro*.
30. A method of suppressing expression of a target gene of interest in a plant or phytopathogen of the plant, comprising introducing an isolated double-stranded (dsRNA) molecule of claim 1 into the plant; wherein said at least one antisense RNA sequence is a sequence for regulating a target gene of interest in the plant or phytopathogen.
31. A method of suppressing expression of a target gene of interest in a plant or phytopathogen of the plant, comprising introducing an isolated double-stranded RNA (dsRNA) molecule of claim 6 into the plant; wherein said at least one antisense RNA sequence is a sequence for regulating a target gene of interest in the plant or phytopathogen.
32. A method of introducing the isolated dsRNA molecule of claim 1 into a seed comprising contacting said seed with the isolated dsRNA molecule under conditions which allow penetration of said dsRNA molecule into said seed, thereby introducing said dsRNA molecule into said seed.
33. A seed comprising the isolated double-stranded RNA (dsRNA) molecule of claim 1.
34. The seed of claim 33, further comprising secondary siRNA products complementary to at least one strand of said dsRNA molecule.
35. The seed of claim 34, wherein said secondary siRNA products are produced in said seed using said dsRNA as a template.
36. The seed of claim 33, wherein said dsRNA is devoid of a heterologous promoter capable of driving expression of said dsRNA molecule in said plant.
37. A seed comprising the isolated double-stranded RNA (dsRNA) molecule of claim 6.
38. The seed of claim 37, further comprising secondary siRNA products complementary

- to at least one strand of said dsRNA molecule.
39. The seed of claim 38, wherein said secondary siRNA products are produced in said seed using said dsRNA as a template.
40. The seed of claim 37, wherein said dsRNA is devoid of a heterologous promoter capable of driving expression of said dsRNA molecule in said plant.
41. A plant or plant part generated from the seed of claim 33.
42. A plant or plant part generated from the seed of claim 37.
43. A seed containing device comprising a plurality of the seeds of claim 33.
44. A seed containing device comprising a plurality of the seeds of claim 37.
45. A sown field comprising a plurality of the seeds of claim 33.
46. A sown field comprising a plurality of the seeds of claim 37.
47. A method of producing a plant comprising:
- a. obtaining a seed of claim 33; and
 - b. germinating said seed to produce said plant.
48. A method of producing a plant comprising:
- a. obtaining a seed of claim 37; and
 - b. germinating said seed to produce said plant.
49. A method of suppressing gene expression in a plant or a phytopathogen of the plant comprising:
- a. contacting a seed with an isolated double-stranded RNA (dsRNA) molecule of claim 1 under conditions which allow penetration of said dsRNA molecule into said seed, thereby introducing said dsRNA molecule into said seed; and optionally
 - b. generating a plant of said seed.
50. The method of claim 49, wherein said dsRNA molecule penetrates a cell of said seed selected from the group consisting of an endosperm cell, an embryo cell, and combinations thereof.
51. The method of claim 49, wherein said contacting is effected by inoculating said seed with said dsRNA molecule.
52. The method of claim 51, further comprising priming said seed prior to said contacting.
53. The method of claim 49, wherein said priming comprises:

- (i) washing said seed with a liquid prior to said contacting; and
 - (ii) drying said seed following step (i).
54. The method of claim 53, wherein said liquid is double deionized water.
55. The method of claim 53, wherein said seed is washed for 2 to 6 hours.
56. The method of claim 53, wherein said seed is washed at 4 °C to 28 °C.
57. The method of claim 53, wherein said seed is dried at 25 °C to 30 °C for 10 to 16 hours.
58. The method of claim 49, wherein said dsRNA molecule in a solution having a final concentration of 0.1-100 µg/µl dsRNA molecule.
59. The method of claim 58, wherein said final concentration of said dsRNA molecule is 0.1-0.5 µg/µl.
60. The method of claim 49, further comprising treating said seed with an agent selected from the group consisting of a pesticide, a fungicide, an insecticide, a fertilizer, a coating agent and a coloring agent following said contacting.
61. The method of claim 60, wherein said treating comprises coating said seed with said agent.
62. The method of claim 49, wherein said penetration provides for accumulation of said dsRNA molecule in said seed.
63. The method of claim 58, wherein said concentration of said dsRNA molecule is adjusted according to a parameter selected from the group consisting of seed size, seed weight, seed volume, seed surface area, seed density and seed permeability.
64. The method of claim 49, wherein said seed is contacted prior to breaking of seed dormancy and embryo emergence.
65. The method of claim 49, wherein said seed is a primed seed.
66. A method of suppressing expression of a target gene in a phytopathogen of a plant comprising providing to said phytopathogen a plant, or plant part, of claim 41, thereby suppressing expression of the target gene in the phytopathogen.
67. The method of claim 66, wherein said phytopathogen is selected from the group consisting of a fungus, a nematode, an insect, a bacteria, and a virus.
68. A method of suppressing expression of a target gene in a phytopathogen of a plant comprising providing to said phytopathogen a plant, or plant part, of claim 42, thereby suppressing expression of the target gene in the phytopathogen.

69. The method of claim 68, wherein said phytopathogen is selected from the group consisting of a fungus, a nematode, an insect, a bacteria and a virus.
70. An isolated double-stranded RNA (dsRNA) molecule comprising:
- a first RNA strand having a nucleic acid sequence comprising in a sequential order from 5' to 3',
 - an endovirus 5' UTR sequence,
 - an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence;
 - a multiple cloning site;
 - an endovirus 3' UTR sequence; and
 - a second RNA strand that is a reverse complement of said first RNA strand.
71. An isolated dsRNA molecule comprising:
- a first RNA strand having a nucleic acid sequence comprising in a sequential order from 5' to 3',
 - an endovirus 5' untranslated region (UTR) sequence;
 - an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence;
 - an antisense nucleic acid sequence for regulating a target gene;
 - an endovirus 3' UTR sequence; and
 - a second RNA strand that is a reverse complement of said first RNA strand.
72. The isolated dsRNA molecule of claim 70, wherein said endovirus 5' UTR sequence, said endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence and said endovirus 3' UTR sequence are capable of autonomous replication when introduced into a plant cell.
73. The isolated dsRNA molecule of claim 71, wherein said endovirus 5' UTR sequence, said endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence and said endovirus 3' UTR sequence are capable of autonomous replication when introduced into a plant cell.
74. The isolated dsRNA molecule of claim 70, wherein said 5' UTR sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 14.
75. The isolated dsRNA molecule of claim 71, wherein said 5' UTR sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 14.

76. The isolated dsRNA molecule of claim 70, wherein said 3' UTR sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 22.
77. The isolated dsRNA molecule of claim 71, wherein said 3' UTR sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 22.
78. The isolated dsRNA molecule of claim 70, wherein said endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 23.
79. The isolated dsRNA molecule of claim 71, wherein said endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence shares between 100% and 90% sequence identity to a nucleic acid sequence of SEQ ID NO: 23.
80. The dsRNA of claim 71, wherein said nucleic acid sequence for regulating a target gene is 17 to 600 base pairs (bp) long.
81. The dsRNA of claim 71, wherein said nucleic acid sequence for regulating a target gene is selected from the group consisting of a miRNA and an siRNA.
82. The dsRNA of claim 71, wherein said nucleic acid sequence for regulating a target gene is a miRNA.
83. The isolated dsRNA molecule of claim 72, wherein said plant is a crop plant.
84. The isolated dsRNA molecule of claim 73, wherein said plant is a crop plant.
85. A method of regulating expression of a target gene of interest in a plant comprising introducing an isolated dsRNA molecule of claim 73, and wherein said antisense RNA sequence is for regulating said target gene of interest in said plant, thereby regulating expression of said target gene of interest in said plant.
86. A kit for introducing an isolated dsRNA molecule to seeds comprising:
- (i) the isolated dsRNA molecule of claim 1; and
 - (ii) a priming solution.
87. The kit of claim 86, wherein said dsRNA molecule and said priming solution are provided in separate containers.
88. A kit for introducing an isolated dsRNA molecule to seeds comprising:
- (i) the isolated dsRNA molecule of claim 6; and
 - (ii) a priming solution.

89. The kit of claim 88, wherein said dsRNA molecule and said priming solution are provided in separate containers.
90. A pesticidal composition comprising the isolated dsRNA molecule of claim 1.
91. A pesticidal composition comprising the isolated dsRNA molecule of claim 6.
92. The seed of claim 1, said seed comprising RNA dependent RNA polymerase activity capable of amplifying said dsRNA molecule.
93. The seed of claim 6, said seed comprising RNA dependent RNA polymerase activity capable of amplifying said dsRNA molecule.
94. The plant of claim 33, said plant comprising RNA dependent RNA polymerase activity capable of amplifying said dsRNA molecule.
95. The plant of claim 37, said plant comprising RNA dependent RNA polymerase activity capable of amplifying said dsRNA molecule.
96. The seed of claim 1, wherein said seed is a hybrid seed.
97. The seed of claim 6, wherein said seed is a hybrid seed.
98. A DNA transcription template comprising
- a double stranded DNA sequence, that when transcribed produces a transcript having a first RNA strand having at least one antisense RNA sequence for regulating a target gene of interest in a plant or a phytopathogen of the plant; at least one heterologous smRNA-binding sequence for binding to a first smRNA expressed in said plant;
 - a first promoter on a first end of said double stranded sequence, and
 - a second promoter on a second end of said double stranded sequence.
99. The DNA transcription template of claim 98, wherein said first smRNA comprises a nucleic acid sequence that has between 100% and 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 to 288, and complements thereof.
100. The DNA transcription template of claim 98, wherein said transcript further comprises a helicase binding sequence having a helicase binding site.
101. The DNA transcription template of claim 98, wherein said at least one heterologous RNA sequence corresponding to a first smRNA is 5' or 3' to said antisense RNA sequence.
102. The DNA transcription template of claim 98, further comprising a second

heterologous smRNA-binding sequence for binding to a second smRNA expressed in said plant and said first heterologous smRNA and said second heterologous smRNA flank said at least one antisense RNA sequence.

103. The DNA transcription template of claim 102, wherein said second smRNA comprises a nucleic acid sequence that has between 100% and 90% sequence identity to a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 to 288, and complements thereof.
104. The DNA transcription template of claim 98, wherein said first promoter is a T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, or SP6 DNA-dependent RNA Polymerase promoter.
105. The DNA transcription template of claim 98, wherein said second promoter is a T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, or SP6 DNA-dependent RNA Polymerase promoter.
106. The DNA transcription template of claim 98, wherein said first and said second promoters are the same promoter selected from the group consisting of T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, and SP6 DNA-dependent RNA Polymerase promoter.
107. A DNA transcription template comprising
- a double stranded DNA sequence, that when transcribed produces a transcript having a first RNA strand having a nucleic acid sequence comprising in a sequential order from 5' to 3',
 - an endovirus 5' untranslated region (UTR) sequence;
 - an endovirus RNA Dependent RNA Polymerase (RDRP) coding sequence;
 - an antisense nucleic acid sequence for suppressing a target gene;
 - an endovirus 3' UTR sequence;
 - a first promoter on a first end of said double stranded sequence; and
 - a second promoter on a second end of said double stranded sequence.
108. The DNA transcription template of claim 107, wherein said transcript further comprises a helicase binding sequence having a helicase binding site.
109. The DNA transcription template of claim 107, wherein said at least one heterologous RNA sequence corresponding to a first smRNA is 5' or 3' said antisense RNA sequence.

110. The DNA transcription template of claim 107, further comprising a second heterologous RNA sequence corresponding to a second smRNA expressed in said plant and said first heterologous smRNA and said second heterologous smRNA flank said at least one antisense RNA sequence.
111. The DNA transcription template of claim 107, wherein said first promoter is a T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, or SP6 DNA-dependent RNA Polymerase promoter.
112. The DNA transcription template of claim 107, wherein said second promoter is a T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, or SP6 DNA-dependent RNA Polymerase promoter.
113. The DNA transcription template of claim 107, wherein said first and said second promoters are the same promoter selected from the group consisting of T7 DNA-dependent RNA Polymerase promoter, a T3 DNA-dependent RNA Polymerase promoter, and SP6 DNA-dependent RNA Polymerase promoter.

FLORESCENT siRNA IN RICE (ORYZA SATIVA)- TIME COURSE EXOGENOUS

TREATED 24 H

TREATED 5.5 H

TREATED 3.5 H

TREATED 10 MIN

CONTROL

5

4

3

2

1

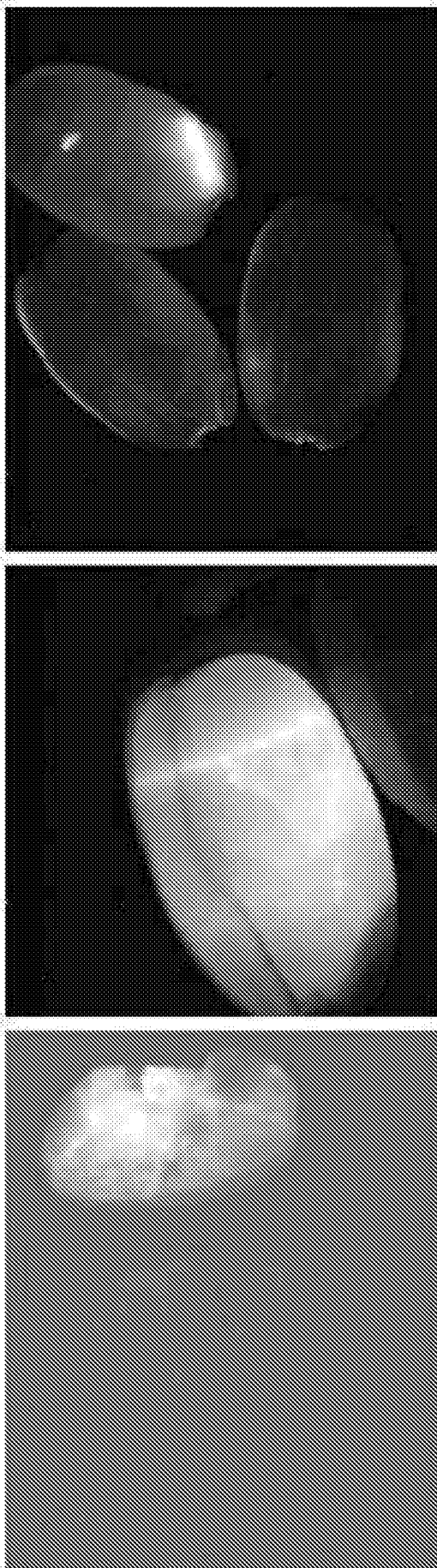


FIG. 1

SOLANUM LYCOPERSICON TAS3:

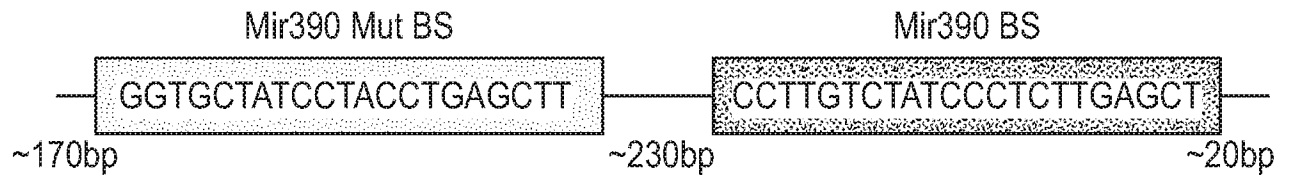


FIG. 2A

dsRNA CONSTRUCT #1 - EXOGENOUS TRIGGER CONTROL

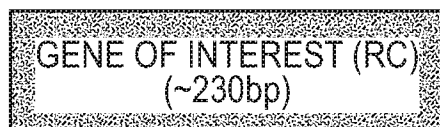


FIG. 2B

dsRNA CONSTRUCT #2 - DUAL Mir390BS
ON SENSE STRAND + EXOGENOUS SEQUENCE



FIG. 2C

3/22

dsRNA CONSTRUCT #3 - DUAL Mir390BS BOTH ON THE SENSE AND ANTISENSE STRANDS



FIG. 3

dsRNA CONSTRUCT #4 - miR390BS AS OVERHANGS
SENSE 5'->3'



ANTI SENSE 3'->5'



FIG. 4

dsRNA CONSTRUCT #5 - DUAL Mir390BS + HELICASE INSIDE

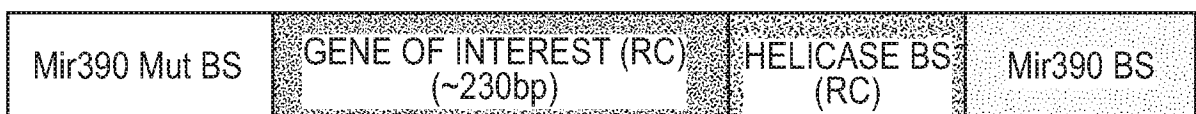


FIG. 5

4/22

dsRNA CONSTRUCT #6 - Mir390BS
ON BOTH STRANDS + HELICASE OVERHANG

SENSE 5'->3'



ANTI SENSE 3'->5'

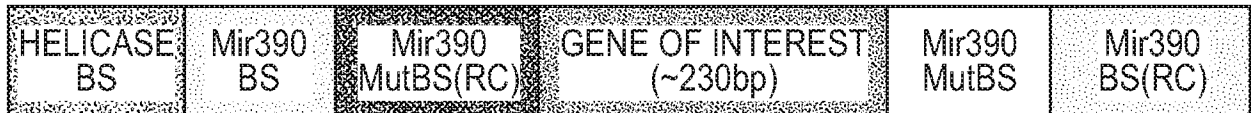


FIG. 6

dsRNA CONSTRUCT #7 - SENSE DUAL Mir390BS
COUPLED WITH ANTISENSE Mir4376BS



FIG. 7

dsRNA CONSTRUCT #8 - ENDOGENOUS TRIGGER CONTROL

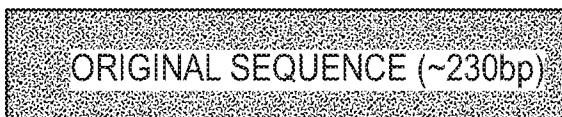


FIG. 8

dsRNA CONSTRUCT #9 - Mir390BS + ENDOGENOUS INSERT

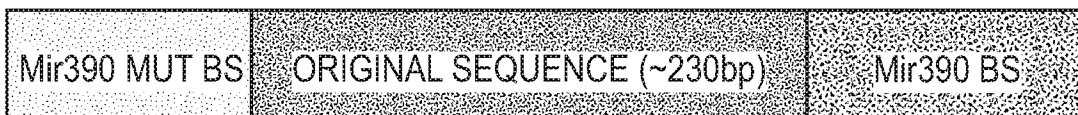


FIG. 9

5/22

* GFP234



FIG. 10A

* TASMir390

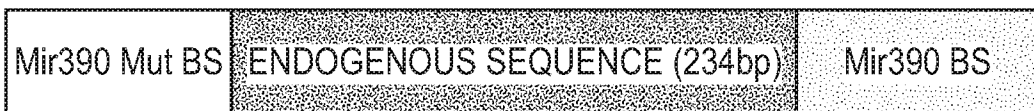


FIG. 10B

* GFP234Mir390 - Mir390BS ON SENSE STRAND



FIG. 10C

* GFP234Mir390 HELICASE

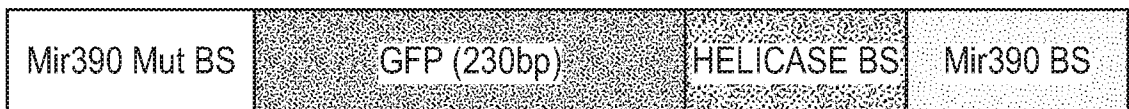


FIG. 10D

* GFP234Mir390_Mir4376

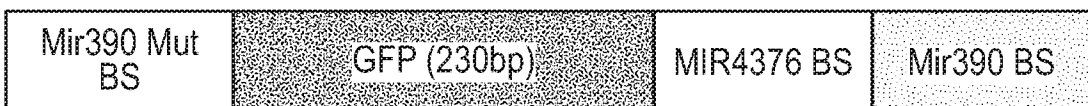


FIG. 10E

6/22

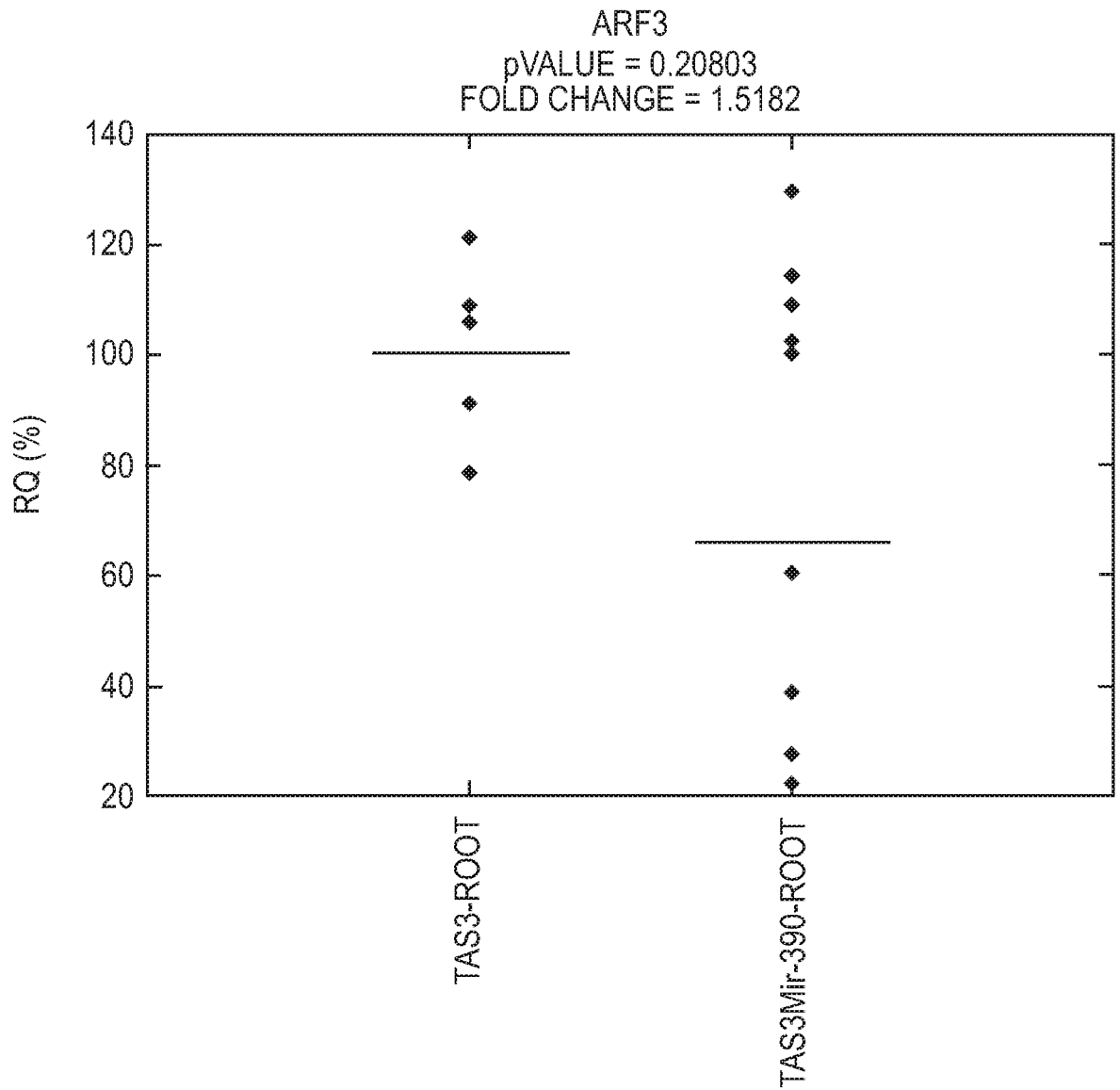


FIG. 11A

7/22

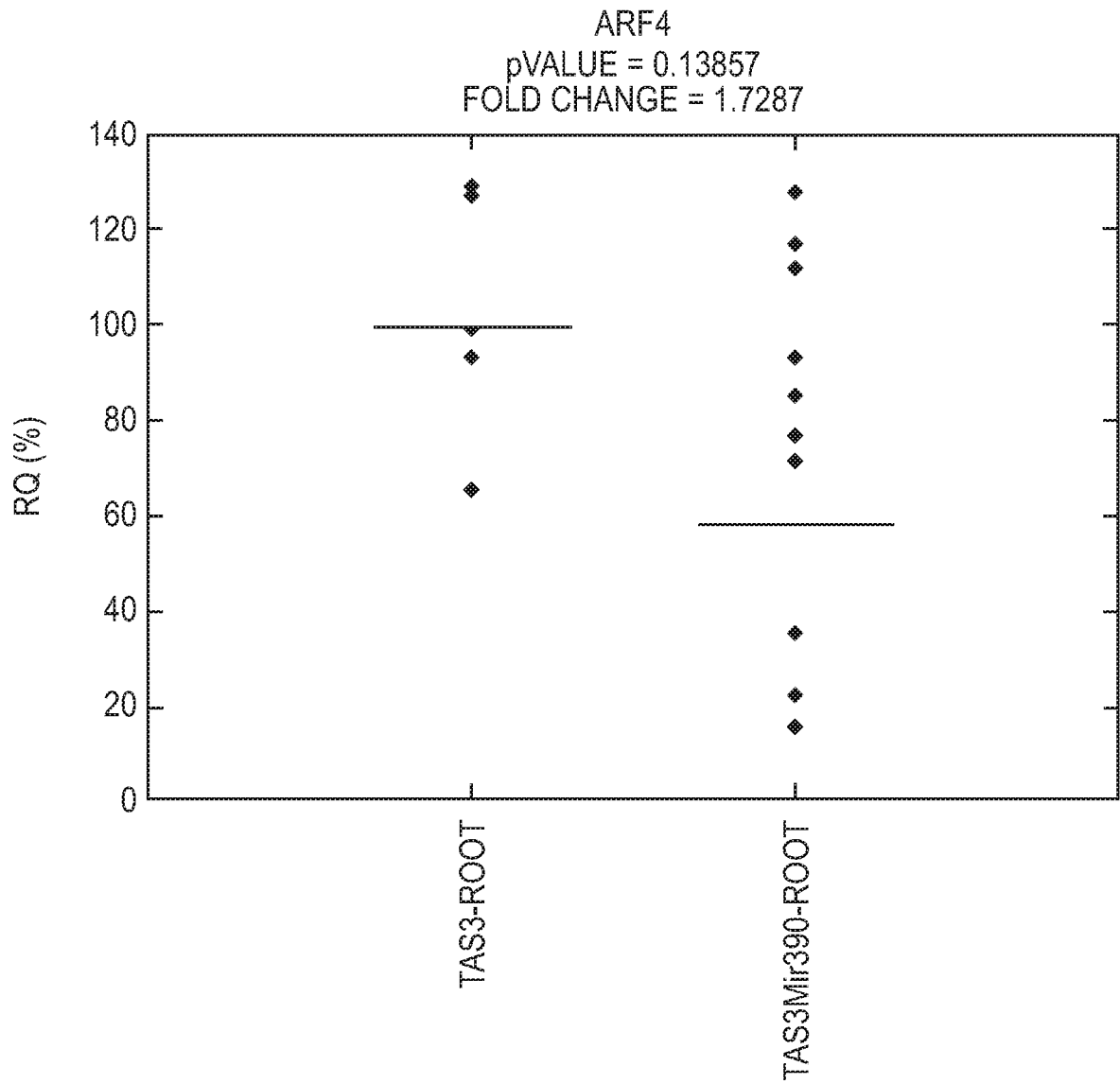


FIG. 11B

8/22

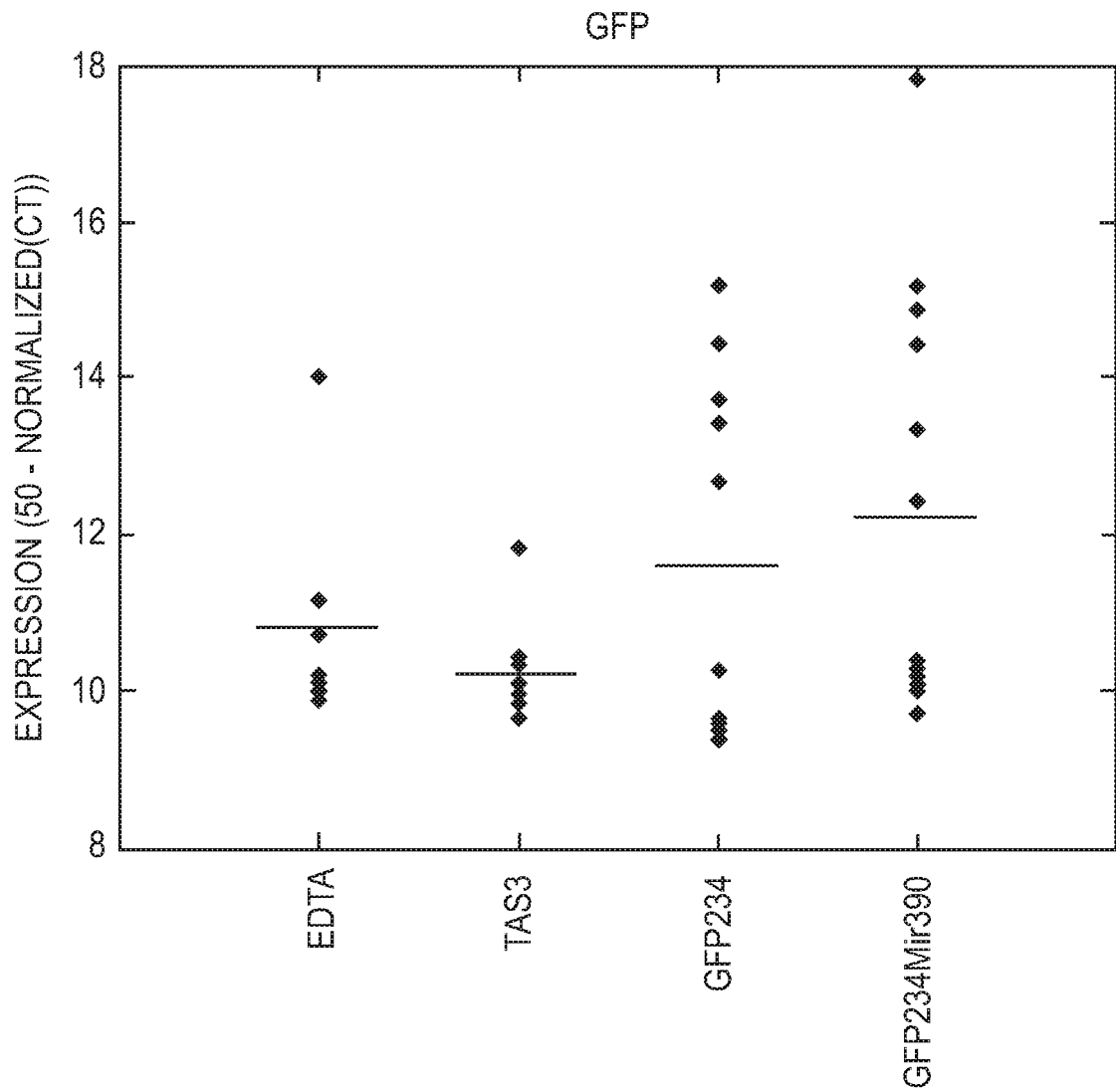


FIG. 11C

11/22

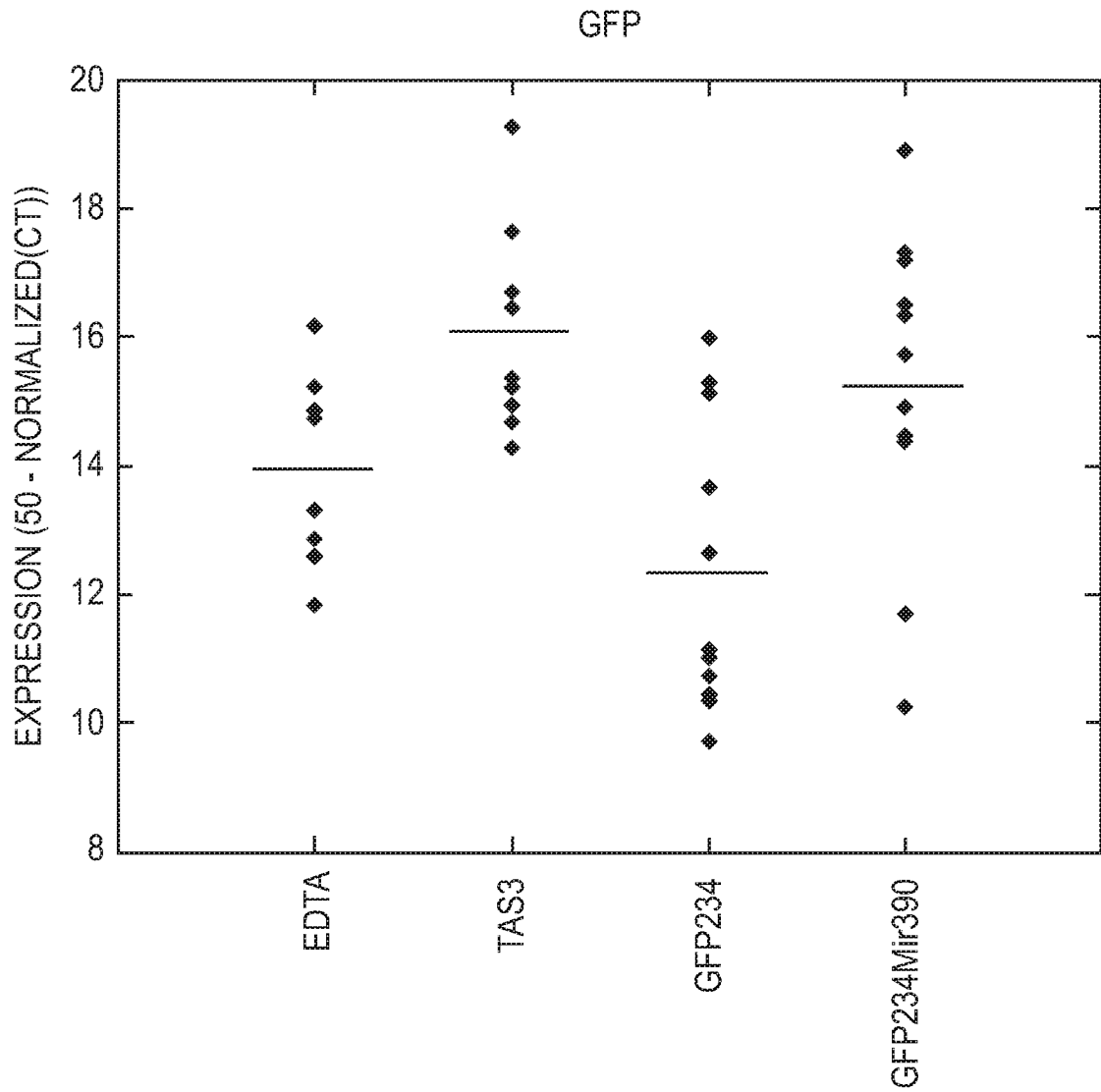


FIG. 11F

12/22

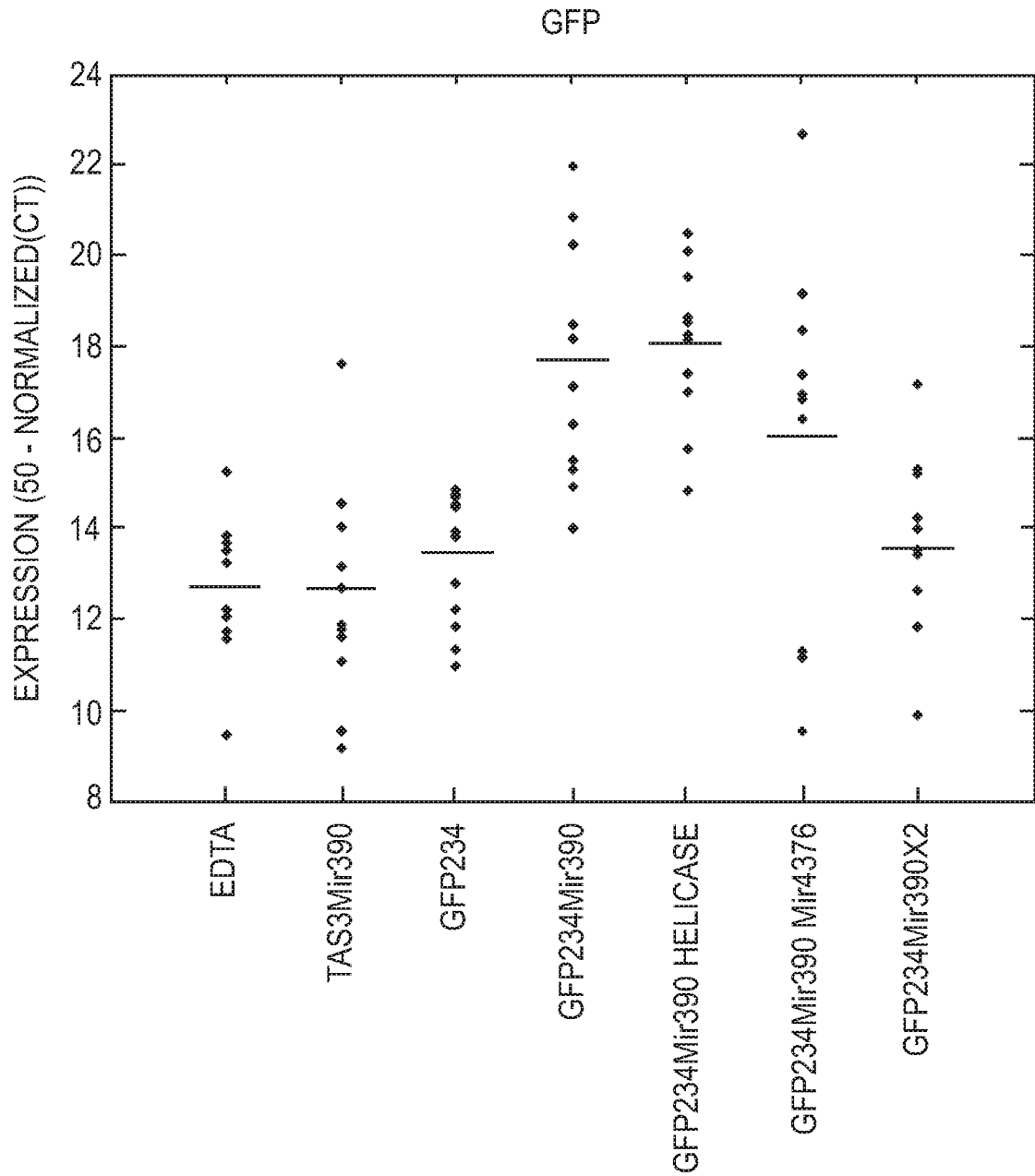


FIG. 12A

13/22

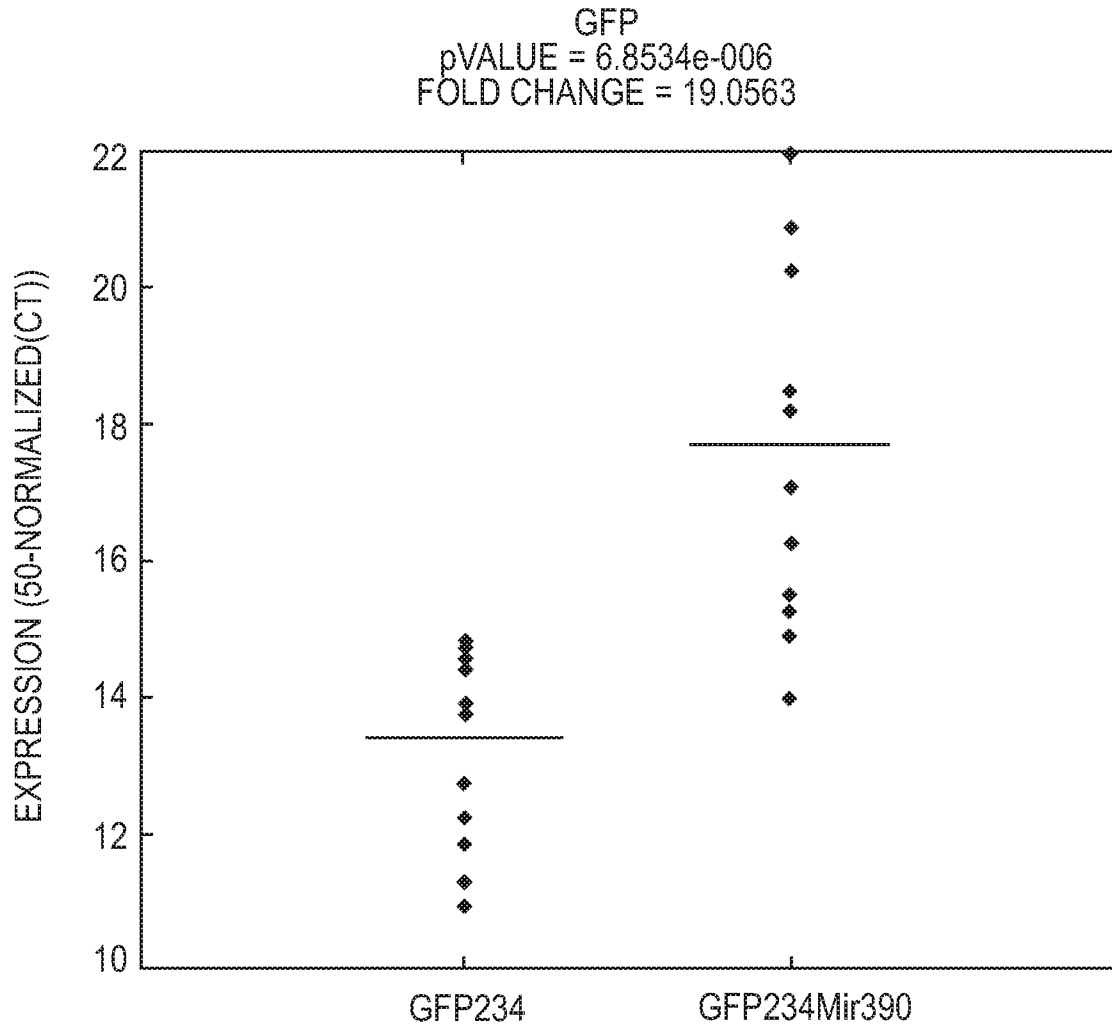


FIG. 12B

14/22

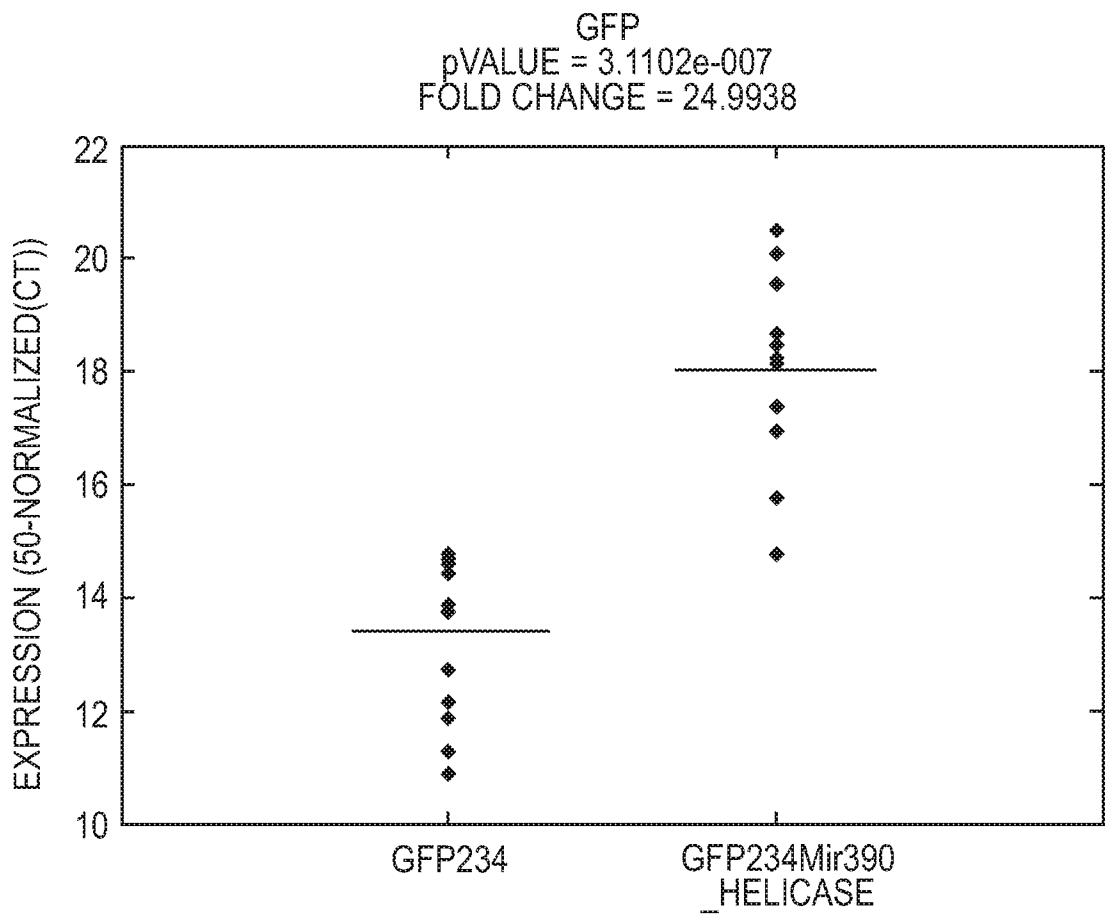


FIG. 12C

16/22

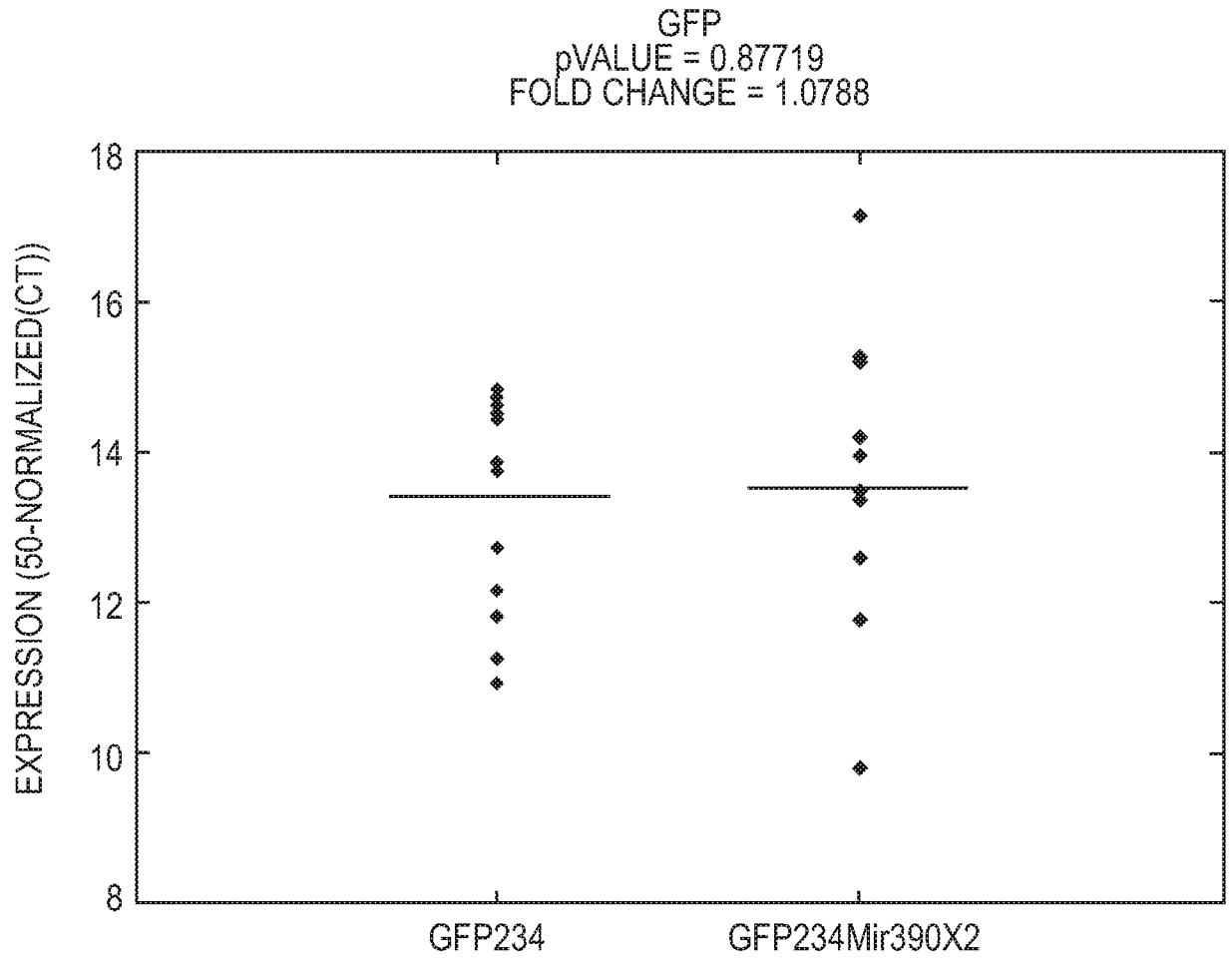


FIG. 12E

17/22

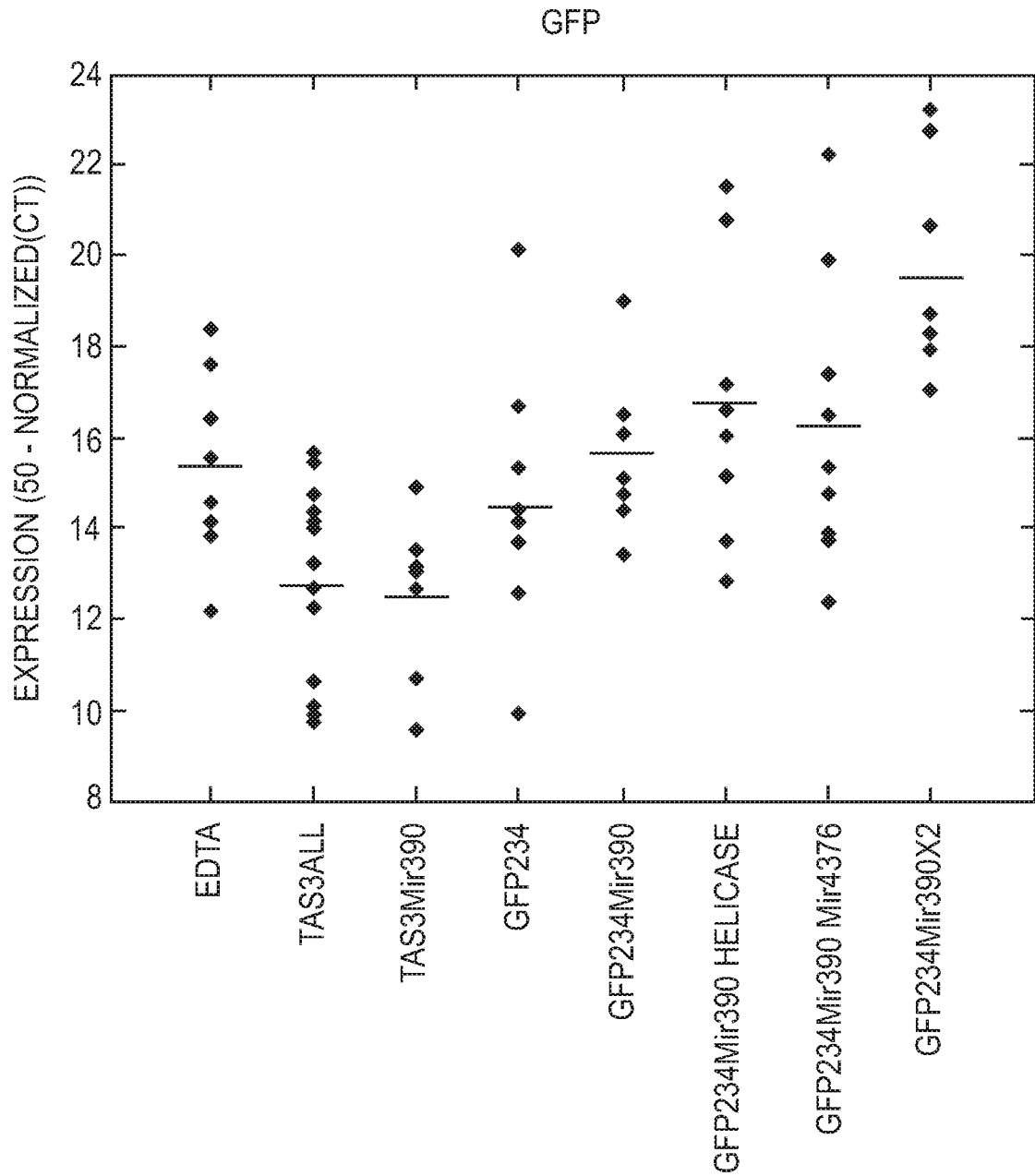


FIG. 13A

18/22

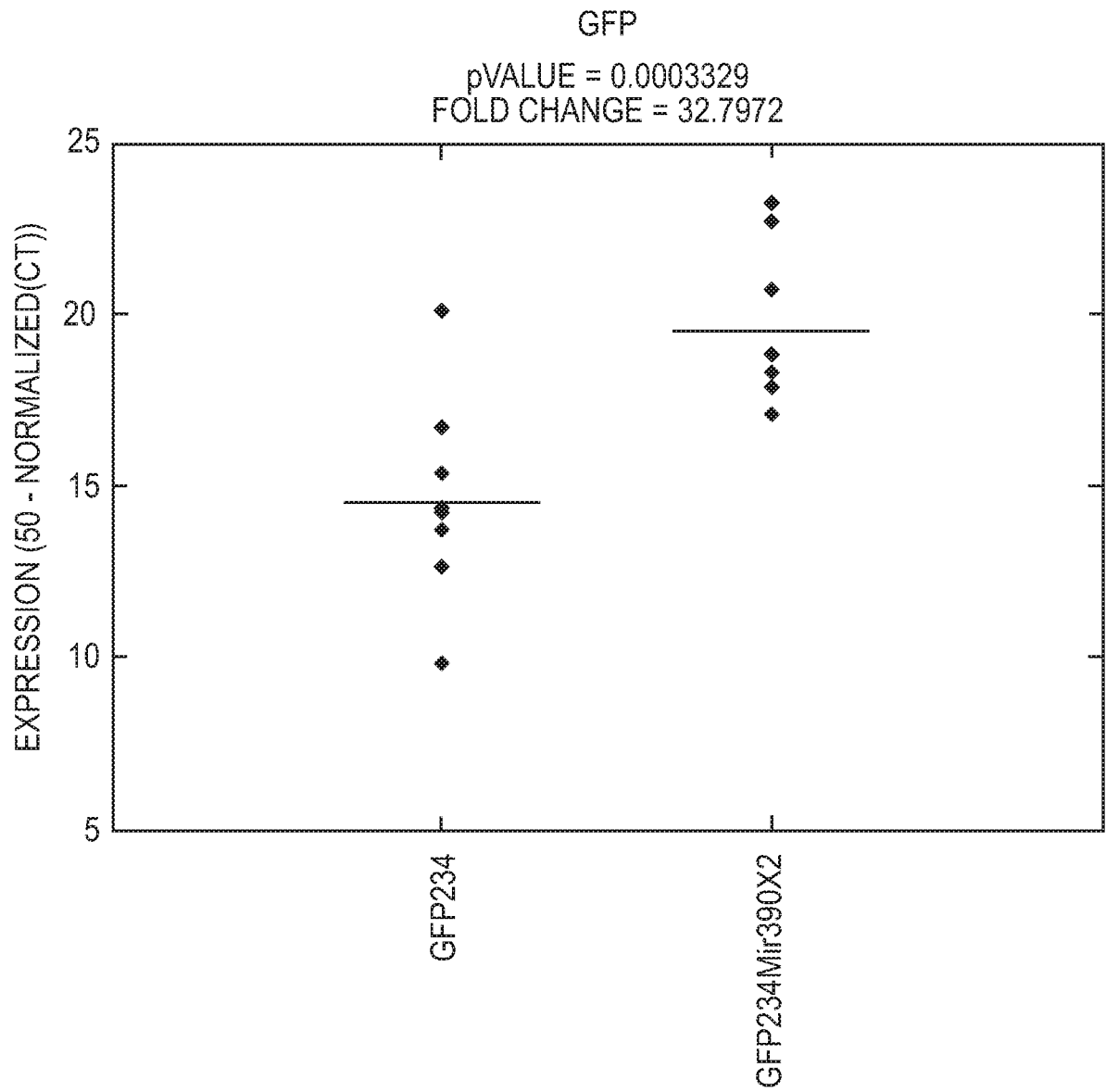


FIG. 13B

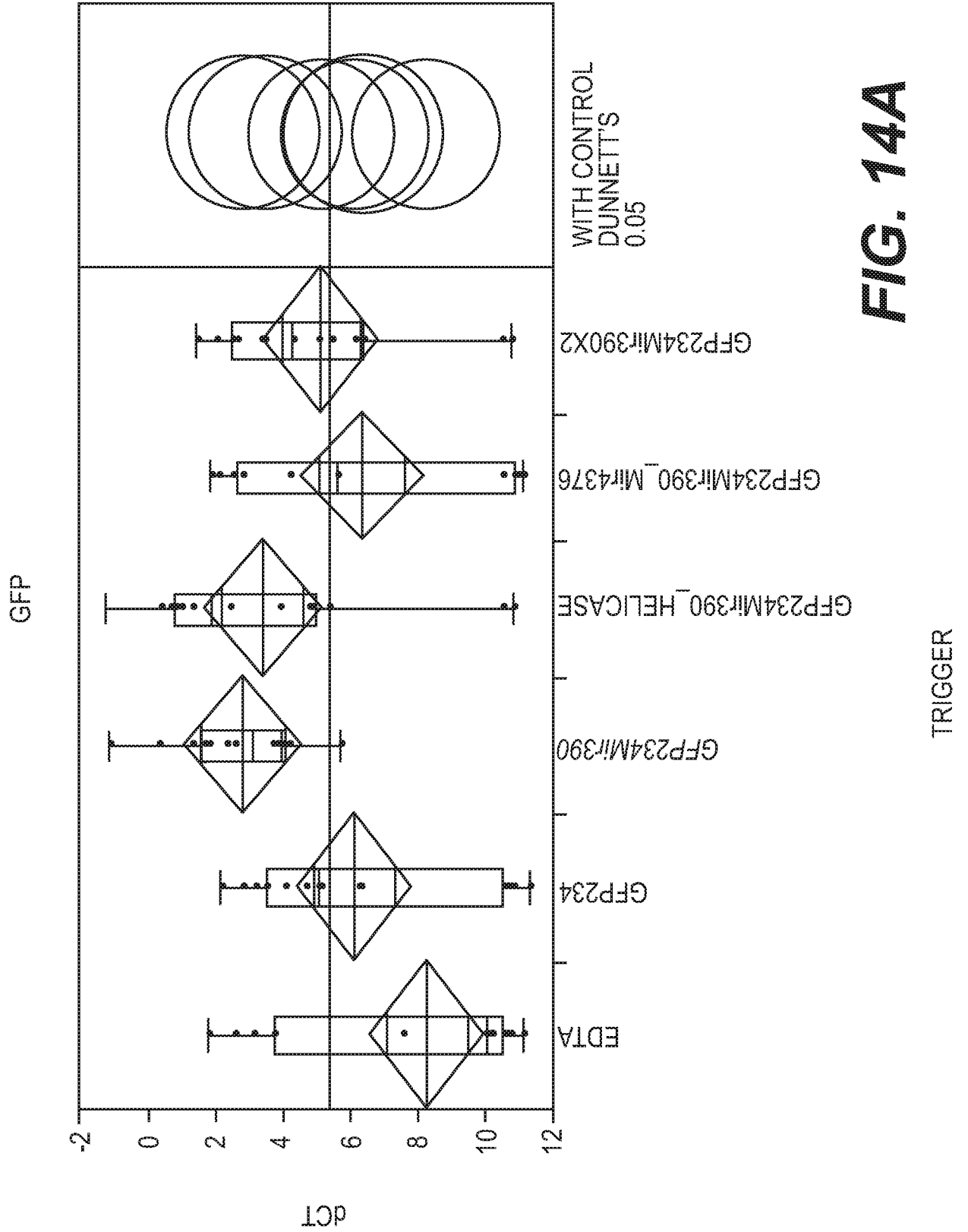


FIG. 14A

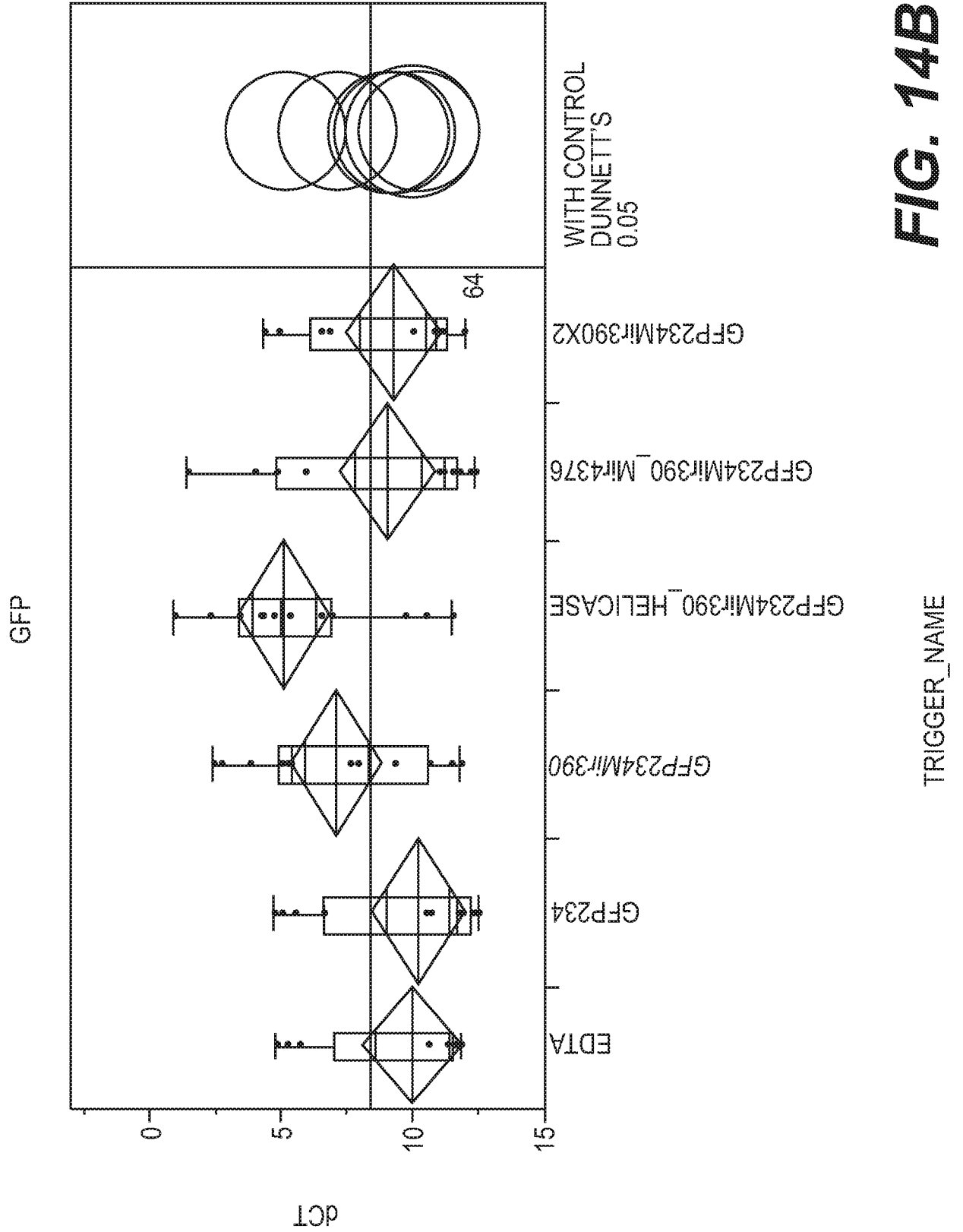
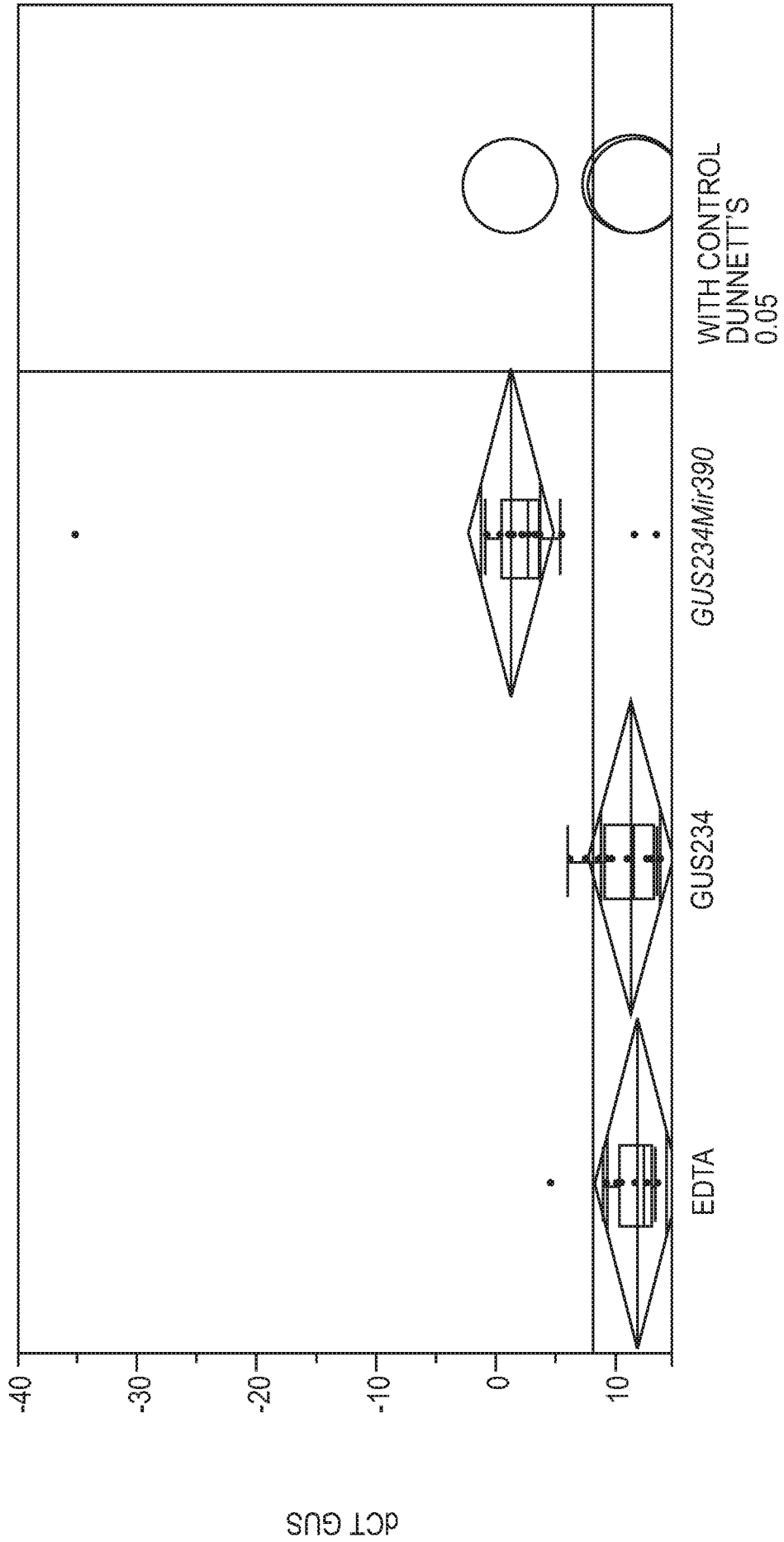


FIG. 14B

21/22

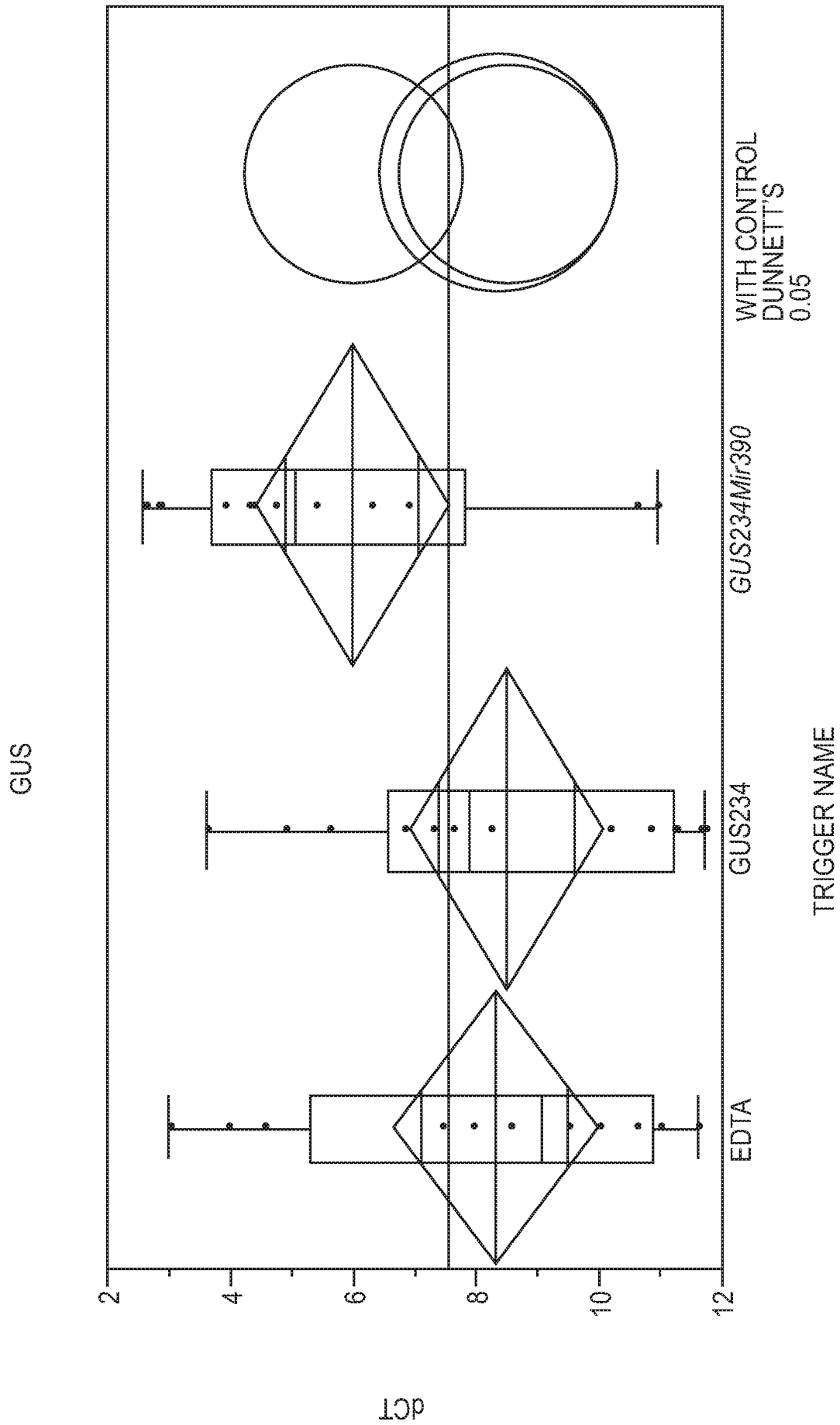
GUS



TRIGGER_NAME

FIG. 15A

22/22



WITH CONTROL
DUNNETT'S
0.05

FIG. 15B