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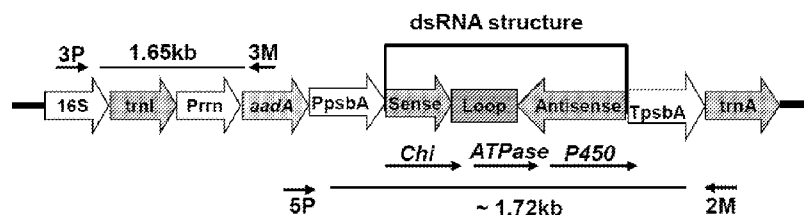


Fig. 1A

(57) Abstract: Compositions and methods for down-regulating genes through oral administration of RNAi molecule encapsulated in plant cells are provided.

**COMPOSITIONS AND METHODS THEREOF FOR DOWN-REGULATION OF
GENES FOLLOWING ORAL DELIVERY OF AN RNAi MOLECULE
BIOENCAPSULATED WITHIN PLANT CELLS**

BY

5

HENRY DANIELL

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Application No. 62/119,172, filed February 21, 2015, the entire disclosure being incorporated by reference herein as though set forth in full.

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**STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY
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FIELD OF THE INVENTION

The present invention relates to methods and compositions for down-regulating genes for conferring desired agronomic traits or for various biomedical applications, including oral delivery of RNAi effective to down-regulate dysfunctional genes associated with human disease.

BACKGROUND OF THE INVENTION

20 Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

In the past two decades, the concept of chloroplast genetic engineering has been advanced to achieve hyper-expression of foreign proteins, with recent advances in conferring
25 novel agronomic traits (Clarke and Daniell, 2011; Lee et al., 2011; Jin et al., 2012), biomass/biofuel enhancement (Verma et al., 2010, 2013; Agarwal et al., 2011; Jin et al., 2011) or

for various biomedical applications for oral delivery of vaccines (Davoodi-Semiromi et al., 2010; Lakshmi et al., 2013; Arlen et al., 2008), autoantigens (Ruhlman et al., 2007; Verma et al., 2010; Sherman et al., 2014) or biopharmaceuticals (Kwon et al., 2013 a, c; Ruhlman et al., 2010; Boyhan and Daniell, 2010; Kohli et al., 2014; Shenoy et al., 2014; Shil et al., 2014). While
5 application of RNAi technology through plant nuclear transformation has several limitations, delivery of small RNA prepared in more efficient systems as human therapeutics are also severely limited by their methods of delivery (Rothschild 2014). Chloroplast transformation offers several advantages over nuclear transformation including expression of large amounts of foreign proteins, up to 70% of the total leaf protein (De Cosa et al., 2001; Oey et al., 2009;
10 Ruhlman et al., 2010). This is possible due to the polyploidy nature of the plastid genetic system with up to 10,000 copies of the chloroplast genome in each plant cell, resulting in the ability to sustain a very high number of functional gene copies (Ruiz et al., 2011). In addition, transgene containment due to maternal inheritance, lack of transgene silencing and position effect are unique advantages of chloroplast genetic engineering (Verma and Daniell, 2007; Daniell, 2007;
15 Verma et al., 2008).

However, chloroplast has the exceptional ability to produce abundant transcripts (up to 200,000 copies per ng total RNA) but this genome has never been exploited to produce and deliver siRNA or miRNA or dsRNA. As described below, there is a great need to down regulate harmful genes to confer protection against various plant pests. In addition, down regulation of
20 dysfunctional genes causing cancer or autoimmune diseases or immune disorders in human health is highly desired. Due to the high level of chloroplast transcription, a large amount of the dsRNA could be synthesized and orally delivered via bioencapsulation in plant cells to target harmful genes (Kwon et al., 2013c). To the best of our knowledge, there is no report on the expression the dsRNAs via the chloroplast genome modification for this purpose.

25 Introduction of transgenic technology in crop plants has generated insect-resistant plants to reduce yield loss and pesticide utilization (Bale et al., 2008; Kos et al., 2009). For example, expression of *Bacillus thuringiensis* (Bt) Cry toxin in crop plants has resulted in great success both economically and ecologically (Qaim and Zilberman 2003; Wu et al., 2008). However, there is emerging population of insect resistance to biopesticides in transgenic crops and
30 outbreak of non-target pests (Bravo and Soberon, 2008; Gahan et al., 2001; Lu et al., 2010). Thus, new approaches which are more effective and environmental friendly are needed. Since

the discovery of dsRNA which could silence genes (Fire et al., 1998), RNA interference (RNAi) has been developed as an efficient and powerful tool in plants and animals to silence expression of harmful genes (Wesley et al., 2001; Aravin et al., 2001). Several insect genes have been down-regulated by injection of dsRNA (Bettencourt et al., 2002; Ohnishi et al., 2006). Oral
5 delivery of high concentrations of dsRNA in the artificial diet is required (Turner et al., 2006) because they are degraded in the digestive system. Bioencapsulation within plant cells should protect dsRNA and increase their efficacy. Therefore, it is important to develop an efficient method of delivery, which could be used for large scale pest control in the field. Several dsRNA specific to target genes of insects have shown protection from these herbivores (Mao et al., 2007;
10 Baum et al., 2007). But, advances in commercial development are limited by low abundance of siRNA transcripts.

DsRNA transgenic plant-mediated RNAi was used for silencing the hemipteran insect midgut genes and the results demonstrated the potential of dsRNA-mediated RNAi for field-level control of planthoppers (Zha et al., 2011). Viral vectors have been used to produce dsRNA in
15 *Nicotiana attenuata* to provide a transient and rapid silencing of midgut genes of the plant's lepidopteran herbivore, *Manduca sexta* (Kumar et al., 2012). The insect resistance is significantly improved in transgenic tobacco plants expressing dsRNA from the cotton bollworm (Zhu et al., 2012). Wuriyangan and Falk (2013) used tobacco mosaic virus (TMV) as vector to produce dsRNA in tobacco, tomato and tomatillo plants. Tomatillo plants infected with recombinant
20 TMV containing V-ATPase or *B. cockerelli* actin sequences decreased *B. cockerelli* progeny production. Recently, Xiong et al., (2013) developed transgenic tobacco plants expressing dsRNA for a molt-regulating transcription factor gene (HaHR3) and showed increase in cotton bollworm resistance. El-Shesheny et al., (2013) reported that silencing the abnormal wing disc gene of Asian citrus psyllid by direct application of dsRNA disrupted the adult wing
25 development and increased the nymph mortality. It has also been shown that silencing of the HaHMG-CoA reductase gene by RNAi inhibited oviposition of the cotton bollworm (Wang et al., 2013). Despite a decade of these studies (mostly in model systems), commercial development is limited by use of viruses or prohibitively expensive application of ds RNA in the field.

30 Cotton bollworm Cytochrome P450 monooxygenase gene located in the midgut of insects plays a central role in adaptation of the cotton metabolite-gossypol. The RNAi target to

P450 gene (CYP6AE14) has been expressed in tobacco and cotton (Hodgson et al., 1995; Mao et al., 2007, 2011). The feeding of plant cells expressing RNAi specific to Cytochrome P450 monooxygenase gene of cotton bollworm decreased transcript in the midgut, retarded larval growth and impaired cotton bollworm tolerance to the gossypol (Mao et al., 2007). Feeding of transgenic corn expressing dsRNAs target to V-ATPase gene showed a significant larval stunting and mortality (Baum et al., 2006). Pest chitin synthases (CHS) are key enzymes for trachea, cuticle and midgut development (Merzendorfer, 2006). They are encoded by CHSA and CHSB genes. CHSA genes are specifically expressed in ectodermal cells, including tracheal and epidermal cells, while CHSB genes are expressed specifically in gut epithelial cells that produce the peritrophic matrix of the midgut (Merzendorfer and Zimoch, 2003). It is well known that chitin is the main component of fungi and arthropods body skeleton, which is absent in vertebrates and plants (Zimoch et al., 2005). So, chitin synthases could be used as an ideal insect growth regulatory target for RNAi technology (Chen et al., 2008; Tian et al., 2009).

For most nuclear transformation of biopesticide genes, including those currently under commercial cultivation, the expression level is less than 1% of the total soluble protein (Ripoll et al. 2003; Saha et al., 2006). However, chloroplast transformation has resulted in much higher levels of expression and greater protection against resistant insects (Kota et al., 1999; DeCosa et al., 2001; Dufourmantel et al., 2007; Jin et al., 2011, 2012).

Since its recent discovery, siRNA has rapidly become the standard tool for gene silencing in vitro. siRNA is able to silence genes to below detectable levels with the delivery of only a small number of molecules to a cell. A well-designed siRNA can target a mutated diseased gene while sparing the wild-type counterpart, making it a highly effective therapeutic option in terms of efficacy and specificity. Billions of dollars have been spent developing a delivery mechanism for siRNA, but the major remaining hurdle is a safe and efficient delivery system. A recent seminal study demonstrated that plant miRNAs are capable of surviving the conditions of the digestive tract, entering circulation, and silencing gene expression through a plant diet. Other recent findings, such as the discovery of specific subsets of miRNA in the microvesicles of breast milk, suggest that there may be an innate pathway for uptake and processing of miRNAs through the digestive tract.

Since the 1971 National Cancer Act, billions of dollars have been invested in cancer research, yet progress in therapeutics has been relatively limited. Despite intensive efforts,

cancer remains one of the leading and most devastating causes of death in the western world. Traditional chemotherapy targets dividing cells non-specifically, making the killing of cancer cells while sparing essential non-cancerous tissue a significant challenge. Newer, targeted therapies such as those using monoclonal antibodies offer a high degree of specificity, but are
5 dependent in their action and lethality upon binding a target. Many of the most significant cancer targets are currently undruggable due to lack of extracellular targets for antibody binding or binding pockets for small molecules. There remains a great need for specific targeting of oncogenes in cancer cells.

10 SUMMARY OF THE INVENTION

In accordance with the present invention, recombinant plant plastid and viral expression vectors are disclosed which encode silencing RNA molecules which down modulate target genes of interest. Exemplary vectors comprise, as operably linked components in the 5' to 3' direction of translation, a promoter operable in said plant, a heterologous polynucleotide sequence coding
15 for at least one RNAi molecule selected from the group comprising dsRNA, siRNA, shRNA, and miRNA and a transcription terminator sequences functional in said plant, wherein the vector optionally encodes a nucleic acid encoding a selectable marker. In a particularly preferred embodiment of the invention, the vector comprises a heterologous polynucleotide sequence coding for at least one RNAi molecule that down-regulates a target gene, including, without
20 limitation, a P450 monooxygenase gene, a Vac ATPase A gene, and a chitin synthase B gene.

In yet another embodiment the heterologous polynucleotide is an RNAi molecule that down-regulates a dysfunctional gene causing or regulating cancer or immune disorders, particularly autoimmune disorders. In a particularly preferred embodiment, the RNAi down regulates K-ras and is useful for the treatment of cancer, particularly pancreatic cancer.

25 Transformed edible plant cells, plants derived therefrom and plant portions harvested from such plants also comprise an aspect of the invention and are useful in methods of treatment of cancer and immune disorders. Plants or plant portions within the scope of the invention, include for example, maize, lettuce, rice, grass, rye, barley, oat, wheat, soybean, peanut, grape, potato, sweet potato, pea, canola, tobacco, tomato or cotton.

In yet another aspect of the invention, a method of treating cancer or an immune disorder is provided. An exemplary method comprises oral administration to a subject an edible plant or plant portion comprising a therapeutically effective amount of an RNAi molecule expressed from a plant chloroplast selected from the group comprising dsRNA, siRNA, shRNA, and miRNA, said RNAi molecules targeting a gene which aberrantly causes or regulates the cancer or immune disorder. In a preferred embodiment of the method, the RNAi molecule down-regulates a cancer oncogene such as K-ras.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-Fig. 1F. Chloroplast dsRNA vectors, evaluation of transgene integration and homoplasmy. **Fig. 1A** shows the pLD-dsRNA chloroplast transformation vector map. The dsRNA structure for the target genes is the typical "sense-loop-antisense" structure. For the P450 gene, the sequence of stem-loops is shown as below :

"GCAACGAGGTCGAAATGAGTTCAAGAGACTCATTTCGACCTCGTTGC" (SEQ ID NO:

1). For the Chi and ATPase genes, the RNAi vectors also have the similar "sense-loop-antisense" structures. The sizes of these "sense-loop-antisense" structures are ~70 nt. **Fig. 1B** are schematic representations of the chloroplast flanking sequences used for homologous recombination, probe DNA sequence (0.81 kb), when digested with SmaI. **Fig. 1C** shows a PCR analysis of the wild type and transplastomic lines using the primer pair 3P/3M (Upper) and 5P/2M (Lower). Primer annealing sites are shown in 1A. Lanes 1-5: P450:RNAi ; 5-10: Chi: RNAi; 10-15 ATPase: RNAi transplastomic lines. P: positive control; N: negative control. M: DNA marker. **Fig. 1D** shows a Southern blot analysis of P450: RNAi transplastomic lines hybridized with chloroplast flanking sequence probe (WT, wild type and 11 transplastomic lines). **Fig. 1E** shows a Southern blot analysis of Chi: RNAi transplastomic lines (WT, wild type; 1-5, transplastomic lines). **Fig. 1F** shows a Southern blot analysis of ATPase: RNAi lines (WT, wild type; A2, A4 are negative lines; A5, B1 and B3 are heteroplasmic lines; A1, A3, A7, A8, B2, B4, A6 and C1 are homoplasmic lines).

Fig. 2A-Fig. 2E. - Northern blot and Real-Time qRT-PCR analysis of ds RNA in transplastomic plants: For the northern blot, equal amount of 2 µg total RNA of every sample was loaded and separated on 1.0 % denaturing agarose gel. The PCR amplified product from 3' psbA UTR was

used as probe for northern blot. **Fig. 2A** show hybridization signals of dsRNAs (**Fig. 2A** is unprocessed and **Fig. 2B** is cleaved) were detected in P450: RNAi transplastomic lines (2.3, 2.4, 2.6, 2.11 and 10.4), whereas the cleaved dsRNA product was absent in wild type plants. **Fig. 2C** shows hybridization signals of dsRNAs (unprocessed and cleaved) were detected in A1, A3, A6, B2, B4, B5 and B14 ATPase: RNAi transplastomic lines whereas the cleaved dsRNA product was absent in wild type. **Fig. 2D** are hybridization signals of dsRNAs were detected in 1-5 RNAi: Chi transplastomic lines whereas the cleaved dsRNA product was absent in wild type plants. Note: the expected dsRNAs were indicated by the arrow in figure 2A-C. **Fig. 2E** shows a transcript abundance of dsRNA in the wild type and two independent transplastomic lines-2.6 and 10.4.

Fig. 3A-Fig. 3B. Strategy for P450: RNAi transplastomic plant insect bioassay. Two steps are needed for this insect bioassay. In the first step, third-instar larvae of *Helicoverpa armigera* were fed on P450: RNAi leaves for 4 days. **Fig. 3A** - In this step, most of the P450 transcripts should be degraded siRNA. **Fig. 3B** - Then, these larvae were transferred to the following diet or leaves: artificial diet with 3mg/g gossypol or without gossypol; cotton leaves with glands; glandless cotton leaf and wild type tobacco leaf. In the second step, the gossypol in the artificial diet or leaves should affect larval growth and development. The weight change of larvae was measured at this step.

Fig. 4A-Fig. 4B. Effect of gossypol and siRNA on larval growth. The third- instar larvae were fed on P450: RNAi leaves for 4 days and were then transferred to the artificial diet or leaves (see the protocol in Figure 3). **Fig. 4A** shows a net weight increase of larvae reared on the artificial diet supplemented with gossypol (1 to 4 mg/g). The third-instar larvae were fed on the artificial diet for 5 days and then the weight increase was measured. **Fig. 4B** shows a net weight increase of larvae reared on artificial diet with (3mg/g) or without gossypol, cotton leaves with or without glands and wild type tobacco leaves. Net weight increase values consist of means $\pm$ standard deviation. Asterisk indicates $P < 0.05$ compared with wild type control group.

Fig. 5A-Fig. 5B. Quantity of P450 transcript in the midgut of tested larvae after feeding on the wild type and transplastomic leaves. The third- instars larvae were fed on transplastomic P450:

RNAi or wild type tobacco leaves for 4 days or 7 days and then the midgut of these insects were isolated under stereomicroscope and then washed with ddH₂O to remove all the debris. The cleaned midgut tissues were stored in 70% ethanol at - 20°C. RNA was isolated from these midgut tissues and used for Real-Time qRT-PCR analysis. **Fig. 4A** shows a P450 transcription level of the tested larvae fed with wild type and two transplastomic line L2.6 and L10.4 for 4 days. **Fig. 4B** shows a P450 transcription level of the tested larvae fed with wild type and two transplastomic lines L2.6 and L10.4 for 7 days. The primers used for quantification of dsRNAs of P450 in the transplastomic plants leaves were: upstream primer (5'-AGAGACTCATTTCGACCTCGTTGCT-3; SEQ ID NO: 2) and downstream primer (5'-GGAGCAATAGCACCTCTTGATAGAA-3'; SEQ ID NO: 3). The upstream primer anneals with P450 and the downstream primer anneals with TpsbA. All the results were obtained from at least three independent biological replicates. Transcription values consist of means $\pm$ standard deviation. Asterisk indicates $P < 0.05$ when compared with wild type control group. Two asterisks indicate $P < 0.01$ when compared with wild type control group.

Fig. 6A-Fig. 6B. Net weight changes of *Helicoverpa armigera* feeding on transplastomic leaves expressing ATP and Chi dsRNAs. **Fig. 6A** shows a net weight increase of *Helicoverpa armigera* fed with ATP: RNAi transplastomic tobacco leaves for 4 days. **Fig. 6B** shows a net weight increase of *Helicoverpa armigera* fed with Chi: RNAi transplastomic tobacco leaves for 4 days; Net weight increase values consist of means $\pm$ standard deviation. Asterisk indicates $P < 0.05$ compared with wild type control group.

Fig. 7A-Fig. 7D. Larval development and insect mortality bioassay. **Fig. 7A** shows a pupation rate of larvae fed with P450: RNAi tobacco leaves transferred to different diets or leaves. Gossypol: artificial diet containing 3 mg/g gossypol; No gossypol: artificial diet without gossypol; Glanded: glanded cotton leaves; Glandless: cotton leaves without glands; WT: wild type tobacco leaves. **Fig. 7B-Fig. 7C** shows a pupation rate of larvae after feeding on ATP: RNAi and Chi: RNAi tobacco leaves. **Fig. 7D** shows a larval mortality and pupal phenotype. 1: Pupation failure led to mortality after feeding artificial diet containing 3 mg/g gossypol; 2: dead larva after feeding glanded cotton leaves; 3: dead larva after feeding with ATP: RNAi tobacco leaves; 4: dead larva after feeding with Chi: RNAi tobacco leaves; 5: successful pupation of

insect fed on wild type tobacco leaves all the time during the bioassay. Pupation values consist of means $\pm$ standard deviation. Asterisk indicates $P < 0.05$ compared with wild type control group.

Fig. 8. Experimental design: shRNA oligos inserted into pGreen vector, co-transformed into Agrobacterium with pSoup helper vector, introduced into *Nicotiana Benthamiana* by Agrobacterium infiltration, and fed to GFP mice by oral gavage.

Fig. 9. Optimization of the shRNA stem-loop structure.

Fig. 10. Modified shRNA constructs to contain higher AT content.

Fig. 11. shRNA constructs optimized to contain a 2nt TT 3' overhang.

Fig. 12. Example of GFP shRNA constructed by oligo annealing for insertion into pGreen vector.

Fig. 13. Example of stem-loop primer design. 6nt segment overlaps with 3' end of mature miRNA.

Fig. 14A-Fig. 14B. Design of **Fig. 14A** 'chimeric' structure of miR156a plant miRNA backbone with GFP targeting sequence substituted (blue box) and **Fig. 14B** GFP shRNA construct.

Fig. 15: GFP shRNA sequence confirmed by DNA sequence of the final vector.

Fig. 16: DNA sequence confirmation of the final KrasG12D2 transformation vector.

Fig. 17: DNA sequence confirmation of the final Kras transformation vector

DETAILED DESCRIPTION OF THE INVENTION

In the past two decades, chloroplast genetic engineering has been advanced to achieve high level protein accumulation but not for down-regulation of targeted genes. Therefore, in this report, lepidopteran chitin synthase (Chi), cytochrome P450 monooxygenase (P450) and V-ATPase dsRNAs were expressed via the chloroplast genome to study RNA interference (RNAi) of target genes in intended hosts. PCR and Southern blot analysis confirmed homoplasmy and site-specific integration of transgene cassettes into the chloroplast genomes. Northern blots and Real-Time qRT-PCR confirmed abundant processed and unprocessed dsRNA transcripts (up to 3.45 million copies of P450 dsRNAs/ μ g total RNA); the abundance of cleaved dsRNA was greater than the endogenous psbA transcript. Feeding of leaves expressing P450, Chi and V-ATPase dsRNA decreased transcription of the targeted gene to almost undetectable levels in the insect midgut, likely after further processing of dsRNA in their gut. Consequently, the net weight of larvae, growth and pupation rates were significantly reduced by chloroplast-derived dsRNAs. Taken together, successful expression of dsRNAs via the chloroplast genome for the first time opens the door to study RNA interference/processing within plastids. Most importantly, dsRNA expressed in chloroplasts can be utilized for gene inactivation to confer desired agronomic traits or for various biomedical applications, including down regulation of dysfunctional genes responsible for a variety of different medical disorders, including without limitation, cancer and autoimmune disorders, after oral delivery of dsRNA bioencapsulated within plant cells.

Oral administration is the preferred route of drug delivery due to ease of administration and patient compliance. A plant expression system would make production of drugs inexpensive and increase access of medicine to patients unable to afford current therapies. Successful delivery of RNAi would also allow targeting of currently undruggable targets such as inflammatory genes, oncogenes (ie. KRAS) and transcription factors, and could be used as a preventative measure in many diseases.

DEFINITIONS

For purposes of the present invention, "a" or "an" entity refers to one or more of that entity; for example, "a cDNA" refers to one or more cDNA or at least one cDNA. As such, the terms "a" or "an," "one or more" and "at least one" can be used interchangeably herein. It is also noted that the terms "comprising," "including," and "having" can be used interchangeably. Furthermore, a compound "selected from the group consisting of" refers to one or more of the

compounds in the list that follows, including mixtures (i.e. combinations) of two or more of the compounds.

According to the present invention, an isolated, or biologically pure molecule is a compound that has been removed from its natural milieu. As such, "isolated" and "biologically
5 pure" do not necessarily reflect the extent to which the compound has been purified. An isolated compound of the present invention can be obtained from its natural source, can be produced using laboratory synthetic techniques or can be produced by any such chemical synthetic route.

The term "genetic alteration" as used herein refers to a change from the wild-type or reference sequence of one or more nucleic acid molecules. Genetic alterations include without
10 limitation, base pair substitutions, additions and deletions of at least one nucleotide from a nucleic acid molecule of known sequence.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular
15 modifications that would not affect the functional and novel characteristics of the sequence.

With regard to nucleic acids used in the invention, the term "isolated nucleic acid" is sometimes employed. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the
20 "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryote or eukaryote.

An "isolated nucleic acid molecule" may also comprise a cDNA molecule. An isolated nucleic acid molecule inserted into a vector is also sometimes referred to herein as a recombinant nucleic acid molecule.

25 With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form.

By the use of the term "enriched" in reference to nucleic acid it is meant that the specific DNA or RNA sequence constitutes a significantly higher fraction (2-5 fold) of the total DNA or RNA present in the cells or solution of interest than in normal cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that "enriched" does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been significantly increased.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment (compared to the natural level, this level should be at least 2-5 fold greater, e.g., in terms of mg/ml). Individual clones isolated from a cDNA library may be purified to electrophoretic homogeneity. The claimed DNA molecules obtained from these clones can be obtained directly from total DNA or from total RNA. The cDNA clones are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection of the cells carrying the cDNA library. Thus, the process which includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones yields an approximately 1-6-fold purification of the native message. Thus, purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. Thus, the term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest.

The term "complementary" describes two nucleotides that can form multiple favorable interactions with one another. For example, adenine is complementary to thymine as they can form two hydrogen bonds. Similarly, guanine and cytosine are complementary since they can form three hydrogen bonds. Thus if a nucleic acid sequence contains the following sequence of

bases, thymine, adenine, guanine and cytosine, a "complement" of this nucleic acid molecule would be a molecule containing adenine in the place of thymine, thymine in the place of adenine, cytosine in the place of guanine, and guanine in the place of cytosine. Because the complement can contain a nucleic acid sequence that forms optimal interactions with the parent nucleic acid molecule, such a complement can bind with high affinity to its parent molecule.

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single stranded nucleic acid molecules of varying complementarity are well known in the art. For instance, one common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989): $T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$.

As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C . The T_m of a DNA duplex decreases by $1-1.5^{\circ}\text{C}$ with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C . The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are $20-25^{\circ}\text{C}$ below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately $12-20^{\circ}\text{C}$ below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 $\mu\text{g/ml}$ denatured salmon sperm DNA at 42°C , and

washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in IX SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X
5 Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

The term "oligonucleotide" or "oligo" as used herein means a short sequence of DNA or DNA derivatives typically 8 to 35 nucleotides in length, primers, or probes. An oligonucleotide can be derived synthetically, by cloning or by amplification. An oligo is defined as a nucleic
10 acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide. The term "derivative" is intended to include any of the above described variants when comprising an additional chemical moiety not normally a part of these molecules. These chemical moieties can have varying purposes including, improving
15 solubility, absorption, biological half-life, decreasing toxicity and eliminating or decreasing undesirable side effects.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a
20 nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are
25 selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the
30 probe, with the remainder of the probe sequence being complementary to the target strand.

Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either
5 single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions
10 such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient
15 complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence
20 may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

25 Polymerase chain reaction (PCR) has been described in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

An "siRNA" refers to a molecule involved in the RNA interference process for a sequence-specific post-transcriptional gene silencing or gene knockdown by providing small interfering RNAs (siRNAs) that has homology with the sequence of the targeted gene. Small
30 interfering RNAs (siRNAs) can be synthesized in vitro or generated by ribonuclease III cleavage from longer dsRNA and are the mediators of sequence-specific mRNA degradation. Preferably,

the siRNA of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Applied Biosystems (Foster City, CA, USA), Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK). Specific siRNA constructs may be between 15-35 nucleotides in length, and more typically about 21 nucleotides in length.

The term "vector" relates to a single or double stranded circular nucleic acid molecule that can be infected, transfected or transformed into cells and replicate independently or within the host cell genome. A circular double stranded nucleic acid molecule can be cut and thereby linearized upon treatment with restriction enzymes. An assortment of vectors, restriction enzymes, and the knowledge of the nucleotide sequences that are targeted by restriction enzymes are readily available to those skilled in the art, and include any replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element. A nucleic acid molecule of the invention can be inserted into a vector by cutting the vector with restriction enzymes and ligating the two pieces together.

Many techniques are available to those skilled in the art to facilitate transformation, transfection, or transduction of the expression construct into a prokaryotic or eukaryotic organism. The terms "transformation", "transfection", and "transduction" refer to methods of inserting a nucleic acid and/or expression construct into a cell or host organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt, an electric field, or detergent, to render the host cell outer membrane or wall permeable to nucleic acid molecules of interest, microinjection, peptide-tethering, PEG-fusion, and the like. The term "promoter element" describes a nucleotide sequence that is incorporated into a vector that, once inside an appropriate cell, can facilitate transcription factor and/or polymerase binding and subsequent transcription of portions of the vector DNA into mRNA. In one embodiment, the promoter element of the present invention precedes the 5' end of a nucleic acid molecule that encodes a dsRNA. Host cell machinery then translates mRNA into a polypeptide.

Those skilled in the art will recognize that a nucleic acid vector can contain nucleic acid elements other than the promoter element and the dsRNA encoding nucleic acid molecule. These other nucleic acid elements include, but are not limited to, origins of replication, ribosomal binding sites, nucleic acid sequences encoding drug resistance enzymes or amino acid metabolic enzymes, and nucleic acid sequences encoding secretion signals, localization signals, or signals useful for polypeptide purification.

A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, plastid, phage or virus that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radio immunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like. The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The terms "recombinant organism," or "transgenic organism" refer to organisms which have a new combination of genes or nucleic acid molecules. A new combination of genes or nucleic acid molecules can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. The term "organism" relates to any living being comprised of a least one cell. An organism can be as simple as one eukaryotic cell or as complex as a mammal. Therefore, the phrase "a recombinant organism" encompasses a recombinant cell, as well as eukaryotic and prokaryotic organism.

The term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, addition of stabilizers, or compounding into, for example, immunogenic preparations or pharmaceutically acceptable preparations.

A "specific binding pair" comprises a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which in normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies, ligands and receptors and complementary nucleotide sequences. The skilled person is aware of many other examples. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a large molecule. In embodiments in which the specific binding pair comprises nucleic acid sequences, they will be of a length to hybridize to each other under conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

The terms "agent" and "test compound" are used interchangeably herein and denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Biological macromolecules include siRNA, shRNA, antisense oligonucleotides, small molecules, antibodies, peptides, peptide/DNA complexes, and any nucleic acid based molecule.

In certain embodiments of the invention, the dsRNAi produced as described herein will be administered to a patient in combination with conventional chemotherapeutic agents to increase beneficial therapeutic effects. Such agents include, for example, paclitaxel (Taxol®), cisplatin, docetaxol, carboplatin, vincristine, vinblastine, methotrexate, cyclophosphamide, CPT-11, 5-fluorouracil (5-FU), gemcitabine, estramustine, carmustine, adriamycin (doxorubicin), etoposide, arsenic trioxide, irinotecan, and epothilone derivatives.

The following materials and methods are provided to facilitate the practice of the present invention.

Generation and molecular characterization of transplastomic plants

Young tobacco leaves (4 weeks old) were bombarded with chloroplast dsRNA vectors using the biolistic device PDS1000/He and transplastomic lines were recovered as described previously (Jin et al., 2011). Bombarded leaves were then subjected to three rounds of selection. First two rounds of selection were performed on the regeneration medium of plants (RMOP) and the third round of selection was made on hormone free half MS medium (1/2 MSO) medium. Growth media were supplemented with 500 mg/L spectinomycin. After selection, putative transplastomic shoots were transferred to pots in the greenhouse to increase biomass.

Tobacco genomic DNA was isolated from the untransformed and spectinomycin-resistant transplastomic lines using DNeasy plant mini kit (Qiagen). PCR analysis was used to confirm transgene integration into the chloroplast genome using two sets of primers- 3P-3M and 5P-2M as described previously (Jin et al., 2011, 2012). The 3P primer (5'-AAAACCCGTCCTCAGTTCGGATTGC-3'; SEQ ID NO: 2) anneals with the native chloroplast genome and 3M primer (5'-CCGCGTTGTTTCATCAAGCCTTACG-3'; SEQ ID NO: 3) anneals with the aadA gene. Therefore this pair of primers was used to check site-specific integration of the selectable marker gene into the chloroplast genome. The 5P primer (5'-CTGTAGAAGTCACCATTGTTGTGC-3'; SEQ ID NO: 4) anneals with the selectable marker

gene aadA and 2M primer (5'-TGACTGCCCCACCTGAGAGCGGACA-3'; SEQ ID NO: 5) anneals with the trnA gene, which were used to confirm integration of the transgene expression cassette.

Southern blot analysis of putative transplastomic lines was performed according to our previous protocol (Singh et al., 2009; Jin et al., 2011, 2012). In brief, total genomic DNA (~2 µg) isolated from third round of selection was digested with SmaI and separated on a 0.8% agarose gel and then transferred to a nylon membrane. The pUC-Ct vector DNA was digested with BglII and BamHI to generate a 0.81 kb fragment of the flanking sequence. The digested DNA bands were labeled with ³²P α[dCTP] and the membrane was hybridized using Stratagene QUICK-HYB hybridization solution followed by manufacturer's protocol.

Northern hybridization, real-Time PCR (qRT-PCR) analysis of small RNAs

Total RNA was isolated from fully expanded wild type and transplastomic tobacco leaves by Qiagen RNeasy Mini Kit (Qiagen) and quantified by Nanodrop. For the northern blot, equal amount of total RNA (2 µg) was separated on 1.0% denaturing agarose gel and transferred to the nylon membrane (Nytran SPC; Whatman). The PCR amplified product from 3' psbA UTR was used as the probe for northern blot of P450:RNAi, Chi: RNAi and ATPase: RNAi transplastomic lines. Membrane was hybridized with PCR amplified DNA fragment labeled with ³²P[dCTP] using the QUICK-HYB hybridization solution following manufacturer's protocol (Stratagene).

For the Real-Time qRT-PCR, total RNA was isolated using TRIzol reagent (Invitrogen, USA). The third-instar larvae were fed on transplastomic P450: RNAi or wild type tobacco leaves for 4 days or 7 days and then the midguts of these tested insects were isolated under stereomicroscope and then washed with ddH₂O to remove any debris. The cleaned midgut tissues were stored in 70% ethanol at - 20°C until use. After removing the genomic DNA using DNase I, 1.0-2.0 µg of total RNA from tobacco leaves or cotton bollworm midgut tissue was reverse-transcribed to cDNA using a commercially available kit (Applied Biosystems, USA). Quantitative real-time PCR was performed with My-IQ²Cycler (Bio Rad, USA) using 2× SYBR Green master mixes. The primers used for quantification of dsRNAs of P450 in the transplastomic plants leaves were: upstream primer (5'-AGAGACTCATTTCGACCTCGTTGCT-3'; SEQ ID NO: 6) and downstream primer

(5'- GGAGCAATAGCACCTCTTGATAGAA-3'; SEQ ID NO: 7). The upstream primer anneals with P450 and the downstream primer anneals with TpsbA. Quantification was performed by the delta cycle time method with *Helicoverpa zea* β -Actin using as an internal standard for normalization. All the results were obtained from three independent biological replicates.

Insect culture and feeding bioassays

Cotton bollworm (*Helicoverpa armigera*) eggs were purchased from Benzon research laboratory. See the world wide web at benzonresearch.com/insectlist.htm. They were hatched at 25°C and all the larvae were fed with the artificial diet (Southland Products Incorporated on the world wide web at tecinfo.com/~southland/. All dishes were kept at 25 °C on a 14-h-day/10-h-night cycle. For the gossypol tolerance experiments, third instars larvae were transferred to the artificial diet supplemented with gossypol (0-4 mg) for 4 d and the increase in weight was recorded.

For the P450: RNAi bioassay, the larvae were reared on artificial diet until they developed to third-instar stage. These third instar larvae were fed with tobacco leaves expressing P450 RNAi for 3 d. Then, the larvae were transferred to the following diets or leaves: (A) artificial diet; (B) artificial diet supplemented with 1-3 mg g⁻¹ of gossypol; (C) glanded (gossypol containing) cotton leaves; (D) glandless cotton leaves respectively for 4 days. After feeding for 4 days, the increase in weight of the larvae from these four groups was recorded. For the Chi and ATPase RNAi feeding bioassay, third-instar larvae were fed on the transplastomic leaves expressing dsRNA targeted to Chi and ATPase genes for 4 days. The increase in weight of the larvae was recorded. For each treatment, 10 larvae were used and the experiment was repeated 3 times. After different feeding experiments, all larvae were put back on the artificial diet until pupae emerged. The mortality and pupation rate were recorded at the end of these experiments.

Statistical analysis

All statistical analyses were performed with SAS software (SAS Institute Inc.). Significance of variance was determined after the one way ANOVA ($P > 0.05$) and is presented in all graphs as mean $\pm$ S.E.

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

Engineered chloroplast dsRNA silences cytochrome P450 monooxygenase, V-ATPase and chitin synthases genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation

Construction of chloroplasts transformation dsRNA vectors

Three target genes from *Helicoverpa armigera* were used for RNA interference (RNAi) induced through dsRNA P450 gene (P450, GenBank: DQ986461.1, CYP6AE14), Vac ATPase A gene (ATPase, GenBank: HM629434.1) and chitin synthase B gene (Chi, EST cloned from midgut RNA of *Helicoverpa armigera*). The candidate target sites of these genes were obtained by the GenScript siRNA Target Finder on the world wide web at genscript.com/siRNA_target_finder.html#.

Sequences were filtered to eliminate candidates with unfavorable thermodynamic properties. Length of siRNA target site was set as 21mers. Low GC content (<60) was maintained to enhance siRNA functionality. For each target, free energy of sense and antisense was calculated and sequences with strong internal structures were eliminated.

Likewise, tandem repeats were removed. Candidates were ranked based on algorithm using ΔE

The dsRNA structure for the target genes is the typical "sense-loop-antisense" structure. The

loop structure for these dsRNA is 'TTCAAGAGA'. The sequence of stem-loops for the P450, ATPase, Chi genes are shown below and the loop sequence is indicated by underline: 5'-

GCAACGAGGTCGAAATGAGTTCAAGAGACTCATTTTCGACCTCGTTGC-3' (SEQ ID NO:

1); 5'-GTCACGTGACGTAGTGCTGGTTCAAGAGACCAGCACTACGTCAGTGAC-3'(SEQ ID

NO: 8); 5'-GGTGAGGACCGATGGCTCTTTCAAGAGAAGAGCCATCGGTCCTCACC-

3'(SEQ ID NO: 9). More details on dsRNA primers and vector construction are shown in Table

1.

Table 1. DsRNA structure for RNAi cassettes and primers for dsRNA synthesis

Target genes	dsRNA structure for the target genes (sense-loop-antisense)
Cytochrome P450	GCAACGAGGTCGAAATGAGT TTCAAGAG ACTCATTTTCGACCTCGTTGC *(1)
ATPase A	GTCAGTACGCTAGTGCTGGT TTCAAGAG ACCAGCACTACGTCAGTGAC (8)
Chitin synthase B	GGTGAGGACCGATGGCTCT TTCAAGAGA AGAGCCATCGGTCCTCACC (9)
Primers for dsRNA vectors construction	
Primer	Sequence (5'-3')
Upstream primer: Pa (psbA promoter)	TTCC GTCGAC GCTAGAGAAGTCCGTATTTTTC (10) <i>Sall</i>
Downstream primer : Pb-1 (P450 dsRNA for psbA promoter)	GTCGAAATGAG TCTCTTGAA CTCATTTTCGACCTCGTTGCC AACAGTATAACATGACTTATATACTCGTGTCA (11)
Downstream Pb-2 (ATPase dsRNA for psbA promoter)	GACGTAGTGCTGGT TCTCTTGAA ACCAGCACTACGTCAGTGA CCAACAGTATAACATGACTTATATACTCGTGTCA (12)
Downstream Pb-3 (chitin synthase dsRNA for psbA promoter)	CCGATGGCTCT TCTCTTGAA AGAGCCATCGGTCCTCACCC AACAGTATAACATGACTTATATACTCGTGTCA (13)
Downstream Pc (psbA terminator)	CAGTTGAC CTGCAG CCCCAAACAAATACAAAATCA (14) <i>PstI</i>
Upstream Pd-1 (P450 dsRNA for psbA terminator)	TGAG TTCAAGAG ACTCATTTTCGACCTCGTTGCTTTTTTCT AGAGATCCTGGCCTAGT (15)
Upstream Pd-2 (ATPase dsRNA for psbA terminator)	TGGT TTCAAGAG ACCAGCACTACGTCAGTGACTTTTTTCT AGAGATCCTGGCCTAGT (16)
Upstream Pd-3 (Chitin synthase dsRNA for psbA terminator)	TCT TTCAAGAGA AGAGCCATCGGTCCTCACCTTTTTTCTA GAGATCCTGGCCTAGT (17)

*numbers in parentheses are SEQ ID NOS:

5 The primer Pa and Pb were used to amplify the psbA promoter and part of dsRNA sequence by PCR. Similarly, primer Pc and Pd were used to amplify part of dsRNA and the psbA terminator. Because the 3' of PCR product of Pa & Pb primers have a 23~25bp complementary sequence with the 5' of PCR product from Pc & Pd primers, they should anneal into a dsRNA expression cassette (PpsbA:: ds RNA::TpsbA) (Table 1). Formation of such ds

RNA stem loop structures is very common in chloroplast 5' UTR and 3' UTR transcripts (Zou et al., 2003; Merhige et al., 2005; Ruhlman et al., 2010). Two restriction enzyme sites (Sall & PstI) were also introduced into this dsRNA expression cassettes by PCR (Table 1). The dsRNA expression cassettes were double digested by Sall & PstI and then subcloned into the tobacco
 5 chloroplast transformation vector-pLD vector (Jin et al., 2011; Jin et al., 2012; Verma and Daniell, 2007)

The chloroplast transformation vectors were constructed based on the pLD vector (Daniell et al., 1998; 2001). In pLD-Chi: RNAi, pLD-P450: RNAi and pLD-ATPase: RNAi chloroplast vectors, the dsRNAs are regulated by the psbA promoter and its 5' and 3' untranslated regions (Figure 1a). Spectinomycin-resistance gene (*aadA*) was used as the
 10 selectable marker for plant regeneration and is driven by the Prn constitutive rRNA operon promoter with GGAG ribosome-binding site. The inverted repeat region trnI/trnA was used as flanking sequences for homologous recombination into the chloroplast genome (Figure 1a).
 Generation and molecular characterization of dsRNA transplastomic lines

Transplastomic plants were created as described previously (Jin et al., 2011; Verma et al., 2008). Several shoots emerged after bombardment of chloroplast vectors with gold particles coated with each pLD-RNAi plasmid in the first round of selection. The second round of selection advanced shoots towards homoplasmy and the third round of selection in root induction medium established independent transplastomic lines. After third round selection and
 20 confirmation by the PCR and Southern analysis, the transplastomic lines were moved to the greenhouse for increasing biomass and further characterization. Two sets of primers, 5P/2M and 3P/3M were used for PCR analysis to confirm site-specific integration of transgenes into the chloroplast genome. The 3P/3M primers pair annealed to the native chloroplast genome upstream of the site of integration and the *aadA* gene, resulting in a 1.65 kb PCR product. The 5P
 25 and 2M primers were used to confirm integration of dsRNA expression cassettes, which produced a 1.72kb PCR product. PCR results confirmed the expected size products in all of the transplastomic lines which were absent in the wild type control plants (Figure 1a,1c).

Southern blot analysis was used to determine homoplasmy and to confirm site-specific integration of transgenes into the chloroplast genome. Total plant DNA digestion with SmaI,
 30 generated 5.6 kb fragments from transplastomic lines after hybridization with the [32P]-labeled trnI-trnA flanking sequence probe. This confirmed site-specific integration of the transgenes into

the spacer region between the trnI and trnA genes (Figure 1b). Furthermore, the absence of the 4.0 kb fragments in pLD-Chi: RNAi, pLD-p450: RNAi transplastomic lines confirmed that homoplasmy has been achieved (Figure 1d, 1e). However, in pLD-ATPase: RNA lines, A2 and A4 showed 4.0 kb fragment similar to wild type suggesting these two lines are negative lines while other three lines A5, B3 and B5 showed two bands, suggesting heteroplasmy (Figure 1f). All other transplastomic lines generated a single 5.6 kb hybridizing fragment suggesting they are homoplasmic.

Higher level transcription level of dsRNA via the chloroplast genome

To investigate transcription and processing of the dsRNA via the tobacco chloroplast genome, northern hybridization analysis was done in the Southern blot-positive transplastomic lines, using 3' psbA UTR as the probe. Expected transcript sizes were detected in P450: RNAi (2.3, 2.4, 2.6, 2.11 and 10.4), ATPase: RNAi (A1, A3, A6, B2, B4, B5, B14) and Chi: RNAi (1 to 5) transplastomic lines while no dsRNA product was observed in WT (Figure 2a-c). Both processed and unprocessed dsRNA transcripts were observed in all three dsRNA transplastomic lines. It is interesting to note that dsRNA transcripts (cleaved and unprocessed) were several fold higher than the highly expressed endogenous psbA gene. This may be due to double the copy number of transgenes (because of transgene insertion within the inverted repeat region) and transcription driven by the psbA promoter as well as the 16S rRNA promoter driving the entire ribosomal operon. Also, the cleaved dsRNA transcript was more abundant than the endogenous psbA transcript. The Northern blot positive transplastomic plants were used for insect feeding bioassays.

Real-Time PCR (qRT-PCR: Real-Time Quantitative Reverse Transcription PCR) analysis was performed to quantify the transcription level of dsRNAs. In this report, dsRNA of p450 gene was chosen for qRT-PCR analysis. These results showed that there was more than three million (3.19-3.45 millions) copies of P450 dsRNA in each micro-gram total RNA from transplastomic leaves, further confirming results observed in northern blots. However, cleaved dsRNA was not detected in the wild-type tobacco leaves (Figure 2d), confirming probe specificity

P450 monooxygenase suppression via chloroplast dsRNA feeding reduced the *Helicoverpa armigera* tolerance to gossypol

Gossypol and related sesquiterpene biosynthesized in cotton are toxic to many organisms. However, most *Helicoverpa* insects resist gossypol because the P450 monooxygenases in the midgut of these insects permit them to tolerate the gossypol. Therefore, in order to suppress P450 gene, two step insect bioassay was conducted. In the first step, third-instar larvae of *Helicoverpa armigera* were fed on P450: RNAi expressed leaves for four days. Then, all these larvae were transferred to the following diet or leaves: artificial diet with 3mg/g gossypol or without gossypol; cotton leaves with glands/glandless and wild-type tobacco leaves. Addition of gossypol in the artificial diet or in leaves affects the growth and development of the larvae (Figure 3). For the gossypol tolerance test, the third-instar larvae of *Helicoverpa armigera* were fed on artificial diet supplemented with 0-4 mg/g of gossypol. These results showed that gossypol mediated toxicity to larvae were moderate. The weight increases (163-190mg) on low concentration gossypol diet (1-2 mg/g) were comparable to respective controls (246 mg). However, the larval growth was dramatically suppressed at higher concentrations (3-4 mg/g) of gossypol diet with net weight increase of 15.1-55.4 mg (Figure 4a). Thus, *Helicoverpa armigera* insects can tolerate relatively low concentration of gossypol. Therefore, 3mg/g of gossypol concentration was used for subsequent insect bioassays.

Suppression of the P450 gene by dsRNA expressed via the chloroplast genome has been demonstrated herein. The transcription level of this targeted gene was assessed by qRT-PCR. After feeding on the two independent transplastomic lines (L2.6 and L10.4) for four days, the midgut of tested larvae were isolated and used for qRT-PCR analysis. The transcription level of the P450 gene located in the insect midgut was significantly decreased in both L2.6 ($P<0.01$) and L10.4-fed insects ($P<0.05$) (Figure 5a). Similarly, the transcription level in both groups continued to decrease and the transcription of P450 in the L2.6 was too low to be detected in some samples (Figure 5b).

In parallel experiments, other insects were subjected to the insect bioassay by feeding on different diet/leaves. The larval growth was significantly affected by the gossypol after four days of feeding on transplastomic leaves expressing dsRNA (Figure 4b). The net weight increase of larvae fed on the diet supplemented with 3mg/g of gossypol was 45mg, which was one sixth of the net weight increase when larvae fed on the diet without gossypol. Most *Gossypium* species

store gossypol in the pigmented glands, but some glandless cultivars do not accumulate this toxin (Luo et al., 2001). Larvae fed with glanded leaves grew slower than those on the glandless leaves (net weight increase 56.5 mg vs 214 mg).

5 Helicoverpa armigera showed stunted development after feeding on leaves expressing dsRNA-Chi and dsRNA-ATPase via the chloroplast genome

For the Chi and ATPase: RNAi feeding bioassay, third-instar larvae were fed with the transplastomic leaves expressing dsRNA for 4 days. The bioassay results showed that siRNA significantly reduced *H. armigera* growth. The net weight increase of larvae fed on dsRNA-ATPase leaves was 57.7 mg within four days whereas it was 130.7 mg when fed on wild type untransformed leaves (Figure 6a). Similarly, net weight increase of larvae fed with dsRNA-Chi expressing leaves was significantly ($P<0.05$) lower than those fed with wild type untransformed leaves. The average net increase of larvae fed on dsRNA-Chi leaves was 47.6 mg, whereas it was 136.7 when larvae were fed with wild type untransformed leaves (Figure 6b).

15 Silencing the P450, ATPase and Chi1 genes lead to the higher larvae mortality and lower pupation rate

The transformation from larvae to pupae is a key step in the metamorphosis of *Helicoverpa* insects. In addition to the net weight increase of insects after feeding on different diet or leaves, the larval mortality and pupation rate were also observed in all treatment groups. After feeding with different diet or leaves for 4 days, all larvae were transferred to the artificial diet until pupation. During this transfer, some larvae failed to form pupae and finally died (Figure 7d, 1-4 dead larvae). Only a few larvae developed into pupation stage (Figure 7d, number 5 live pupa). The larvae (with P450 monooxygenase silencing) fed on the gossypol diet or glanded leaves showed significantly lower pupation rate ($P<0.05$) when compared to larvae fed on non-gossypol or glandless or wild type untransformed leaves (Figure 7a).

The larval population decreased when the larvae were fed with transplastomic leaves expressing dsRNA-ATP and dsRNA-Chi for 4 days. During this growth period, some larvae moved slowly and stopped feeding and finally failed to complete the pupation. Only 56.7% of the larvae were successfully transformed into pupa after feeding with dsRNA-ATPase leaves whereas 83.3% of the larvae transformed to pupa when fed with wild type untransformed leaves (Figure 7b). Similar bioassay results were obtained for insects fed with dsRNA-Chi expressing

leaves. The pupation rate in the control group (80%) was significantly higher ($P < 0.05$) than the dsRNA-Chi fed group (46.7%, Figure 7c).

DISCUSSION

5 The expression of dsRNA via the chloroplast genome was successfully achieved for the first time as described herein. Most importantly, the qRT-PCR data provided direct proof of higher transcription level of dsRNA in the transplastomic cells, up to 3.5 million copies of dsRNA in one microgram (106 pg) RNA. Cleaved dsRNA is more abundant than the highly expressed endogenous chloroplast *psbA* gene. Higher accumulation of dsRNAs in plant cells is a requirement for the effective silencing of targeted genes.

10 The data presented here demonstrate that RNAi is a powerful tool for gene silencing and mediated post-transcriptional down-regulation of chitin synthases, P450 monooxygenase and V-ATPase gene transcripts and impact on development of larvae fed with transplastomic leaves. In the P450 bioassays, we observed that the P450 targeted transcripts in the insect midgut decreased significantly over time. The P450 transcript level of transplastomic lines 7-day-after feeding declined up to 92% when compared to respective controls. The correlation between dosage and effectiveness of RNAi have been shown in several siRNA injection studies (Boisson et al., 2006; Whyard et al., 2009; EI-Shesheny et al., 2013). When compared to the in vitro spray/micro-injection of siRNA solution or feeding the artificial diet supplemented with siRNA, the transplastomic plant mediated RNAi offers several advantages: 1) higher doses of chloroplast-derived transcripts 2) stable expression throughout plant life cycle and 3) higher RNAi activity free from degradation of siRNA in vitro or in the digestive system due to bioencapsulation within plant cells.

25 Most importantly, we describe high abundance of dsRNA transcripts in transplastomic lines and their processing. Several lines of evidence support processing of dsRNA within chloroplasts, although unprocessed RNA is more abundant than cleaved transcripts. When primers specific for dsRNA transcript (P450/*TpsbA*) was used in qRT-PCR, dsRNA transcript products were observed in transplastomic lines but not in untransformed wild-type plants. Processed, cleaved and stabilized dsRNA with the 3'*psbA* UTR should be 272 nucleotides. Correct size of this transcript was observed in all three dsRNA transplastomic lines but not in untransformed wild-type plants. Because no inter-cistronic processing element was engineered,

in the absence of processing only dicistron or polycistron transcripts should have been observed without any dsRNA cleavage and processing. According to our current understanding of processing of transgene transcripts (Martinez de Alba et al., 2013), after generation of primary transcripts, RISC complex cleaves mRNA and cleaved products are stabilized by SGS3; RDR6
5 then generates dsRNA and DCL2/4 form siRNA duplexes. However, none of these proteins are known to be present in plastids and yet we observed cleaved, stabilized and protected dsRNA products. So, it is possible that dsRNA processing occurs via a different mechanism in chloroplasts. Formation of stem-loop structure by dsRNA could leave single stranded regions for ribonucleolytic cleavage (Hotto et al., 2010). In addition, plastid non-coding RNAs (pncRNA)
10 biogenesis relies on the assembly of RNases that generate mRNAs and rRNAs and are protected by secondary structures as well as RNA binding proteins (Hotto et al., 2012). In this case, dsRNA is further protected by the psbA 3'UTR, stabilizing this partially processed dsRNA product. Clearly, this ideal delivery system in which dsRNA is protected by bioencapsulation within plant cells represents an advance in the art of RNAi technology.

15 Recent development in transcriptomics, specifically strand-specific RNA sequencing by next generation sequence technology, have allowed high-throughput, comprehensive detection of low-abundance transcripts typical of the non-coding RNAs studied in eukaryotes and bacteria. Most recently, a few plastid pncRNAs had been identified (Zhao et al., 2007; Germain et al., 2011; Zhelyazkova et al., 2012) and even fewer had been investigated for functional role in gene
20 regulation (Hegeman et al., 2005; Hotto et al., 2010; Zghidi-Abouzid et al., 2011). Another important source of pncRNA identification includes profiling 21–24 nt miRNAs and siRNAs (Shah et al., 2010; Hotto et al., 2012). Using RNA-Seq technology, pncRNAs of <40 nt were identified from Arabidopsis leaves, tomato fruit and flower barley leaf plastids and rice (*Oryza sativa*) leaves, respectively (Mohorianu et al., 2011; Ruwe and Schmitz-Linneweber, 2012;
25 Zhelyazkova et al., 2012). Although there has been more pncRNA identification and pncRNA biogenesis, their functionality is still not clear. Till now, there is no evidence for an RNA interference pathway in plastids. However, bacteria could generate a size class of 20–50 nt ncRNAs termed (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs), which are involved in silencing foreign genes.

30 Plant mediated RNAi has been an important crop protection strategy targeting insect pests with greater specificity than currently available pesticides or Bt toxin. Especially in the

case of lepidopteran insect control, this technology has exhibited great potential. CYPs (e.g P450) was first successfully used to targeted genes to control lepidopteran insects, which could detoxify synthetic insecticide compounds such as gossypol in *Gossypium* plants. Several past reports showed that silencing these CYPs would suppress transcript levels of the targeted genes, attenuate their function, and finally decrease larval growth or survival (Mao et al., 2007, 2011; Zha et al., 2011; Kumar et al., 2012). Since CYPs belong to a multigene family, they have been proposed to be ideal targets for RNAi strategy (Kumar et al., 2012). Therefore, in this first attempt of gene silencing via the chloroplast genome, three dsRNA targets were selected as suitable candidate genes. In the present invention, successful silencing of three midgut lepidopteran insect genes with chloroplasts-derived dsRNA indicates that the plant-mediated RNAi approach is feasible via the chloroplast genome.

As described above, there is a great need to down-regulate harmful genes to confer protection against various plant pathogens. In addition, down regulation of out of control genes causing cancer or autoimmune diseases or immune disorders is highly desired in human health.

Due to the high level of chloroplast transcription, a large amount of the dsRNA could be synthesized and orally delivered via bioencapsulation in plant cells to target disruption of harmful genes. To the best of our knowledge, there is no report on the expression the dsRNAs via the chloroplast genome. Taken together, results reported here demonstrate that the approach of chloroplast derived dsRNA will be useful not only in plant biotechnology but also in various biomedical applications.

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EXAMPLE II

25 Chloroplast produced RNAi for Treatment of Human Disease

RNA interference (RNAi, siRNA, miRNA, shRNA, dsRNA) has great potential to be among the next generation of therapeutics for treating cancer and other genetic and viral diseases. The major obstacle blocking the clinical application of siRNA has been the development of an approach that is able to deliver sufficient siRNA to the cytoplasm of target cells in a form that is capable of eliciting a silencing effect. Prior attempts have focused on various delivery routes for RNAi including gene therapy, engineered nanoparticles, and

conjugation with polymers. Thus far, none of these approaches has emerged as a safe and reliable solution for repeated delivery.

Until recently, the long standing paradigm in the digestion of nucleic acids has been that they are degraded into their basic building blocks and all genetic information is lost during digestion. Recently, results from several groups have challenged this model, demonstrating oral delivery of functional siRNA using nanoparticles and nanocarriers [29, 30]. Other studies have shown that miRNA contained in the exosomes of breast milk are stable in digestive conditions and are able to elicit an expansion of T cells when added to human blood. It has been demonstrated that double stranded RNA is resistant to acidity as low as pH 1 and is resistant to degradation by RNase [1, 26, 30]. Circulating miRNAs have also been shown to exist in a form extremely stable in serum and resistant to degradation by RNase, however naked miRNAs are rapidly degraded upon injection [33]. This suggests that circulating miRNAs may exist in complex with lipoproteins such as LDL or exist within microvesicles and exosomes.

C. elegans has long been known to respond to exogenous RNAi through dietary uptake. Feeding siRNA to *C. elegans* is able to achieve effective systemic silencing. Believed to act as an immune response to environmental pathogens, the uptake of RNAi in *C. elegans* is mediated by the transmembrane gate channel, SID-1 [10, 21]. In *C. elegans*, double-stranded RNA (dsRNA) passively enters cells through SID-1 to achieve systemic silencing of siRNA delivered through feeding [21, 25]. A lesser-studied SID-1 analogue, SIDT1 has been described in mammals to have extremely conserved homology and function as a miRNA transporter in mammals. It has been shown to mediate intercellular communication via miRNA within a tumor microenvironment [28]. Furthermore, overexpression of SIDT1 has been shown to mediate uptake of siRNA in human cancer cells [27, 32]. SIDT1 expression has not been characterized across different tissues in mammals, though several groups have suggested that it may have similar functions in mediating systemic RNAi effects.

RNAi provides a very potent and specific form of cancer therapy. RNAi can target any gene in a cell, including those that are currently undruggable by conventional therapeutics. The mechanism of mRNA degradation through the RNAi pathways allows a single RNAi molecule to destroy thousands of mRNA transcripts, making it an extremely efficient gene silencing mechanism. Currently, the major remaining hurdle for therapeutic use of RNAi is efficient in vivo intracellular delivery. In a recent study by Zhang et al., these investigators report that

miRNA present in ingested rice was absorbed through the digestive tract, disseminated to a number of tissues around the body in microvesicles, and was able to suppress expression of LDLRAP1 (an LDL cholesterol uptake protein) through the silencing action of rice MIR168-a. Importantly, this silencing effect was abrogated upon introduction of an antisense MIR168-a strand, providing further evidence that the silencing of LDLRAP1 was indeed mediated by the exogenous MIR168-a. Other studies have also shown that a specific set of miRNAs are secreted into breast milk and are believed to influence the development of nursing infants.

Bioencapsulation of proteins in plant cells has previously been exploited in the oral delivery of therapeutic biologics [2], suggesting that this kind of protection can be utilized in oral delivery of RNAi. Furthermore, other groups have successfully delivered functional siRNA orally using siRNA encapsulated in engineered polymers and nanocarriers [29, 30], suggesting that targeted gene silencing can be achieved by oral delivery. These startling discoveries show that mammalian gene expression may be altered by the food that we eat, opening the possibility of exploiting these pathways to deliver therapeutic RNAi bioencapsulated in plant cells.

MIR168-a has modest native expression levels in rice, and with a 13 base-pair overlap with the LDLRAP1 gene was able to elicit ~30% reduction in LDLRAP1 levels.

A novel route of oral delivery through the feeding of transgenic plants expressing high copy numbers of engineered RNAi is described herein. We have created RNAi constructs targeting GFP that can be fed to mice globally expressing GFP under a ubiquitin promoter.

Uptake of various forms of RNAi across the GI tract, dissemination to tissues, and physiologic silencing response in mice are then assessed. The mechanism and specificity of uptake of various forms of RNAi (pre-miRNA, plant and animal miRNA, siRNA, shRNA) can be characterized to elucidate the mechanism and optimize design of improved therapeutic RNAi constructs.

Silencing of GFP expression can demonstrate that this is a viable method of delivery for RNAi which can then be applied to a broad range of therapeutics. Delivery Kras RNAi using this delivery route will be assessed in mouse models of colon, lung, and pancreatic cancers. The exciting delivery route described herein for RNAi is cost effective, easily administered, and can solve many unmet medical needs.

RNAi tissue targeting, silencing effects and therapeutic potential in cancer or autoimmune disorders following oral delivery of transgenic plants

Plant tissue from *Nicotiana benthamiana* expressing a GFP shRNA transgene is fed to globally-expressing GFP mice and dissemination and silencing assessed (Fig. 8).

Using a GFP marker and an RNAi construct with perfect complementarity to our target gene, we have a powerful system to study the uptake and dissemination of RNAi through oral delivery. Qualitative visual data through IHC as well as quantitative data from stem-loop QT-PCR, ELISA, and northern and western blots can be generated. The ability of exogenous siRNA from plants to enter circulation and silence expression of a reporter gene should further elucidate the silencing mechanism in mammals.

We have designed shRNA constructs optimized on several parameters (stem-loop structure [47] (Figure 9), high AT content (Figure 10), and a 2nt TT 3' overhang [48] (Figure 11), while avoiding complementary sequences in the genome) targeting the coding region of our target genes. Parallel chloroplast vectors with target sequences are made as described in example 1. The 5' 'seed-sequence' of our shRNA (nt 2-8) should avoid complementarity to the 3' UTR of endogenous genes in the target host to avoid off-target silencing via miRNA pathways. The full length silencing sequence as well as the seed sequence is checked for homologous genes using a BLAST search and online algorithms to search for, and minimize potential off target effects. To confirm that the observed silencing is indeed due to shRNA activity, mismatches can be introduced into the target sequence and to assess whether the silencing effect can be abrogated. Scrambled shRNA and empty vector will also be used as a negative control. We will initially evaluate our shRNA constructs in vitro by testing three silencing constructs in Caco2 cells transfected with a GFP plasmid under a ubiquitin promoter.

In the case of GFP, shRNAs with the maximal silencing efficiency are selected which exhibit minimal off-target effects. To further evaluate off-target effects. To further evaluate our shRNA constructs, in vitro microarray analysis as previously described will be performed[50] (Agilent Human 1A V2 arrays). Using several constructs targeting different parts of the GFP gene, we will assess the targeting potential, effects on cell viability, and off target effects of each construct. Overlapping oligos were designed with 4nt overhangs for the corresponding restriction sites (BamH1 and Sal1) to the pGreen vector (Fig. 12). To avoid the self-annealing of the individual oligo strands, oligos were annealed by heating the mixture to 95° C for 10 minutes

and allowing the mixture to cool overnight in a water bath to minimize self-annealing of the shRNA oligos.

Following ligation into the pGreen vector, the DNA is transformed into JM109 competent cells and cultured overnight in LB medium containing kanamycin. The *E. coli* is amplified and DNA is purified and transformed into LB4404 *Agrobacterium* competent cells by incubating with a pSoup helper plasmid for 5 minutes on ice, 5 minutes in liquid nitrogen, and 5 minutes at 37°C. *Agrobacterium* is grown at 28°C for 48 hours in YEB medium containing kanamycin and streptomycin to select for transformed colonies. Transformed *Agrobacterium* is centrifuged and re-suspended in an MES infiltration medium, which is injected directly into the *N. benthamiana* using a syringe. Using this method, transgenic plants rapidly express high levels of the transcripts. In parallel, transplastomic lines expressing dsRNA are created by bombardment of chloroplast dsRNA vectors.

RNA will be purified from leaves harvested from these GFP shRNA-expressing plants using Trizol and quantified by stem-loop QT-PCR for transcript expression levels and normalized to U6 snRNA and GAPDH to allow us to enable dosage setting. Stem-loop primers have been designed with a stem-loop structure containing a 6nt overlap with the 3' end of the mature miRNA sequence, and a reverse primer overlapping the 12nt on the 5' end (Fig. 13). To get an absolute concentration, a standard curve using known concentrations of synthetic oligos can be generated. The leaf material will be homogenized and suspended in PBS, then fed to genetically engineered mice that express GFP globally under a ubiquitin promoter. To evaluate the degree of GFP silencing and dose-dependency, groups of mice will be orally gavaged with a series of doses (based on dilutions of final concentration of transcript) of 200µL of GFP-targeting transgenic plant material suspended in PBS. Serum will be collected in 2 hour intervals following feeding for 8 hours. Four days after receiving daily gavage, mice will be sacrificed and organs will be perfused and harvested. Protein and RNA will be extracted from tissue and analyzed by stem-loop qRT-PCR for GFP transcripts, as well as GFP ELISA and IHC to analyze protein levels. Silencing will be compared to GFP siRNA contained in liposomes delivered both orally and intravenously as a positive control and mice fed plants with *Agrobacterium* transformed with scrambled RNAi as a negative control.

Silencing of GFP over the course of a 4 day period of feeding GFP shRNA to mice is expected. Given that lyophilization increases the concentration of plant material ~10 fold, we

estimate that the final concentration of RNAi should be ~75 fmol/g in order for this approach to be therapeutically relevant. This is ~13-fold higher than the expression of one of the more highly naturally expressed miRNAs, however this expression level is achievable given the high levels of expression using agroinfiltration [51] and the tightly controlled expression of endogenous
5 miRNAs.

Since it was previously reported that significant silencing of a mammalian gene was achieved by a plant miRNA with imperfect complementarity and expressed at natural levels, we have designed an RNAi molecule with perfect complementarity and expect that overexpressing it will yield improved silencing effects. It has also been reported that siRNA with a 2'-O
10 methylation have a reduction of ~80% in off-target effects [46], so we will include this modification in some of our designs for reducing off-target silencing effects of our RNAi. The 2'-O modification has also emerged as the preferred solution to immunogenicity and increase serum stability of circulating RNAi molecules, which will be a major benefit of using RNAi expressed in plant cells. Given that GFP has a 26 hour half-life, we expect that GFP levels will
15 have dropped within this time period in cells that have received the GFP shRNA. Since this approach will only block the production of newly translated GFP, the transcript levels will be a more direct measure of the temporal efficacy of this experiment.

There is great need for an efficient method of silencing mutant Kras. Pancreatic cancer is among the deadliest types of tumors, the vast majority of which contain an activating mutation in
20 the Kras gene. Although one of the most commonly mutated oncogenes in many forms of cancer, Kras is very difficult to target using conventional methods due to its nature as a GTPase. Based on this, Kras will be the first target tested therapeutically. Furthermore, a mutant-specific shRNA targeting Kras G12D will also be tested as this molecule should ameliorate side-effects associated with silencing wild-type Kras in healthy cells. Several other targets for cancer
25 treatment include mTor, cMet, VEGF/VEGFR, c-kit, PDGF/PDGFR, P13K, HER2, EGFR and several other similar genes. siRNA encoding nucleic acids directed to these gene targets have been previously described. Vascular Endothelial Growth factor promotes angiogenesis and solid tumors. Several other specific targets include Ribonucleotide Reductase M2 (RRM2), kinesin spindle protein and VEGF, Polo-like kinase I (PLK1), tenascin-C, protein kinase N3, HIF-1
30 alpha, furin, etc. P53 is the most commonly activated tumor suppressor in different types of cancers. MDM2 is a negative antagonist of P53. Therefore, blocking MDM2 expression should

be ideal for cancer treatment. Although synthetic siRNA are injected to achieve gene silencing in current research protocols, oral delivery of these dsRNA has not yet been investigated. Likewise, proinflammatory cytokines and other factors stimulate or cause several autoimmune disorders and allergies. There are several cytokines that could be down regulated using dsRNAs.

5 Kras is one among the most commonly mutated oncogenes. It maintains Kras GTPase in GTP-bound form and observed in > 90% of pancreatic cancers as well as other tumors. Kras controls several downstream proliferation and growth pathways. MiR-34 is an ideal Kras target. The siRNA has high specificity to distinguish between Kras and mutant Kras containing a single point mutation.

10 We have generated Kras shRNA-vectors for transient viral expression or stable dsRNA expression via the chloroplast genome. The RNAi constructs can be tested in the PDAC cancer cell line. Recombinant plant material can also be fed to genetically engineered KPC mice which provide a model of pancreatic cancer. adenoCre inducible Kras G12D mouse model of lung cancer, and a Kras/APC/p53 mouse model of colon cancer will also be tested. In addition, 15 we have designed mutant-specific Kras shRNA to target the G12D mutation in genetic mouse models to determine whether we can specifically target a mutant oncogene while sparing the wild type gene.

The constructs will be delivered as described above. Mutant-specific Kras have been previously described [54], taking advantage of the loss of nuclease activity that occurs with a 20 mismatch at the nucleotide corresponding to the cleavage site of the target transcript. Screening for off-target effects can optionally also be performed. We expect that the mouse model for lung and colon cancer will exhibit the greatest physiologic effects using this approach. Since Kras is associated with EMT and metastasis, we expect that tumors will exhibit less epithelial to mesenchymal transition (EMT) in treated mice (upregulation of E-cadherin and downregulation 25 of EMT markers such as Zeb1) and decreased number of metastases. Plant-derived miRNA is modified with a 2'-O methyl group. This modification has been used in the delivery of siRNA to improve the siRNA stability in circulation as well as reducing its immunogenic effects, so we expect that this form of RNAi will have superior pharmacokinetic properties once in circulation as compared with synthetic siRNA, and will minimize immunologic response to the foreign 30 RNAi [45].

Characterization of the mechanism and specificity of RNAi uptake in the GI tract

SID-1 is a transmembrane channel protein known to mediate intestinal RNAi uptake in *C. elegans*, acting as the gateway to systemic RNAi silencing in the worms. A highly homologous protein, SIDT1, is known to exist in mammals including humans. SIDT1 appears to be the most likely candidate responsible for mediating uptake of miRNA in the GI tract, yet this protein remains largely unstudied. Overexpression of SIDT1 has been shown to enhance uptake of siRNA in mammalian cells, but its expression has not been characterized in mammals [27, 32]. SIDT1 expression in the subsections of the small and large intestine will be studied. Since it is believed that miRNA in breast milk is taken up by nursing infants, it is possible that this receptor is overexpressed in infancy. For this reason, we will also compare expression levels of nursing mice to adult mice.

Strategic design of RNAi molecules for delivery through the oral pathway will benefit from a greater understanding of processing of RNAi in plant cells and uptake of various forms of RNAi in the GI tract. In vitro experiments to test the uptake of various forms of RNAi molecules by transfecting Caco-2 intestinal epithelial cells with a SIDT1 using pCMV plasmid as previously described will be performed [28]. We will then expose the cells to homogenized plant tissue expressing various forms of RNAi: plant and animal miRNA in pri-miRNA, pre-miRNA, and mature forms, and shRNA of various lengths (Figure 12 and Figure 13). The final processed form of these constructs will be evaluated in plant cells by step-loop QT-PCR and Northern blot. 'Chimeric' miRNA that we have created which has the secondary structure backbone of plant miR156a with a modified targeting sequence based on our target will also be tested. Furthermore we will test the uptake of naked mature miRNA as compared to mature miRNA in complex with Ago2.

Since SIDT1 has been shown to act as a passive channel for RNAi, we expect that expression of SIDT1 in Caco2 cells would facilitate the uptake of RNAi and transport across the cell. Based on previous studies [1], we expect that the RNAi will be packaged in exosomes shed from the cells. Further, we expect that silencing SIDT1 would abrogate the RNAi uptake effect. Based on previous studies, we believe that mature miRNA will be taken up by the SIDT1 receptor and not pre-miRNA or other forms. We expect that the 'chimeric' miRNA created based on the most abundantly disseminated miRNAs in previous studies will yield the mature miRNA most likely to be taken up in the GI tract (Fig. 14).

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25 While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A recombinant plant plastid or viral expression vector which comprises, as operably linked components in the 5' to 3' direction of translation, a promoter operable in said plant, a heterologous polynucleotide sequence coding for at least one RNAi molecule selected from the group comprising dsRNA, siRNA, shRNA, and miRNA and a transcription terminator functional in said plant, said vector optionally encoding a nucleic acid encoding a selectable marker.
2. The vector of claim 1, suitable for stable or transient plant transformation.
3. The vector of claim 1, wherein said RNAi molecule has been optimized and comprises one or more step loop structures, high AT content, and a 2 nucleotide TT 3' overhang, said RNAi molecule lacking complementarity to 3'UTRs in endogenous plant genes, said optimized RNAi molecule exhibiting reduced off target effects relative to RNAi molecules which has not been optimized.
4. The vector of claim 1, comprising a selectable marker which is an antibiotic resistance marker.
5. The vector of claim 1, comprising a heterologous polynucleotide sequence coding for at least one RNAi molecule that down-regulates a P450 monooxygenase gene, a Vac ATPase A gene, and a chitin synthase B gene.
6. The vector of claim 1, comprising a heterologous polynucleotide sequence coding for an RNAi molecule that down-regulates the P450 monooxygenase gene.
7. The vector of claim 1, comprising a heterologous polynucleotide sequence coding for an RNAi molecule that down-regulates the Vac ATPase A gene.
8. The vector of claim 1, comprising a heterologous polynucleotide sequence coding an RNAi molecule that down-regulates the chitin synthase B gene.
9. The vector of claim 1, comprising a heterologous polynucleotide sequence coding for an RNAi molecule that down-regulates dysfunctional genes in cancer or immune disorder.

10. The vector of claim 9, comprising a heterologous polynucleotide sequence coding for an RNAi molecule that down-regulates Kras.
11. The vector of claim 1, wherein the plastid is selected from the group consisting of chloroplasts, chromoplasts, amyloplasts, proplastids, leucoplasts and etioplasts.
- 5 12. A plant cell transformed with the plastid transformation and expression vector of claim 1.
13. An edible transformed plant or portion thereof, which comprises the vector of claim 1.
14. The plant portion as claimed in claim 13, wherein said portion is selected from the group consisting of seeds, leaves, flowers, roots and stems.
15. A transformed plant of claim 13 which is edible for mammals and humans.
- 10 16. A transformed plant of claim 13 which is a monocotyledonous or dicotyledonous plant.
17. A transformed plant of claim 13 which is maize, lettuce, rice, grass, rye, barley, oat, wheat, soybean, peanut, grape, potato, sweet potato, pea, canola, tobacco, tomato or cotton.
18. The transformed plant or plant portion of claim 13 which is lettuce.
19. A transformed plant of claim 13 in which the chloroplasts are homoplasmic.
- 15 20. A transformed plant of claim 13 in which the chloroplasts are heteroplasmic.
21. A method of treating cancer or an immune disorder, comprising oral administration to a subject an edible plant or plant portion comprising a therapeutically effective amount of an RNAi molecule selected from the group comprising dsRNA, siRNA, shRNA, and miRNA, expressed from a chloroplasts, said RNAi molecules targeting a gene which aberrantly causes or regulates
- 20 said cancer or immune disorder.
22. The method of claim 21, wherein said plant portion is selected from seeds, leaves, flowers, roots and stems.
22. The method of claim 21, wherein said RNAi molecule down-regulates a cancer oncogene expressed from a chloroplast.

23. The method of claim 22, wherein said oncogene is Kras.

24. The method of claim 23 for the treatment of pancreatic cancer.

25. The method of claim 21, wherein the plant or plant portion is maize, lettuce, rice, grass, rye, barley, oat, wheat, soybean, peanut, grape, potato, sweet potato, pea, canola, tobacco, tomato or
5 cotton.

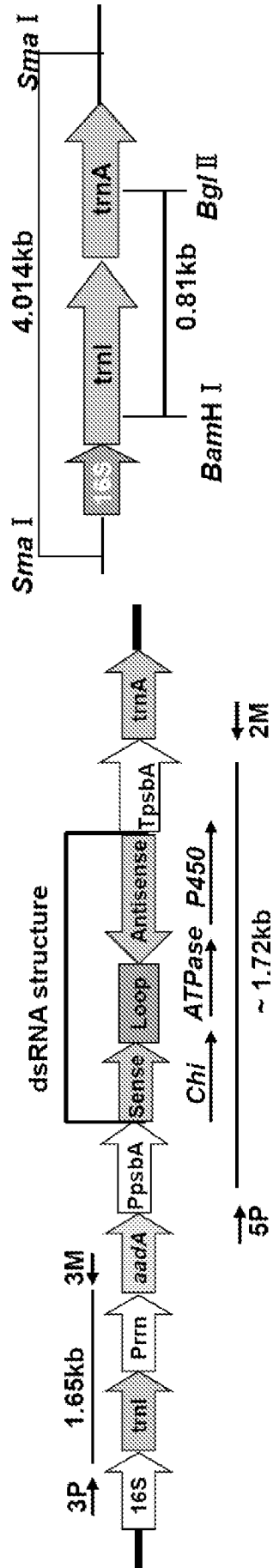


Fig. 1A

Fig. 1B

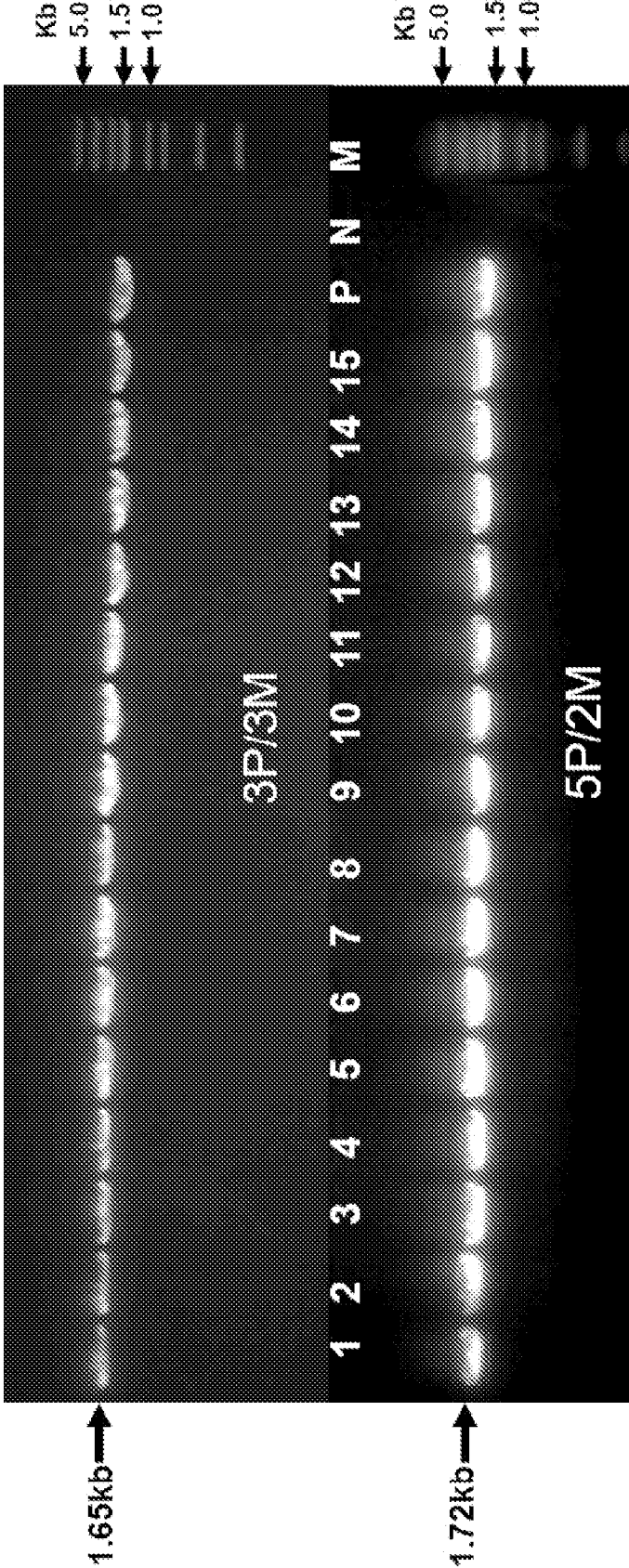


Fig. 1C

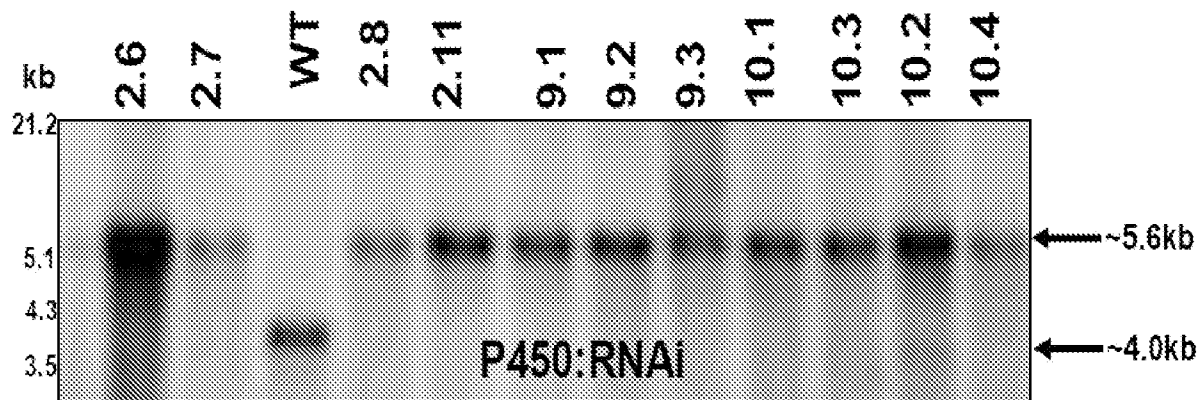


Fig. 1D

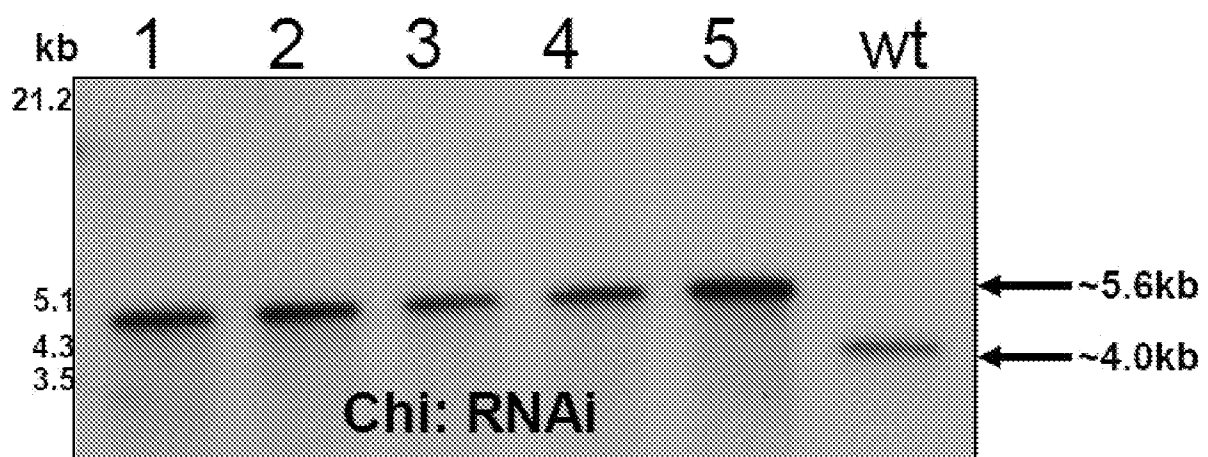


Fig. 1E

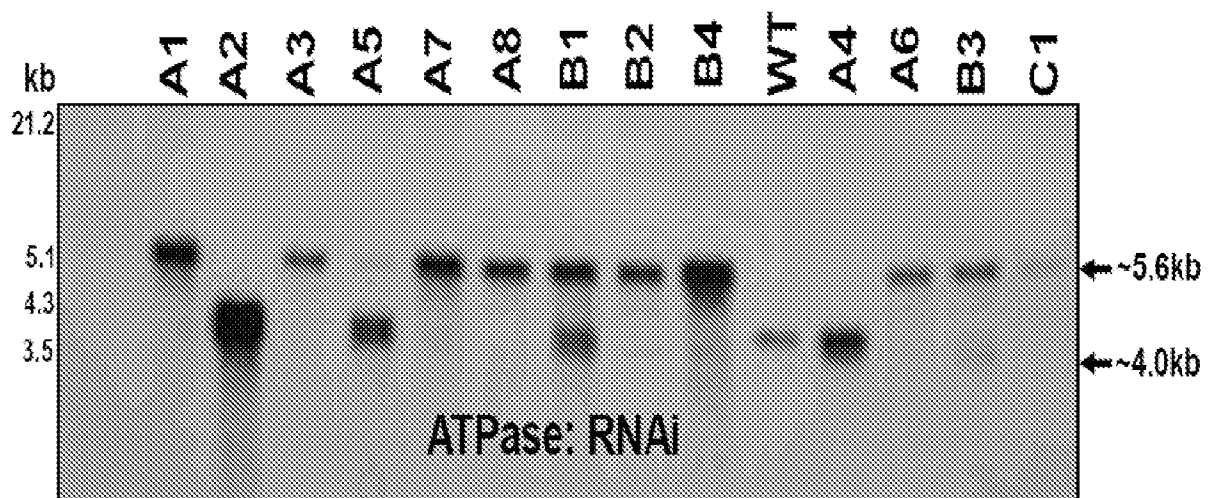


Fig. 1F

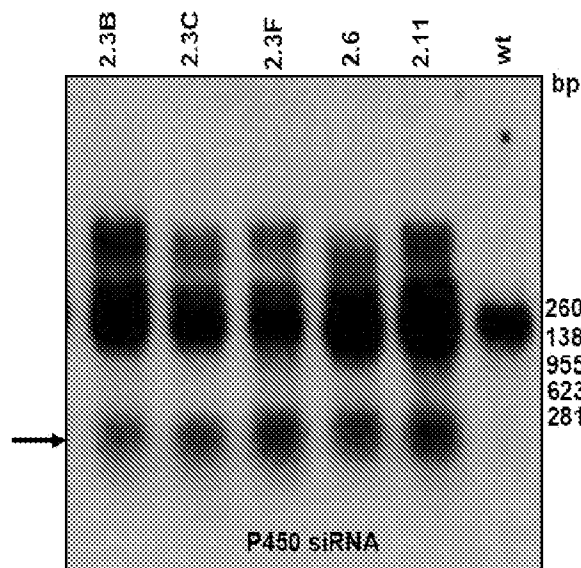


Fig. 2A

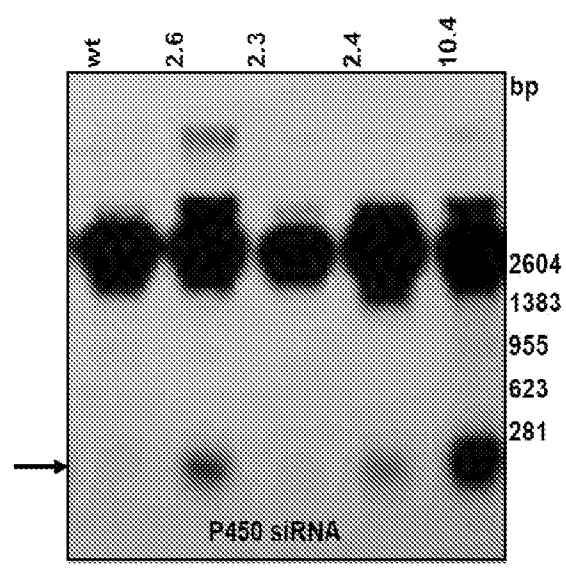


Fig. 2B

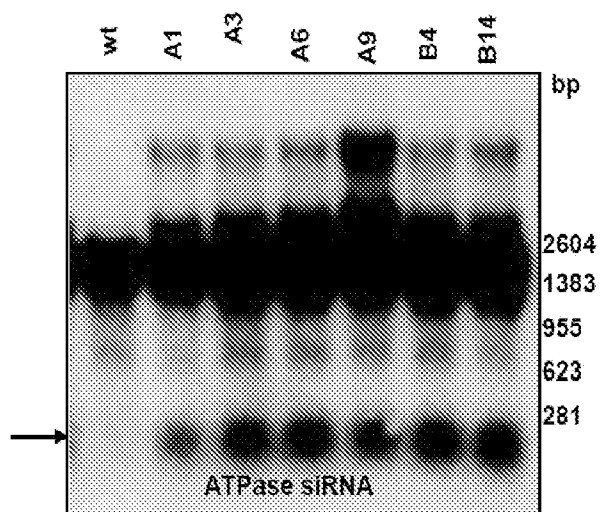


Fig. 2C

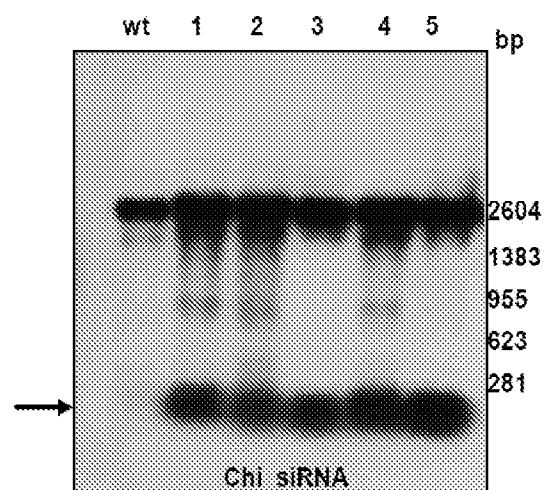


Fig. 2D

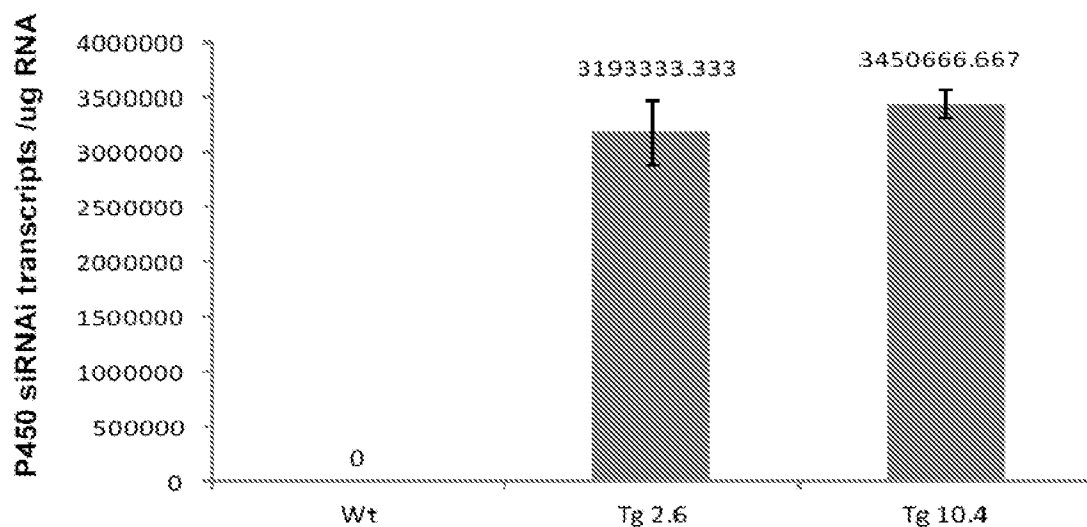
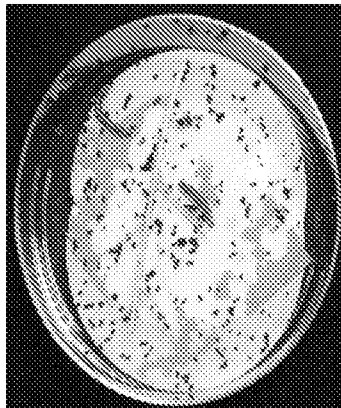
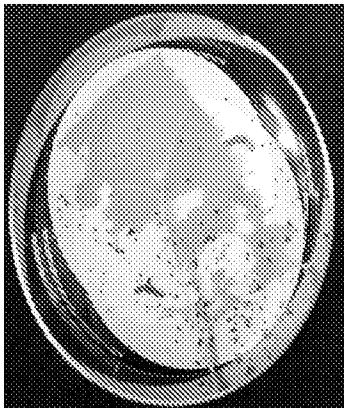


Fig. 2E

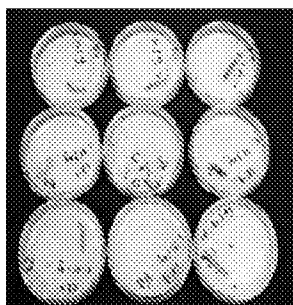
Step one: Synchronous third-instar larvae were fed with siRNA leaves for 4 days



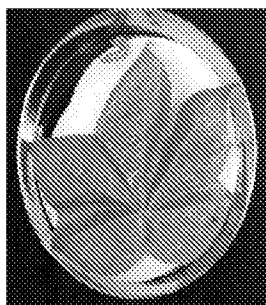
All the transcript of p450 was supposed to be suppressed by siRNA at this stage

Step two: The larvae were transferred to the following plates fed with different diet of leaves

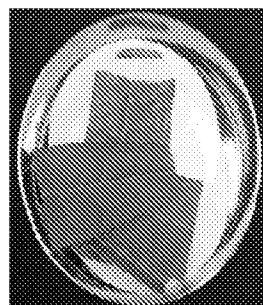
Fig. 3A



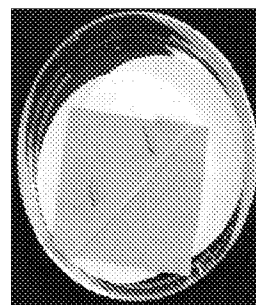
Artificial diet with
3mg/g gossypol or
without gossypol



Cotton leaf with
gland



Glandless
cotton leaf



Wild type
tobacco leaf

Fig. 3B

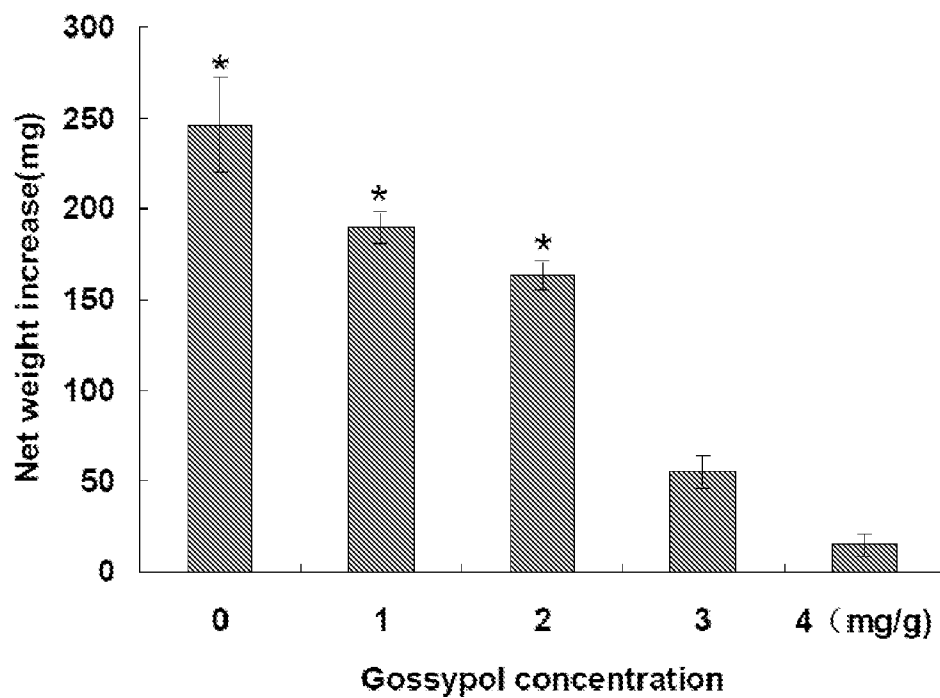


Fig. 4A

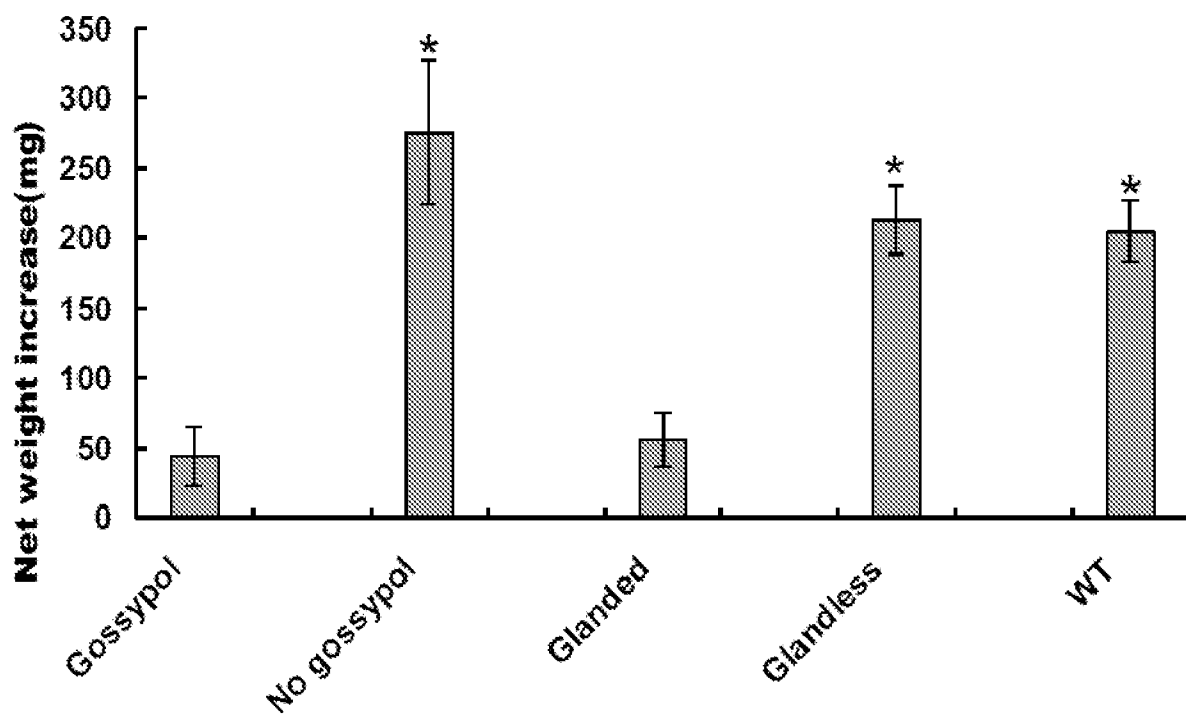


Fig. 4B

Four days after feeding

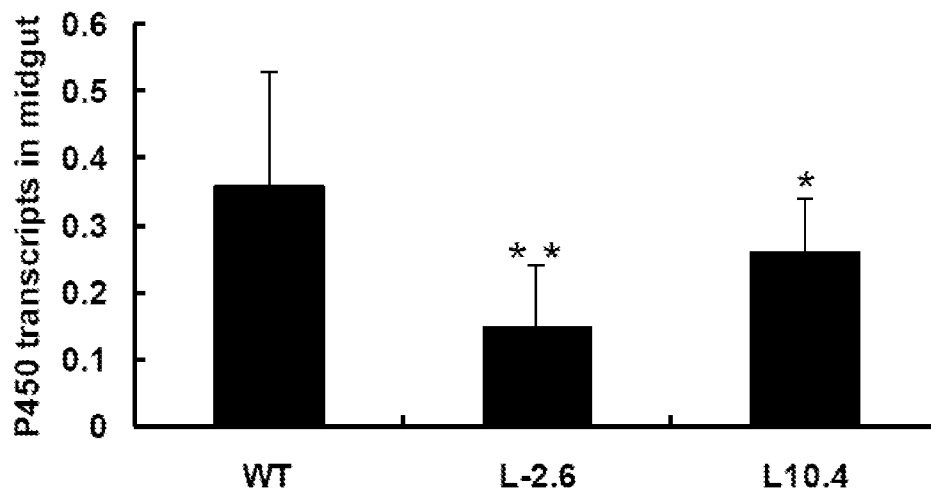


Fig. 5A

Seven days after feeding

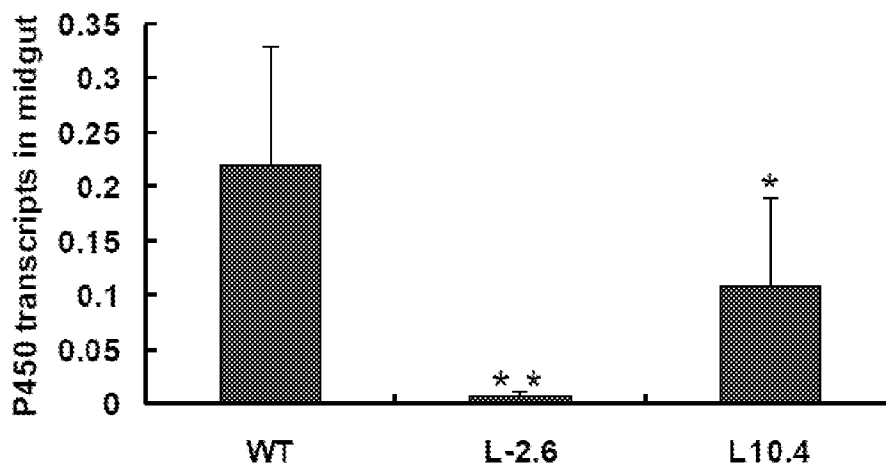


Fig. 5B

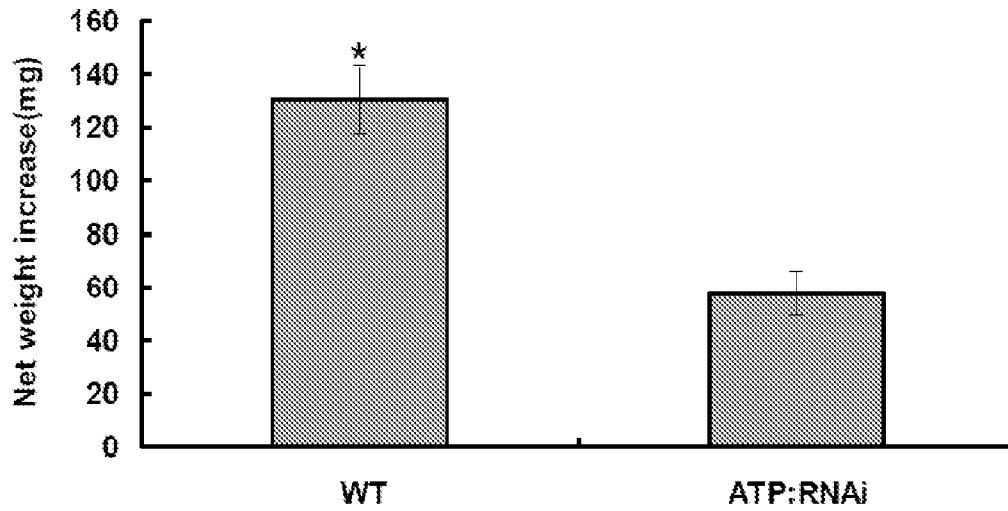


Fig. 6A

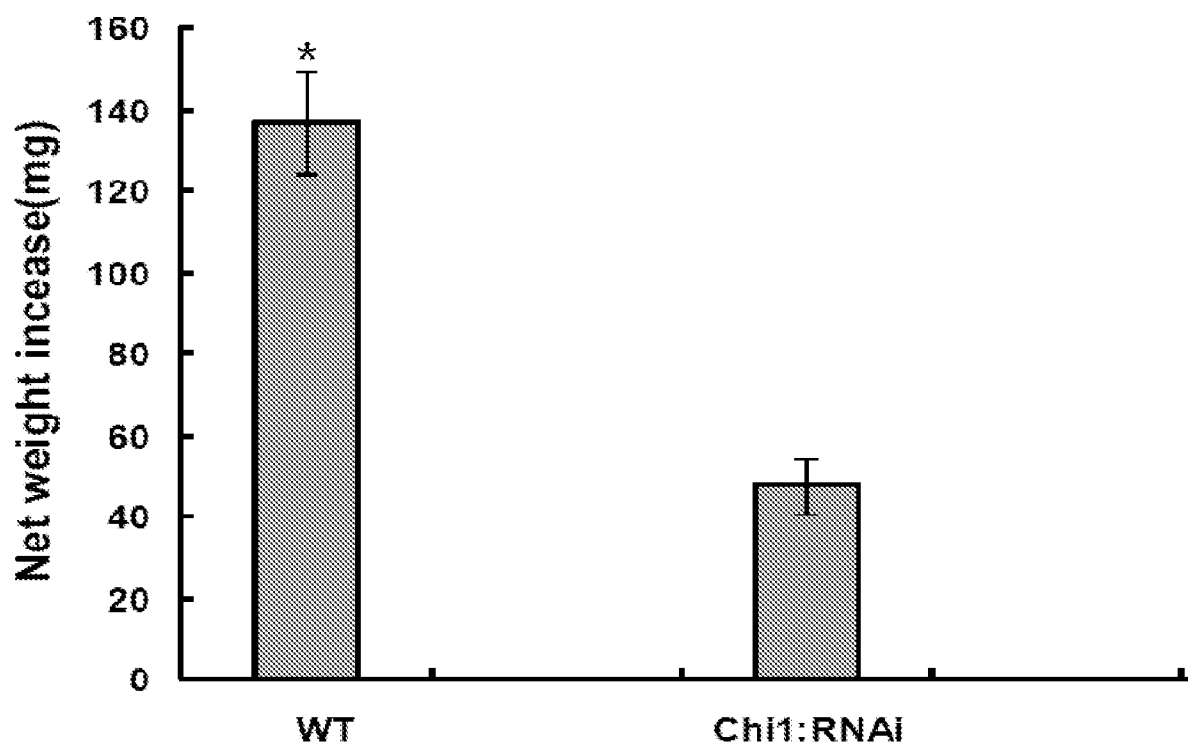


Fig. 6B

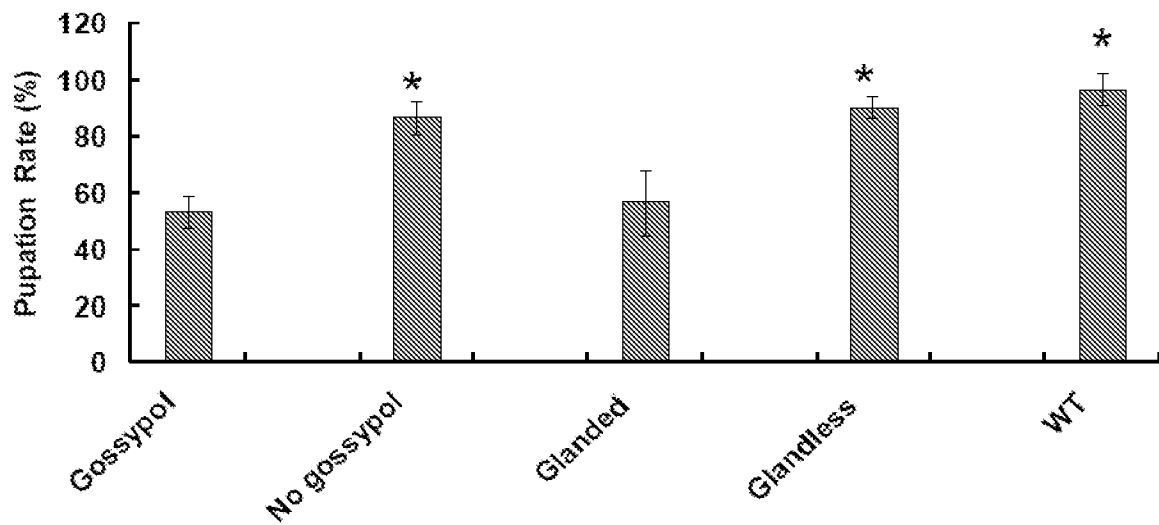


Fig. 7A

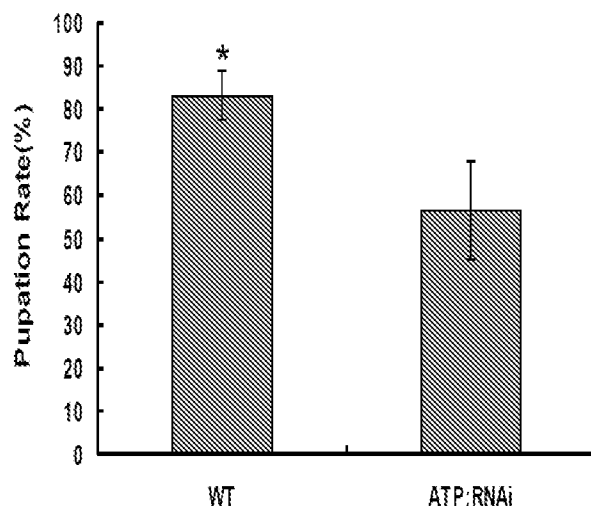


Fig. 7B

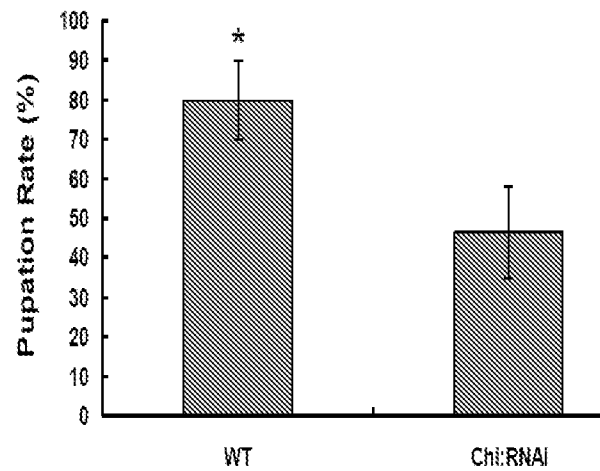


Fig. 7C

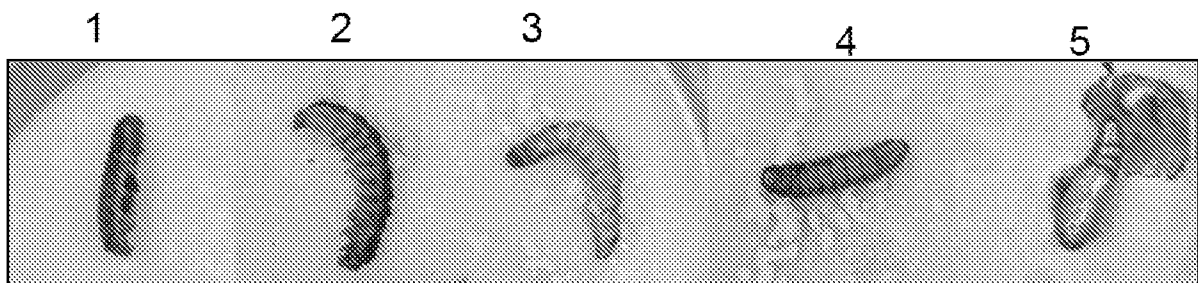


Fig. 7D

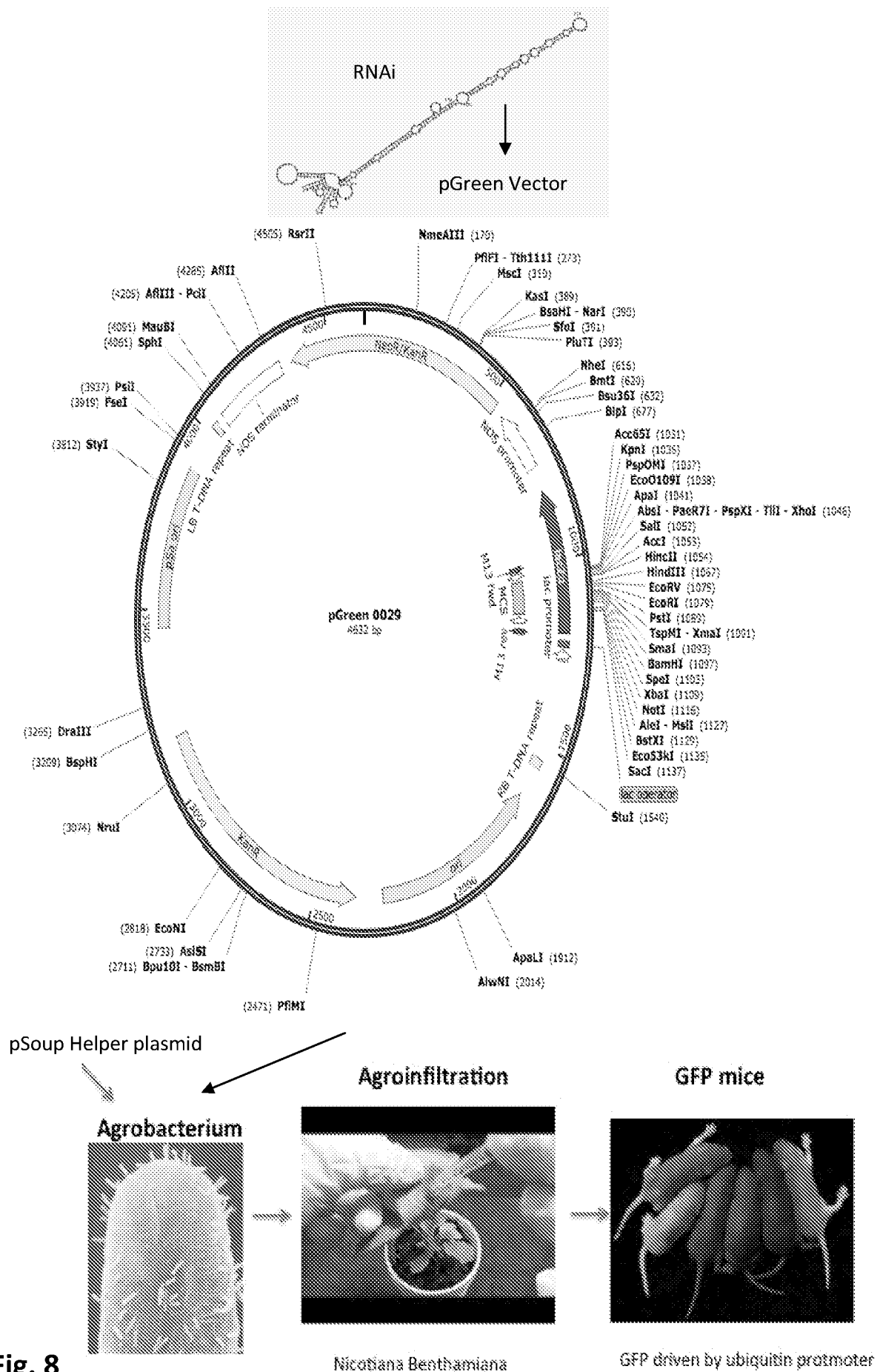
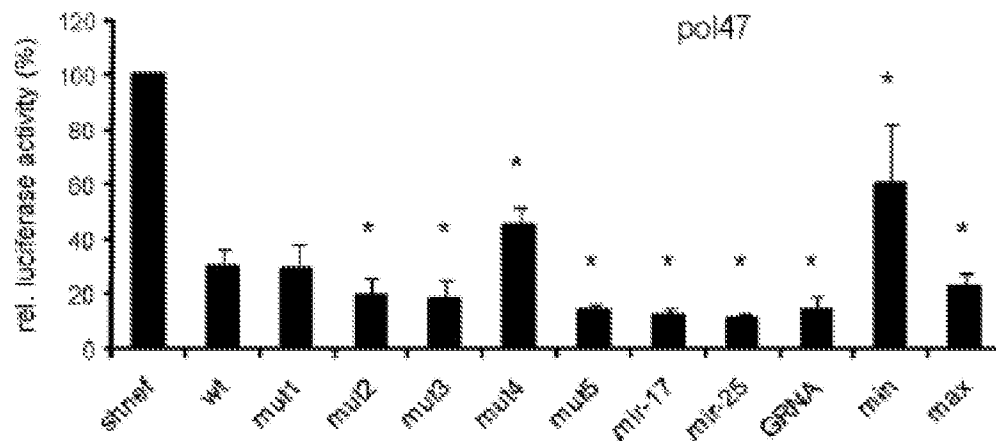
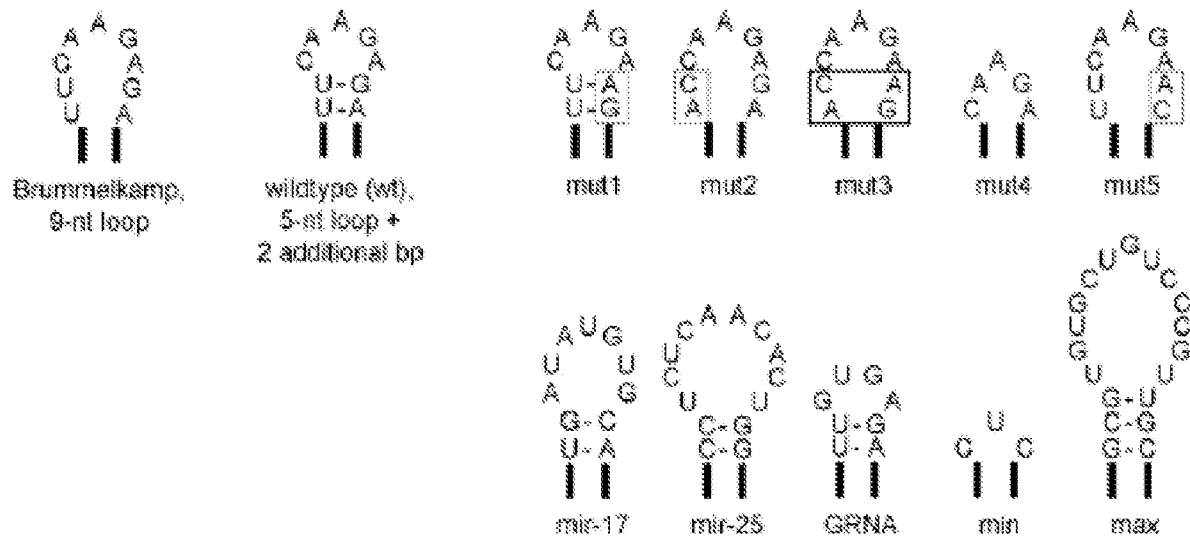


Fig. 8

Stem-loop optimization



- UGAUAUGUGCA loop optimal with 2 matched base pairs
- ACCAAGAAG loop optimal with no matched base pairs

Fig. 9

Increasing AT content

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGA
GCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG
GCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGC
TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCT
TCAGCCGCTACCCCGACCATGAAGGCACGACTTCTTCAAGTCCGCCATGCC
GAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACA
GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC
TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCGAGTA
CAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCA
TCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTC
GCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCC
GACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGA
GCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCG
CATGGACGAGCTGTACAAGTCCGGACTCAGATCTCGAGCTCAAGCTTCGAAT
CTGCAGTCGACGGTACCGCGGGCCGGGATCCACCGGATCTAGATA

Targets contain 5' AA to generate 3' UU on guide strand

AAGCAGCACGACTTCTTCAAG – 47.6%

AAGCTGGAGTACAACTACAAC – 42.8% GC

Fig. 10

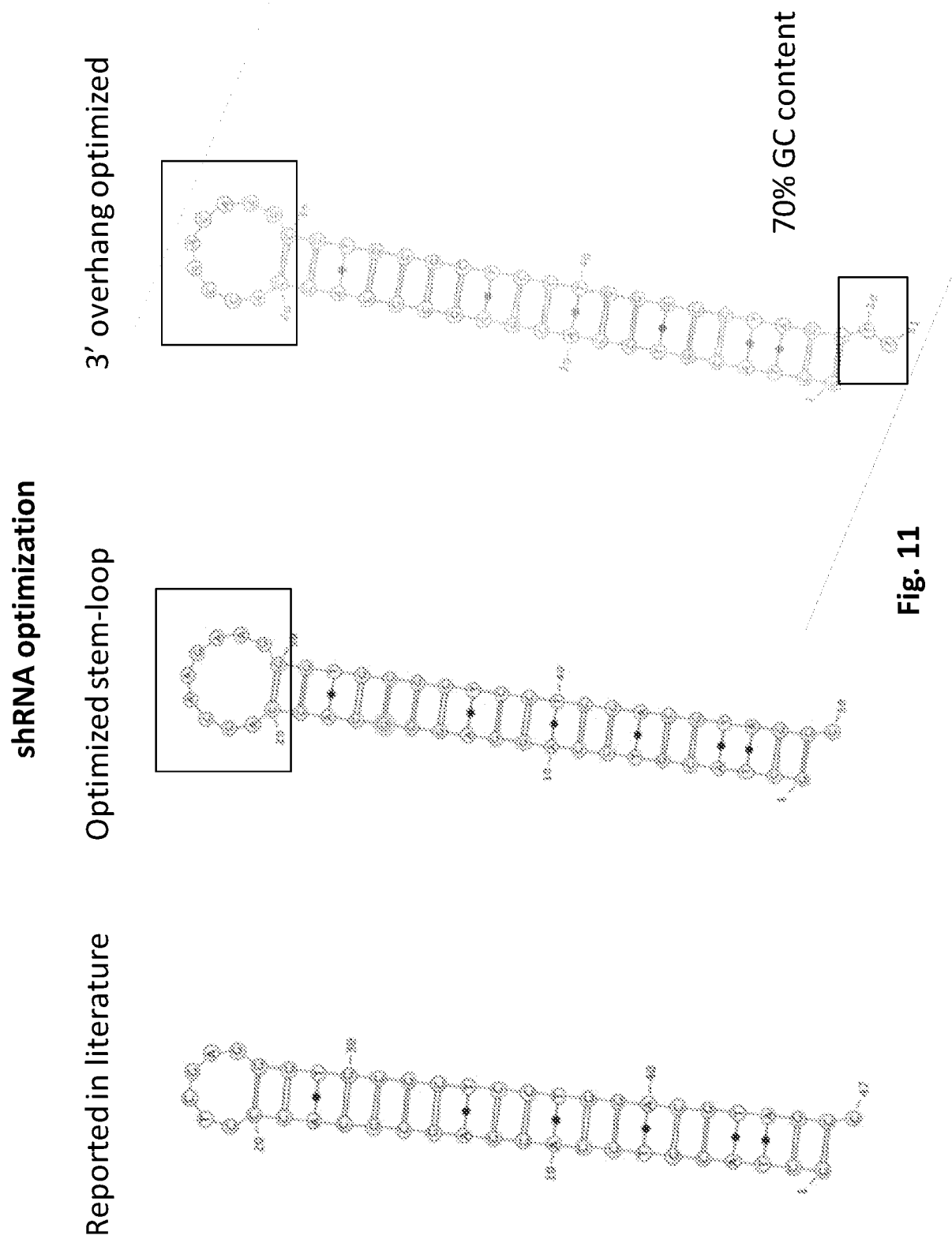


Fig. 11

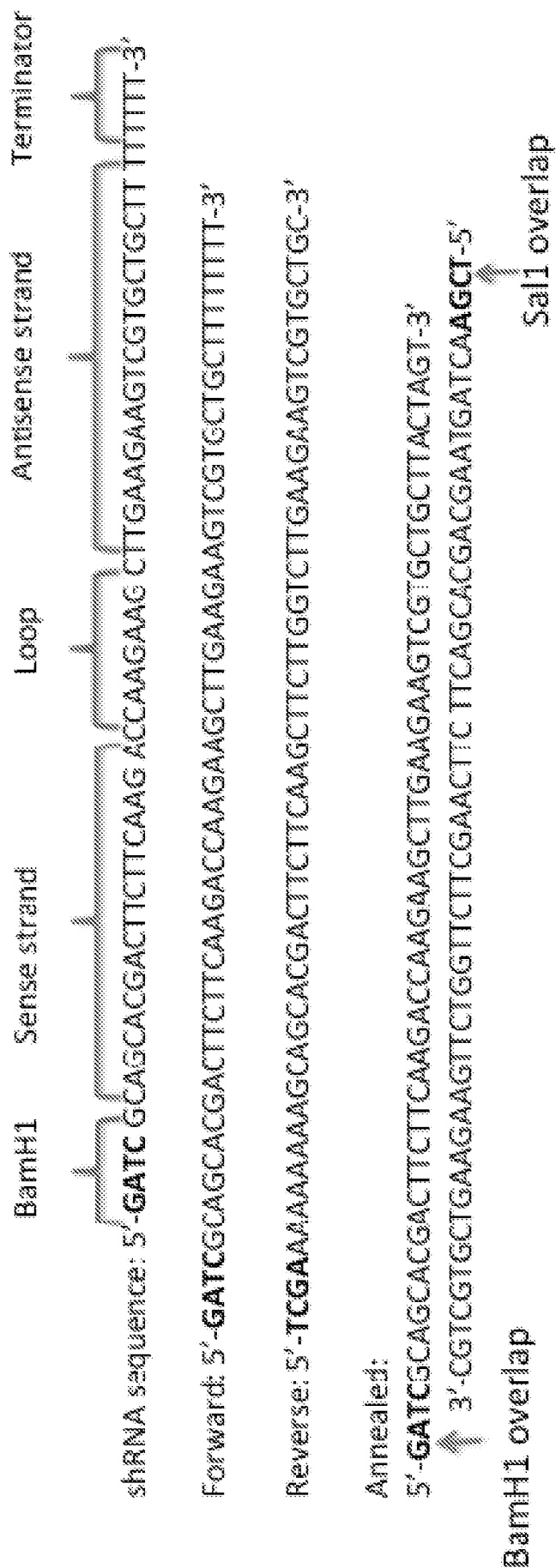
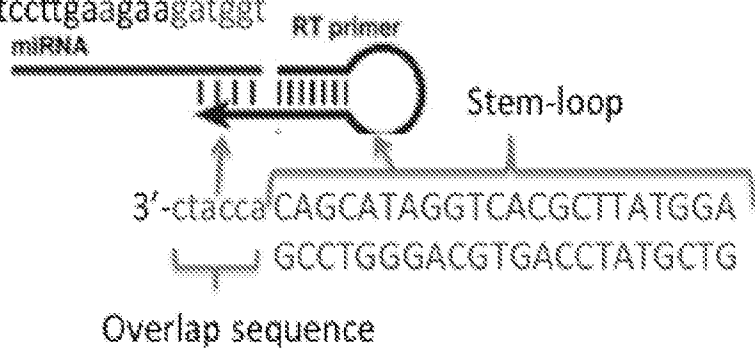


Fig. 12

GFP gene sequence: 5'-accatcttcttcaaggacgac

Mature miRNA: 5'-gtcgtccttgaagaagatggt



Forward primer: 5'-CAACGCAgtcgtccttgaag

Fig. 13

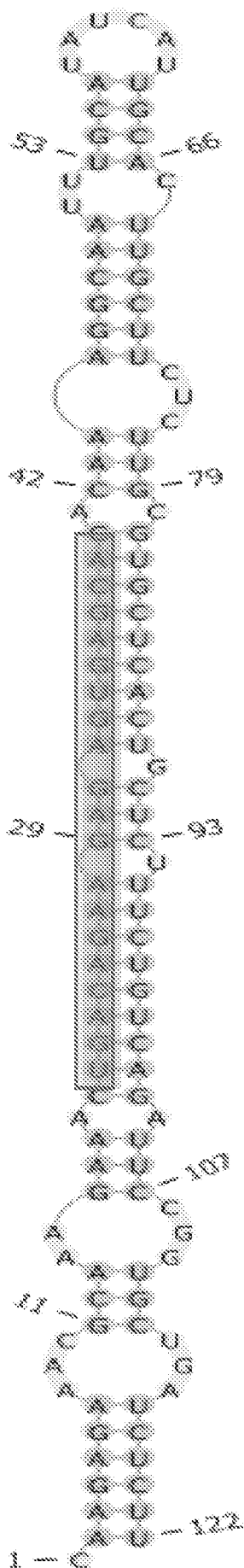


Fig. 14A

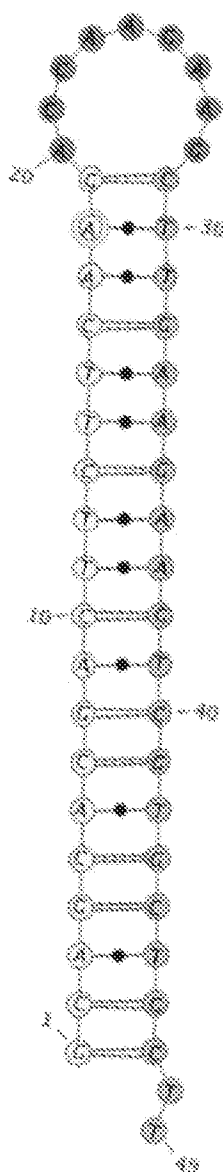


Fig. 14B

Optimized shRNA sequence

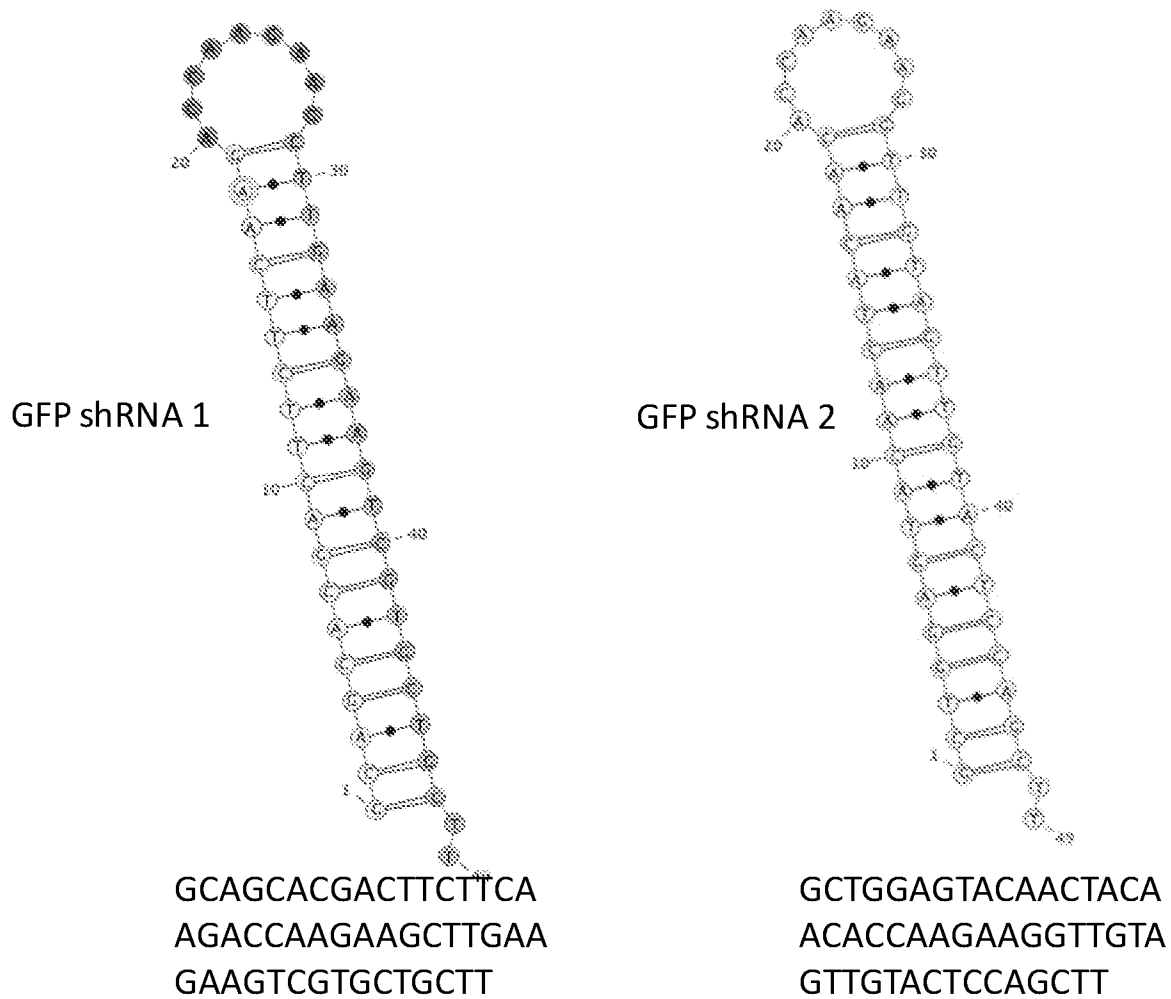


Fig. 15

Kras G12D2 vector sequence

ASCCYTTCWTCGCTTCTTGMGAGTTCTTCTGAGCGGGACTCTGGGGTTCGGACTCTAGCTAG
AGGATCAATTCGGTACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTATTTTCTCC
ATAAATAATGTGTGAGTAGTTTCCCGATAAGGGAAATTAGGGTTCTTATAGGGTTTCGCTCATG
TGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTT
CTAATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATCGATCCACCGCGGAACTCGACTTGC
CTCCGCACAATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTTCATACAGTTTTTTTTTG
TTTATCAGCTTACATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTTCCATTCGGAGT
TTTTGTATCTTGTTTCATAGTTTGTCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATT
TGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAAGGCCCTGGGAATCT
GAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCAT
TTAAGTTGAAAACAATCTTCAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTA
TATACAGCTAGAGTCGAAGTAGTGATTGTCCCTTCGGGGACATCCGATAAATTGGAATYSGTTG
GAGCTGATGGCGTAGACCAAGAAGCTACGCCATCAGCTCCAACTTTTTTTTTCGAC

Fig. 16

Kras3 Vector sequence

ASCCCATCWTCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGGACTCTAGCTAG
AGGATCATAAGTTACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCtCTcTCTATTTTCTCCATAA
ATAATGTGTGAGTAGTTTCCCGATAAGGGAAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGA
GCATATAAGAAACCCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCT
AAAACCAAAATCCAGTACTAAAATCCAGATCGATCCACCGCGGAACTCGACTTGCCTTCCGCACA
ATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTTCATACAGTTTTTTTTTGTATCAGCTTA
CATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTAACTTTCCATTTCGGAGTTTTTGTATCTTGTT
TCATAGTTTGTCCCAGGATTAGAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACA
TCTTCATTCTTAAGATATGAAGATAATCTTCAAAAGGCCCTGGGAATCTGAAAGAAGAGAAGCA
GGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTT
CAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTA
GTGATTGTCCCTTCGGGGACATCCGATAAAATTGGATYSGTTGGRRGCTGGTGGCGTAGCTTCTT
GGTCTACGCCACCAGCTCCAACTTTTTTTTTCGACGTCCGCAAAAATCACCAGTCTCTCTCTACAAA
TCTATCTCTCTATTTTTCTCCARRAATAATGKTGTGRAGTAG

Fig. 17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/18973

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/113, 15/82, 15/09, 15/10; A61P 35/00, 37/02; A61K 36/28, 31/713, 31/7105 (2016.01)
 CPC - C12N 15/113, 15/8218, 15/8285, 15/8261, 15/8214, 15/8257, 15/8258; A61K 36/28, 31/713, 31/7105
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61P 35/00, 37/02; A61K 36/28, 31/713, 31/7105; C12N 15/09, 15/10, 15/82, 15/113 (2016.01)
 CPC: C12N 15/113, 15/8214, 15/8218, 15/8257, 15/8258, 15/8285, 15/8261; A61K 36/28, 31/713, 31/7105

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; PubMed; Google; Google Scholar; Google Patents; KEYWORDS: plant, expression vector, promoter, heterologous, nucleotide, sequence, siRNA, transcription, terminator

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2014/0007296 A1 (HUANG, X et al.) January 2, 2014; abstract; paragraphs [0023], [0037], [0056], [0066], [0069]-[0071], [0073], [0077], [0080], [0092], [0097], [0112], [0118], [0132], [0135]	1-4, 11-17 --- 5-10, 18-21, 22A-B, 23-25
Y	WO 2014/106838 A2 (A.B. SEEDS LTD.) July 10, 2014; abstract; page 12, lines 15-18; page 14, lines 5-11; page 67, lines 20-22; claims 2, 5	5-8
Y	US 2010/0286241 A1 (XIE, F et al.) November 11, 2010; abstract; paragraphs [0019], [0022], [0087], [0141], [0147]	9-10, 21, 22A-B, 23-25
Y	US 2009/0022705 A1 (DANIELL, H) January 22, 2009; abstract; paragraphs [0012], [0013], [0017], [0021], [0024], [0051]	18-21, 22A-B, 23-25

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 June 2016 (27.06.2016)

Date of mailing of the international search report

26 JUL 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
 P.O. Box 1450, Alexandria, Virginia 22313-1450
 Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/18973

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: