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(54) Title: METHOD TO TRIGGER RNA INTERFERENCE

(57) Abstract: A method to generate siRNAs *in vivo* is described, as are constructs and compositions useful in the method. The method does not depend on the use of DNA or synthetic constructs that contain inverted duplications or dual promoters so as to form perfect or largely double-stranded RNA. Rather, the method depends on constructs that yield single-stranded RNA transcripts, and exploits endogenous or *in vivo*-produced miRNAs or siRNAs to initiate production of siRNAs. The miRNAs or siRNAs guide cleavage of the transcript and set the register for production of siRNAs (usually 21 nucleotides in length) encoded adjacent to the initiation cleavage site within the construct. The method results in specific formation of siRNAs of predictable size and register (phase) relative to the initiation cleavage site. The method can be used to produce specific siRNAs *in vivo* for inactivation or suppression of one or more target genes or other entities, such as pathogens.

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METHOD TO TRIGGER RNA INTERFERENCE

STATEMENT OF GOVERNMENT SUPPORT

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REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application number 60/642,126, filed January 7, 2005, the content of which is incorporated herein by reference in its entirety.

FIELD

This disclosure relates to methods of regulating gene expression *in vivo* in plant, fungi, and invertebrate cells, as well as constructs and compositions useful in such methods. Further, it relates to RNAi-inducing nucleic acid constructs having a microRNA or siRNA target sequence (initiator sequence) and one or more siRNA-generating sequences directed to one or more target genes or RNAs, whereby the siRNA-generating sequences are in 21-nucleotide register with the cleavage site guided by the microRNA or siRNA initiator.

BACKGROUND

Mechanisms that suppress the expression of specific cellular genes, viruses or mobile genetic elements (such as transposons and retroelements) are critical for normal cellular function in a variety of eukaryotes. A number of related processes, discovered independently in plants (Matzke *et al., Curr. Opin. Genet. Dev.* 11:221-227, 2001), animals (Fire *et al., Nature*, 391:806-811, 1998) and fungi (Cogoni, *Annu. Rev. Microbiol.* 55:381-406, 2001), result in the RNA-directed inhibition of gene expression (also known as RNA silencing). Each of these processes is triggered by molecules containing double-stranded RNA (dsRNA) structure, such as transcripts containing inverted repeats or double-stranded RNA intermediates formed during RNA virus replication. Non-dsRNAs, also referred to as aberrant RNAs, may also function as initiators of RNA silencing. Such aberrant RNAs may be converted into dsRNAs by silencing-associated RNA-dependent RNA polymerases (RDRs), which have been identified in plants, fungi and *C. elegans* (Tuschl, *ChemBiochem*, 2:239-245, 2001).

Two major classes of small RNAs have been characterized: short interfering RNAs (siRNAs) and microRNAs (miRNAs). The primary transcripts that eventually form miRNAs are transcribed from non-protein-coding miRNA genes. These transcripts form hairpin structures that are then processed by Dicer (or by Dicer-like activities in plants) to yield small RNA duplexes containing 2-base overhangs at each 3' end. The mature single-stranded miRNA approximately 20-22 nucleotides in length forms by dissociation of the two strands in the duplex, and is selectively

incorporated into the RNA-Induced Silencing Complex, or RISC (Zamore, *Science*, 296:1265-1269, 2002; Tang *et al., Genes Dev.*, 17:49-63, 2003; Xie *et al., Curr. Biol.* 13:784-789, 2003).

siRNAs are similar in chemical structure to miRNAs, however siRNAs are generated by the cleavage of relatively long double-stranded RNA molecules by Dicer or DCL enzymes (Zamore, *Science*, 296:1265-1269, 2002; Bernstein *et al., Nature,* 409:363-366, 2001). In animals and plants, siRNAs are assembled into RISC and guide the sequence specific ribonucleolytic activity of RISC, thereby resulting in the cleavage of mRNAs, viral RNAs or other RNA target molecules in the cytoplasm. In the nucleus, siRNAs also guide heterochromatin-associated histone and DNA methylation, resulting in transcriptional silencing of individual genes or large chromatin domains.

MicroRNAs in plants and animals function as posttranscriptional regulators of genes involved in a wide range of cellular processes (Bartel, *Cell* 116:281-297, 2004; He & Hannon, *Nat Rev Genet* 5:522-531, 2004). In the plant *Arabidopsis thaliana*, miRNAs regulate mRNAs encoding at least twelve families of transcription factors, several miRNA metabolic factors, and proteins involved in stress responses, metabolism, and hormone signaling (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Llave *et al.*, *Science* 297:2053-2056, 2002b; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004a; Xie *et al.*, *Curr Biol* 13:784-789, 2003). Plant miRNAs target a disproportionately high number of genes with functions in developmental processes, including developmental timing, control of cell proliferation, meristem cell function, and patterning. Global disruption of miRNA biogenesis or function, or specific disruption of miRNA-target interactions, can result in severe developmental abnormalities (Achard *et al.*, *Development* 131:3357-3365, 2004; Chen, *Science* 303:2022-2025, 2004; Emery *et al.*, *Curr Biol* 13:1768-1774, 2003; Juarez *et al.*, *Nature* 428:84-88, 2004; Kidner & Martienssen, *Nature* 428:81-84, 2004; Laufs *et al.*, *Development* 131:4311-4322, 2004; Mallory *et al.*, *Curr Biol* 14:1035-1046, 2004; Palatnik *et al.*, *Nature* 425:257-263, 2003; Tang *et al.*, *Genes & Dev* 17:49-63 2003; Vaucheret *et al.*, *Genes Dev* 18:1187-1197, 2004), indicating that miRNA-based regulation is crucial for normal growth and development. This idea is reinforced by the conservation of most miRNAs and their corresponding targets through significant evolutionary time (Bartel, *Cell* 116:281-297, 2004). MicroRNAs have been identified by direct cloning methods and computational prediction strategies (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Llave *et al.*, *Plant Cell* 14:1605-1619, 2000a; Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002; Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004).

Plant miRNAs usually contain near-perfect complementarity with target sites, which are found most commonly in protein-coding regions of the genome. As a result, most (but not all) plant miRNAs function to guide cleavage of targets through a mechanism similar to the siRNA-guided mechanism associated with RNAi (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Llave *et al.*, *Science* 297:2053-2056, 2002; Tang *et al.*, *Genes & Dev* 17:49-63 2003). In contrast, animal miRNAs contain relatively low levels of complementarity to their target sites, which are most commonly found in multiple copies within 3' untranslated regions of the target transcript (Lewis *et al.*, *Cell* 115:787-798, 2003; Rajewsky & Socci, *Dev Biol* 267:529-535,

2004; Stark *et al.*, *PLoS Biol* 1:E60, 2003). Most animal miRNAs do not guide cleavage, but rather function to repress expression at the translational or co-translational level (Ambros, *Cell* 113:673-676, 2003; He & Hannon, *Nat Rev Genet* 5:522-531, 2004). At least some plant miRNAs may also function as translational repressors (Aukerman & Sakai, *Plant Cell* 15:2730-2741, 2003; Chen, *Science* 303:2022-2025, 2004). Translation repression is not an inherent activity of animal miRNAs, as miRNAs will guide cleavage if presented with a target containing high levels of complementarity (Doench *et al.*, *Genes Dev* 17:438-442, 2003; Hutvagner & Zamore, *Science* 297:2056-2060, 2002; Yekta *et al.*, *Science* 304:594-596, 2004; Zeng *et al.*, *Proc Natl Acad Sci U S A* 100:9779-9784, 2003).

MicroRNAs form through nucleolytic maturation of genetically defined RNA precursors that adopt imperfect, self-complementary foldback structures. Processing yields a duplex intermediate (miRNA/miRNA*) that ultimately provides the miRNA strand to the effector complex, termed RISC (Khvorova *et al.*, *Cell* 115:209-216, 2003; Schwarz *et al.*, *Cell* 115:199-208, 2003). Plants contain four DICER-LIKE (DCL) proteins, one of which (DCL1) is necessary for maturation of most or all miRNA precursors (Kurihara & Watanabe, *Proc Natl Acad Sci U S A* 101:12753-12758, 2004; Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002; Schauer *et al.*, *Trends Plant Sci* 7:487-491, 2002). The DCL1 protein contains an RNA helicase and two RNaseIII-like domains, a central PAZ domain and C-terminal dsRNA binding motifs. Animal miRNA precursor processing requires Drosha, another RNaseIII domain protein, and Dicer in sequential nucleolytic steps (Lee *et al.*, *Nature* 425:415-419, 2003). HEN1 participates in miRNA biogenesis or stability in plants via a 3' methylase activity (Boutet *et al.*, *Curr Biol* 13:843-848, 2003; Park *et al.*, *Curr Biol* 12:1484-1495, 2002). The dsRNA-binding HYL1 protein is necessary for miRNA biogenesis in cooperation with DCL1 and HEN1 in the nucleus. Based on sequence similarity, HYL1 has been suggested to function like animal R2D2, which is required post-processing during RISC assembly (Han *et al.*, *Proc Natl Acad Sci U S A* 101:1093-1098, 2004; Liu *et al.*, *Science* 301:1921-1925, 2003; Pham *et al.*, *Cell* 117:83-94, 2004; Tomari *et al.*, *Science* 306:1377-1380, 2004; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004a). In animals, Exportin-5 (Exp5) regulates the transport of pre-miRNAs from the nucleus to the cytoplasm by a Ran-GTP-dependent mechanism (Bohnsack *et al.*, *RNA* 10:185-191, 2004; Lund *et al.*, *Science* 303:95-98, 2003; Yi *et al.*, *Genes Dev* 17:3011-3016, 2003). In *Arabidopsis*, HST may provide a related function to transport miRNA intermediates to the cytoplasm (Bollman *et al.*, *Development* 130:1493-1504, 2003). Active miRNA-containing RISC complexes in plants almost certainly contain one or more ARGONAUTE proteins, such as AGO1 (Fagard *et al.*, *Proc Natl Acad Sci USA* 97:11650-11654, 2000; Vaucheret *et al.*, *Genes Dev* 18:1187-1197, 2004). Argonaute proteins in animals were shown recently to provide the catalytic activity for target cleavage (Liu *et al.*, *Science* 305:1437-1441, 2004; Meister *et al.*, *Mol Cell* 15:185-197, 2004).

In addition to miRNAs, plants also produce diverse sets of endogenous 21-25 nucleotide small RNAs. Most of these differ from miRNAs in that they arise from double-stranded RNA (rather than imperfect foldback structures), in some cases generated by the activity of RNA-DEPENDENT

RNA POLYMERASEs (RDRs). *Arabidopsis* DCL2, DCL3, DCL4, RDR1, RDR2 and RDR6 have known roles in siRNA biogenesis (Dalmay *et al.*, *Cell* 101:543-553, 2000; Mourrain *et al.*, *Cell* 101:533-542, 2000; Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004b; Xie *et al.*, *PLoS Biol* 2:642-652, 2004; Yu *et al.*, *Mol Plant Microbe Interact* 16:206-216, 2003). For example, DCL3 and RDR2 cooperate in the heterochromatin-associated RNAi pathway, resulting in ~24-nucleotide siRNAs from various retroelements and transposons, 5S rDNA loci, endogenous direct and inverted repeats, and transgenes containing direct repeats (Xie *et al.*, *PLoS Biol* 2:642-652, 2004; Zilberman *et al.*, *Science* 299:716-719, 2003). RDR6 functions in posttranscriptional RNAi of sense transgenes, some viruses, and specific endogenous mRNAs that are targeted by *trans*-acting siRNAs (ta-siRNAs) (Dalmay *et al.*, *Cell* 101:543-553, 2000; Mourrain *et al.*, *Cell* 101:533-542, 2000; Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004b; Yu *et al.*, *Mol Plant Microbe Interact* 16:206-216, 2003). Ta-siRNAs arise from transcripts that are recognized by RDR6, in cooperation with SGS3, as a substrate to form dsRNA. The dsRNA is processed accurately in 21-nucleotide steps by DCL1 to yield a set of "phased" ta-siRNAs. These ta-siRNAs interact with target mRNAs to guide cleavage by the same mechanism as do plant miRNAs (Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004).

There is a need to develop methods and constructs that can be used to induce targeted RNAi *in vivo*. It is to such methods and constructs, and related compositions, that this disclosure is drawn.

SUMMARY OF THE DISCLOSURE

Provided herein are methods of generating one or more siRNAs *in vivo*; also provided are constructs and compositions useful in the methods. The methods do not depend on DNA or other synthetic nucleic acid molecules that contain inverted duplications (repeats) or dual promoters to form perfect or largely double-stranded RNA. Rather, the methods employ constructs that yield single-stranded RNA transcripts, and take advantage of endogenous (native or heterologous) or *in vivo*-produced miRNAs or siRNAs to initiate production of siRNAs from an engineered RNAi-triggering cassette. The miRNAs or siRNAs guide cleavage of the transcript and set the register (phase) for production of siRNAs (usually 21 nucleotides in length) encoded adjacent to the initiation cleavage site within the construct. The methods result in specific formation of siRNAs of predictable size and register (phase) relative to the initiation cleavage site. The method can be used to produce specific siRNAs *in vivo* for inactivation or suppression of one or more target genes or other entities, such as pathogens or pests (*e.g.*, viruses, bacteria, nematodes). No exogenous hairpin or foldback structure is required in the provided constructs in order to generate siRNAs or to carry out RNAi-like inhibition of target gene(s).

Also provided are methods, and constructs for use in such methods, where the siRNAs are produced in a tissue-specific, cell-specific, or other regulated manner.

Further, transformed cells and organisms that contain a transgene including at least one RNAi-triggering cassette are also provided by this disclosure. For instance, transgenic fungi,

invertebrate animals, and plants are provided that contain at least one RNAi-triggering cassette, which, when transcribed, produces at least one siRNA molecule complementary to a target sequence to be inhibited in that organism.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Refined prediction and validation of miRNA target genes in *Arabidopsis*. (A) Flowchart for miRNA target identification. The number of small RNAs (or targets) passing a filter is shown in parentheses. Predicted targets are classified into 5 bins based on validation data. The false negative rate in Bins 1 and 2 are based on 66 and 28 targets in the 'Rule development set', respectively (see Table 3). (B) Percent of mismatched and G:U base-pairs at each position of the Rule development set targets. Position 1 corresponds to the 5' end of the miRNA. (C) Minimum Free Energy (MFE) ratio of the Rule development set target duplexes. Black circles indicate Rule development set validated targets, open circles indicate rule development set targets only predicted computationally. (D) Number of predicted targets genes for a given miRNA-target duplex score, filtered by duplexes with an MFE ratio ≥ 0.73. Total predicted targets (open triangles) and captured targets in the Rule development set (open circles) are shown. Total targets in the Rule development set (94) is indicated by the dashed line.

Figure 2. miRNA-target duplexes. (A) Target duplexes from Bin 1 validated in this study. (B) Duplexes for predicted miRNA targets in Bin 3.

Figure 3. Validation of miRNA targets by 5'RACE. (A) Protein-coding miRNA targets. The miRNA-target duplex is highlighted, with the fraction of cloned PCR products terminating at a given position in the target validation assay (Llave *et al.*, *Science* 297:2053-2056, 2002) indicated above the duplex. The distribution of cleavage products across all five predicted miR399 target sites is displayed above the schematic representation of At2g33770. (B) Non-coding miRNA targets predicted by the EST database search. Each of these targets corresponds to a ta-siRNA-generating primary transcript.

Figure 4. Characterization and expression profiling of *Arabidopsis* small RNA biogenesis mutants. (A) Phenotype of *hst-15* and *rdr6-15* mutants. Rosettes (Col-0, *rdr6-15, hst-15*), first true leaf (Col-0, *rdr6-15*), bolt and flower (Col-0, *hst-15*) are shown. For array data in (b-e), normalized intensity is plotted as $\log_2$ of the fold change relative to the control sample for each mutant, thus zero represents no change in transcript abundance. (B) Profile of 81 of 94 miRNA target transcripts predicted previously and in this study (Bins 1 and 2, Figure 1). (C) Profile of 12 of 18 miRNA targets genes predicted in this study. The solid lines indicate new targets from existing target families (Bin 3, Figure 1), and the dashed lines indicate novel miRNA targets (Bin 4, Figure 1). Non-validated targets in Bin 5 are not shown. (D) Profile of transcripts significantly co-affected (P>0.01) in *dcl1-7, hen1-1*, and *rdr6-15*. (E) Profile of 93 predicted miRNA target transcripts (light lines), and

PCA component 1 (dark line). (F) Cladogram of the small RNA biogenesis mutant series. The correlation among groups (r x 100) is shown at each node. (G) Scatterplots of all genes showing normalized intensity values representing fold change (*hyl1-2* vs. *hst-15*, *dcl1-7* vs. *hen1-1*, *hyl1-2* vs. *dcl3-1*).

5 **Figure 5.** In-phase processing of *trans*-acting siRNAs directed by miR173 as the initiator. (A-C) Diagrammatic representation of the three *TAS1* and on *TAS2* loci. The naming convention used is TAS (for *Trans*-Acting SiRNA). Ta-siRNAs with functional evidence are shown by the systematic nomenclature (see text for details). The 21 nucleotide phase is indicated by brackets, with the first position starting from the miR173-guided cleavage site. The relative positions from the 10 cleavage site are designated 3'D1, 3'D2, etc. Positions for which small RNAs are represented in the ASRP sequence database are listed with the ASRP ID number. Relative positions of the *At2g39675* and *At3g39680* loci in *Arabidopsis* chromosome 2 are shown in (C). (D) Detection and validation of ta-siRNAs by small RNA blot analysis. Small RNAs were detected using specific oligo probes, except *At3g39680* antisense small RNAs which were detected with a 469 nucleotide radiolabeled 15 RNA transcript. (E) Validation of siR255 target genes by 5' RACE, and predicted *Ag3g39681 (TAS2)*.3'D6(-) targeted PPR genes.

Figure 6. In-phase processing of *TAS3*-derived *trans*-acting siRNAs guided by miR390. (A) Diagrammatic representation of the miR390 target locus, *TAS3* (At3g17185). Labeling is as in Figure 5, but with the 21-nucleotide phased positions designated 5'D1, 5'D2, etc, starting at the 20 miR390-guided cleavage site. The two siRNAs that are predicted to guide cleavage of ARF3 and ARF4 are indicated. (B) Detection and validation of ta-siRNAs from the *TAS3* locus. (C) T-Coffee program alignment of *TAS3* orthologs in plants showing conservation of predicted TAS3 ta-siRNAs and miR390 target site. High levels of conservation are designated by light shading. (D) PLOTCON program similarity score (21 nt window) derived from alignment of 18 *ARF3* and *ARF4* genes from 25 16 species, over a 600 nt region. Two highly conserved regions are indicated by A and B, which are *TAS3* ta-siRNA target sites. Below, validation of small RNA directed cleavage of ARF3 and ARF4 by 5' RACE. The predicted *TAS3*-derived ta-siRNAs are shown below complementary regions of ARF3 and ARF4 sequences. (E) Consensus phylogenetic tree of the ARF family, showing miRNA and ta-siRNA regulated branches. Bayesian posterior probability was 100 except for labeled nodes.

30 **Figure 7.** Model for miRNA-directed formation of ta-siRNAs

Figure 8. Validation of miRNAs in *A. thaliana*. (A) Prediction flowchart for miRNA validation. The number of small RNAs passing a filter is shown in parentheses. (B) Predicted secondary structure of miRNA precuros validated in this study. (C,D) Small RNA blot analysis of miRNAs. miR159 and miR167 are shown as traditional miRNA controls, *AtSN1* is shown as an 35 siRNA control. Ethidium bromide-stained gel (tRNA and 5S RNA zone) is shown at the bottom. Wildtype controls (Col-0 and La-er) are shown next to respective miRNA metabolism mutants (C) and ta-siRNA biogenesis mutants (*rdr6-11* and *sgs3-11*) or transgenic plants expressing viral silencing (D).

Figure 9. Strategy to map *Arabidopsis MIRNA* gene transcription start sites. (A) Schematic representation of a generic *MIRNA* transcript (top), and control *SCL6-IV* mRNA (middle) and miR171-guided cleavage product (bottom). The relative positions of oligonucleotides used in 5'RACE reactions are shown. (B) RLM-5'RACE reactions using poly(A)$^+$-selected RNA that was pretreated with calf intestinal phosphatase (CIP) plus tobacco acid pyrophosphatase (TAP, even-numbered lanes) or with buffer (odd-numbered lanes) prior to adaptor ligation. The 5'RACE products for *SCL6-IV*-specific RNAs (lanes1-4) and three *MIRNA* loci (lanes 5-10) were resolved on a 2% agarose gel. Gene-specific primers used for 5'RACE are indicated above each lane.

Figure 10. *MIRNA* gene transcript start sites and core promoter elements. (A) Base frequency at *MIRNA* transcription initiation sites (n=63). (B) Genomic sequences (-50 to +10 relative to start sites) around 63 start sites (red letters) from 47 *Arabidopsis MIRNA* loci. Putative TATA motifs (bold) are indicated. These sequences correspond to SEQ ID NOs: 286-348. (C) Occurrence of high-scoring TATA motifs within a 250-nucleotide (-200 to +50) genomic context for 63 *MIRNA* transcripts.

Figure 11. Graphic representation of an artificial ta-siRNA construct made in the *TAS1c* context. The construct contains two 21-nt siRNA modules. The represented construct contains siRNAs desiged to target mRNAs for *Arabidopsis* phytoene desaturase (PDS).

Figure 12. Demonstration of artificial ta-siRNA biogenesis and activity in *Nicotiana benthamiana*. Introduction of each construct into *N. benthamiana* in a transient assay resulted in miR173-dependent formation of ta-siRNAs. In the case of 35S:TAS1cGFPd3d4 (A,B), the artificial ta-siRNA construct was co-expressed with a functional GFP gene. Expression of at least one artificial tasiRNA was detected in a miR173-dependent manner, by blot assay using each construct (GFP: A; PDS: C; PID: D). The GFP gene was silenced by the artificial GFP ta-siRNAs in a miR173-dependent manner (B). The same miR173 and tasiRNA255 controls were used for PDS, PID, and GFP siRNA assays)

Figure 13. Artificial ta-siRNA biogenesis and activity in transgenic *Arabidopsis*. The PDS artificial ta-siRNA-generating construct was introduced into wild-type (Col-0) *Arabidopsis* and *rdr6*-15 mutant plants. Both strong and weak loss-of-function PDS phenotypes were detected, but only in wt plants. The rdr6-15 mutant plants lack a critical factor for ta-siRNA biogenesis.

Figure 14. Reconstruction of *TAS1a*, *TAS1b*, *TAS1c*, and *TAS2* ta-siRNA Biogenesis in a Transient Expression Assay using *N. benthamiana*. (A and B) Constructs with wild-type miR173 target sites. Constructs were expressed or coexpressed as indicated above the blot panels. The small RNAs detected in blot assays are shown to the right of each panel. Duplicate biological samples were analyzed for most treatments. (C) Constructs with mutagenized target site or miR173 sequences. Target site and miRNA combinations tested are illustrated schematically above the blot panels. Mutagenized positions are in bold. The miR173res1 probe hybridized to both the miR173 and miR173res1 sequences.

BREIF DESCRIPTION OF THE SEQUENCE LISTING

The nucleic acid sequences listed below are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. § 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included in embodiments where it would be appropriate. In the accompanying sequence listing:

SEQ ID NOs: 1-142 and 281-285 are representative target (initiator) sequences. The initiator sequences are shown as RNA; it is understood that the corresponding DNA sequence would comprise a T in place of any U. The sequences are broken out based on the miRNA complementary to the provided initiator (target) sequences. The corresponding miRNA sequence can be deduced for each target sequence; it is the reverse complement formed of RNA.

SEQ ID NOs: 143-154 are predicted miRNA candidates (shown as RNA) that were tested experimentally, and which are discussed in Example 5.

SEQ ID NOs: 155-206 are miRNA sequences (shown as RNA), which are discussed in Example 5.

SEQ ID NOs: 207-276 are validated miRNA sequences cloned from Arabidopsis small RNA libraries (shown as RNA), and which are discussed in Example 5.

SEQ ID NO: 277 is the nucleic acid sequence of an artificial ta-siRNA locus targeting *Arabidopsis* gene encoding GFP.

SEQ ID NO: 278 is the nucleic acid sequence of an artificial ta-siRNA locus targeting *Arabidopsis* gene encoding phytoene desaturase (PDS).

SEQ ID NO: 279 is the nucleic acid sequence of an artificial ta-siRNA locus targeting *Arabidopsis* gene encoding PINOID (PID).

SEQ ID NO: 280 is an example of a sequence that would be contained in DNA construct containing SEQ ID NO: 1 as an initiator sequence.

SEQ ID NOs: 286-348 are genomic sequences (-50 to +10 relative to start sites) of 63 start sites in 47 *Arabidopsis* miRNA loci. These are shown graphically in Figure 10B.

SEQ ID NOs: 349 to 614 are prminers used in 3'RACE confirmation sequencing.

DETAILED DESCRIPTION

I. Abbreviations

AGO	Argonaute
asRNA	antisense RNA
cDNA	complementary DNA
DCL	dicer-like
dsRNA	double-stranded RNA
GFP	green fluorescent protein
LKR	lysine ketoglutarate reductase
miRNA	microRNA
nt	nucleotide
PID	PINOID
PDS	phytoene desaturase
PTGS	post-transcriptional gene silencing

RDR	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	small interfering RNA
ssRNA	single stranded RNA
ta-siRNA	*trans*-acting siRNA
TGS	transcriptional gene silencing

II. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the invention, the following non-limiting explanations of specific terms are provided:

21-Nucleotide Phasing: An incremental 21-nucleotide register, starting at an initiator cleavage site, in which cleavage is mediated by a RISC guided by either a miRNA or siRNA. Phasing defines a set of 21 nucleotide segments in linear, end-to-end orientation, either to the 5' or 3' side of the initiator cleavage site, or both. Formation of the 21-nucleotide siRNAs in phase with the cleavage site depends on the activity of a DICER or DICER-LIKE enzyme.

Agent: Any substance, including, but not limited to, an antibody, chemical compound, small molecule, therapeutic, nucleic acid, peptide mimetic, peptide, or protein. An agent can increase or decrease the level of miRNA or siRNA expression or production.

Agronomic trait: Characteristic of a plant, which characteristics include, but are not limited to, plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance are agronomic traits. In the plants of this disclosure, the expression of identified recombinant DNA, *e.g.* for gene suppression, confers an agronomically important trait, *e.g.* increased yield. An "enhanced agronomic trait" refers to a measurable improvement in an agronomic trait including, but not limited to, yield increase, including increased yield under non-stress conditions and increased yield under environmental stress conditions. Stress conditions may include, for example, drought, shade, fungal disease, viral disease, bacterial disease, insect infestation, nematode infestation, cold temperature exposure, heat exposure, osmotic stress, reduced nitrogen nutrient availability, reduced phosphorus nutrient availability and high plant density. "Yield" can be affected by many properties including without limitation, plant height, pod number, pod position on the plant, number of internodes, incidence of pod shatter, grain size, efficiency of nodulation and nitrogen fixation, efficiency of nutrient assimilation, resistance to biotic and abiotic stress, carbon assimilation, plant architecture, resistance to lodging, percent seed germination, seedling vigor, and juvenile traits. Yield can also affected by efficiency of germination (including germination in stressed conditions), growth rate (including growth rate in stressed

conditions), ear number, seed number per ear, seed size, composition of seed (starch, oil, protein) and characteristics of seed fill. Increased yield may result from improved utilization of key biochemical compounds, such as nitrogen, phosphorous and carbohydrate, or from improved responses to environmental stresses, such as cold, heat, drought, salt, and attack by pests or pathogens. Recombinant DNA used in this disclosure can also be used to provide plants having improved growth and development, and ultimately increased yield, as the result of modified expression of plant growth regulators or modification of cell cycle or photosynthesis pathways.

Altering level of production or expression: Changing, either by increasing or decreasing, the level of production or expression of a nucleic acid sequence or an amino acid sequence (for example a polypeptide, an siRNA, a miRNA, an mRNA, a gene), as compared to a control level of production or expression.

Antisense, Sense, and Antigene: DNA has two antiparallel strands, a 5' → 3' strand, referred to as the plus strand, and a 3' → 5' strand, referred to as the minus strand. Because RNA polymerase adds nucleic acids in a 5' → 3' direction, the minus strand of the DNA serves as the template for the RNA during transcription. Thus, an RNA transcript will have a sequence complementary to the minus strand, and identical to the plus strand (except that U is substituted for T).

Antisense molecules are molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA. Sense molecules are molecules that are specifically hybridizable or specifically complementary to the minus strand of DNA. Antigene molecules are either antisense or sense molecules directed to a DNA target. An antisense RNA (asRNA) is a molecule of RNA complementary to a sense (encoding) nucleic acid molecule.

Amplification: When used in reference to a nucleic acid, this refers to techniques that increase the number of copies of a nucleic acid molecule in a sample or specimen. An example of amplification is the polymerase chain reaction, in which a biological sample collected from a subject is contacted with a pair of oligonucleotide primers, under conditions that allow for the hybridization of the primers to nucleic acid template in the sample. The primers are extended under suitable conditions, dissociated from the template, and then re-annealed, extended, and dissociated to amplify the number of copies of the nucleic acid. The product of *in vitro* amplification can be characterized by electrophoresis, restriction endonuclease cleavage patterns, oligonucleotide hybridization or ligation, and/or nucleic acid sequencing, using standard techniques. Other examples of *in vitro* amplification techniques include strand displacement amplification (see U.S. Patent No. 5,744,311); transcription-free isothermal amplification (see U.S. Patent No. 6,033,881); repair chain reaction amplification (see WO 90/01069); ligase chain reaction amplification (see EP-A-320 308); gap filling ligase chain reaction amplification (see U.S. Patent No. 5,427,930); coupled ligase detection and PCR (see U.S. Patent No. 6,027,889); and NASBA™ RNA transcription-free amplification (see U.S. Patent No. 6,025,134).

Binding or stable binding: An oligonucleotide binds or stably binds to a target nucleic acid if a sufficient amount of the oligonucleotide forms base pairs or is hybridized to its target nucleic

acid, to permit detection of that binding. Binding can be detected by either physical or functional properties of the target:oligonucleotide complex. Binding between a target and an oligonucleotide can be detected by any procedure known to one skilled in the art, including both functional and physical binding assays. For instance, binding can be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation and the like.

Physical methods of detecting the binding of complementary strands of DNA or RNA are well known in the art, and include such methods as DNase I or chemical footprinting, gel shift and affinity cleavage assays, Northern blotting, dot blotting and light absorption detection procedures. For example, one method that is widely used, because it is simple and reliable, involves observing a change in light absorption of a solution containing an oligonucleotide (or an analog) and a target nucleic acid at 220 to 300 nm as the temperature is slowly increased. If the oligonucleotide or analog has bound to its target, there is a sudden increase in absorption at a characteristic temperature as the oligonucleotide (or analog) and the target disassociate from each other, or melt.

The binding between an oligomer and its target nucleic acid is frequently characterized by the temperature (T_m) at which 50% of the oligomer is melted from its target. A higher (T_m) means a stronger or more stable complex relative to a complex with a lower (T_m).

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and transcriptional regulatory sequences. cDNA may also contain untranslated regions (UTRs) that are responsible for translational control in the corresponding RNA molecule. cDNA is usually synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells or other samples.

Complementarity and percentage complementarity: Molecules with complementary nucleic acids form a stable duplex or triplex when the strands bind, or hybridize, to each other by forming Watson-Crick, Hoogsteen or reverse Hoogsteen base pairs. Stable binding occurs when an oligonucleotide remains detectably bound to a target nucleic acid sequence under the required conditions.

Complementarity is the degree to which bases in one nucleic acid strand base pair with (are complementary to) the bases in a second nucleic acid strand. Complementarity is conveniently described by the percentage, *i.e.*, the proportion of nucleotides that form base pairs between two strands or within a specific region or domain of two strands. For example, if 10 nucleotides of a 15-nucleotide oligonucleotide form base pairs with a targeted region of a DNA molecule, that oligonucleotide is said to have 66.67% complementarity to the region of DNA targeted.

Sufficient complementarity means that a sufficient number of base pairs exist between the oligonucleotide and the target sequence to achieve detectable binding, and disrupt or reduce expression of the gene product(s) encoded by that target sequence. When expressed or measured by percentage of base pairs formed, the percentage complementarity that fulfills this goal can range from as little as about 50% complementarity to full, (100%) complementary. In some embodiments, sufficient complementarity is at least about 50%, about 75% complementarity, or at least about 90%

or 95% complementarity. In particular embodiments, sufficient complementarity is 98% or 100% complementarity.

A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz *et al.*, *Methods Enzymol* 100:266-285, 1983, and by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Complementary: The base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

Control level: The level of a molecule, such as a polypeptide or nucleic acid, normally found in nature under a certain condition and/or in a specific genetic background. In certain embodiments, a control level of a molecule can be measured in a cell or specimen that has not been subjected, either directly or indirectly, to a treatment. A control level is also referred to as a wildtype or a basal level. These terms are understood by those of ordinary skill in the art.

Control plant: A control plant, *i.e.* a plant that does not contain a recombinant DNA that confers (for instance) an enhanced agronomic trait in a transgenic plant, is used as a baseline for comparison to identify an enhanced agronomic trait in the transgenic plant. A suitable control plant may be a non-transgenic plant of the parental line used to generate a transgenic plant. A control plant may in some cases be a transgenic plant line that comprises an empty vector or marker gene, but does not contain the recombinant DNA, or does not contain all of the recombinant DNAs in the test plant.

DICER-LIKE (DCL): Plant homologs of the animal protein DICER. Both DICER and DCL enzymes catalyze formation of small RNA duplexes from larger precursor RNA molecules. By way of example, *Arabidopsis thaliana* contains four *DCL* genes (*DCL1-DCL4*). DCL1 for instance catalyzes processing of fold-back precursors for miRNAs (GenBank Accession No. NM_099986; locus position At1g01040).

DNA (deoxyribonucleic acid): DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid (RNA)). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides (referred to as codons) code for each amino acid in a polypeptide, or for a stop signal. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

Unless otherwise specified, any reference to a DNA molecule is intended to include the reverse complement of that DNA molecule. Except where single-strandedness is required by the text herein, DNA molecules, though written to depict only a single strand, encompass both strands of a double-stranded DNA molecule.

Encode: A polynucleotide is said to encode a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated

to produce the mRNA for and/or the polypeptide or a fragment thereof. The anti-sense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

Expression: The process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (for example, siRNA, transfer RNA and ribosomal RNA). Thus, expression of a target sequence, such as a gene or a promoter region of a gene, can result in the expression of an mRNA, a protein, or both. The expression of the target sequence can be inhibited or enhanced (decreased or increased).

Fluorophore: A chemical compound, which when excited by exposure to a particular wavelength of light, emits light (*i.e.*, fluoresces), for example at a different wavelength than that to which it was exposed. Fluorophores can be described in terms of their emission profile, or "color." Green fluorophores, for example Cy3, FITC, and Oregon Green, are characterized by their emission at wavelengths generally in the range of 515-540 λ. Red fluorophores, for example Texas Red, Cy5 and tetramethylrhodamine, are characterized by their emission at wavelengths generally in the range of 590-690 λ.

Encompassed by the term "fluorophore" are luminescent molecules, which are chemical compounds which do not require exposure to a particular wavelength of light to fluoresce; luminescent compounds naturally fluoresce. Therefore, the use of luminescent signals eliminates the need for an external source of electromagnetic radiation, such as a laser. An example of a luminescent molecule includes, but is not limited to, aequorin (Tsien, *Ann. Rev. Biochem.* 67:509, 1998).

Examples of fluorophores are provided in U.S. Patent No. 5,866,366. These include: 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenyl-azophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein;

nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron .RTM. Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives.

Other fluorophores include thiol-reactive europium chelates that emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-227, 1997; *J. Biol. Chem.* 274:3315-3322, 1999).

Still other fluorophores include cyanine, merocyanine, styryl, and oxonyl compounds, such as those disclosed in U.S. Patent Nos. 5,268,486; 5,486,616; 5,627,027; 5,569,587; and 5,569,766, and in published PCT patent application no. US98/00475, each of which is incorporated herein by reference. Specific examples of fluorophores disclosed in one or more of these patent documents include Cy3 and Cy5, for instance.

Other fluorophores include GFP, Lissamine™, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Patent No. 5,800,996 to Lee *et al.*, herein incorporated by reference) and derivatives thereof. Other fluorophores are known to those skilled in the art, for example those available from Molecular Probes (Eugene, OR).

Gene Silencing: Gene silencing refers to lack of (or reduction of) gene expression as a result of, though not limited to, effects at a genomic (DNA) level such as chromatin re-structuring, or at the post-transcriptional level through effects on transcript stability or translation. Current evidence suggests that RNA interference (RNAi) is a major process involved in transcriptional and posttranscriptional gene silencing.

Because RNAi exerts its effects at the transcriptional and/or post-transcriptional level, it is believed that RNAi can be used to specifically inhibit alternative transcripts from the same gene.

Heterologous: A type of sequence that is not normally (*i.e.* in the wild-type sequence) found adjacent to a second sequence. In one embodiment, the sequence is from a different genetic source, such as a virus or organism, than the second sequence.

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

to the base pairing that occurs between to distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization, though waste times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11, herein incorporated by reference.

The following is an exemplary set of hybridization conditions and is not meant to be limiting.

Very High Stringency (detects sequences that share 90% sequence identity)

Hybridization:	5x SSC at 65°C for 16 hours
Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (detects sequences that share 80% sequence identity or greater)

Hybridization:	5x-6x SSC at 65°C-70°C for 16-20 hours
Wash twice:	2x SSC at RT for 5-20 minutes each
Wash twice:	1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (detects sequences that share greater than 50% sequence identity)

Hybridization:	6x SSC at RT to 55°C for 16-20 hours
Wash at least twice:	2x-3x SSC at RT to 55°C for 20-30 minutes each.

Initiator sequence: A nucleotide sequence of about 21 nucleotides in length that, when present in an RNA molecule, serves as a cleavage site that is recognized by a RISC guided by a miRNA or siRNA. Cleavage at an initiator sequence (usually between the tenth and eleventh nucleotide counted from the 3' end of the initiator sequence) sets the 21-nucleotide phasing within one or both RNA molecules that result after cleavage. These cleavage products, after conversion to double-stranded RNA, are subject to processing by Dicer or DCL enzymesusually in 21-nucleotide intervals upstream and/or downstream of the initiator sequence. In an engineered nucleic acid cassette as described herein, such in-phase cleavages release siRNAs from the cassette. Representative initiator sequences, also referred to as miRNA target sequences, are shown herein, including SEQ ID NOs: 1-142 and 281-285. Additional initiatior sequences will be known to those of ordinary skill in the art. See, for instance, sequences listed in the public database miRBase::Sequences (available on-line through the Sanger Insitute website, microrna.sanger.ac.uk/sequences/index.shtml); sequences in that database through Release 7.1 (October 2005) are included herein by reference.

In the following table of target (initiator) sequences (Table 1), the initiator cleavage site is indicated by a "~" symbol. The sequences are broken out based on the miRNA families. The

corresponding miRNA sequence(s) or miRNA family sequences are largely complementary to the target sequences. The gene name indicates a representative plant species for each sequence: At = *Arabidopsis thaliana*; Gh = *Gossypium hirsutum*; Gm = *Glycine max*; Hv = *Hordeum vulgare*; Le = *Lycopersicum esculentum*; Lj = *Lotus japonicus*; Mc = *Mesembryanthemum crystallinum*; Mt = *Medicago truncatula*; Os = *Oryza sativa*; Pg = *Pennisetum glaucum*; Pt = *Populus tremula*; Pv = *Plumbago zeylanica*; Sb = *Sorghum bicolor* ; So = *Saccharum officinarum*; Tc = *Theobroma cacao*; Ta = *Triticum aesitivum*; Vv = *Vitis vinifera*; Zm = *Zea mays*. Additional plants containing these sequences are discussed below. Validated miRNA target sequences have been confirmed experimentally using a cleavage site assay (Llave *et al.*, *Science* 297:2053-2056, 2002); predicted sequences have yet to be examined experimentally and identified *in vivo*, but were predicted computationally. Sequences that are known only in *Arabidopsis* are indicated.

Table 1

Gene	Target Sequence	Status	SEQ ID #
miR156 family target sequences – all plants			
At1g27370	GUGCUCUCUC~UCUUCUGUCA	Validated	1
At1g53160	CUGCUCUCUC~UCUUCUGUCA	Validated	2
At2g33810	UUGCUUACUC~UCUUCUGUCA	Predicted	3
At3g15270	CCGCUCUCUC~UCUUCUGUCA	Predicted	4
miR159 family target sequences – all plants			
At5g06100	UGGAGCUCCCU~UCAUUCCAAU	Validated	5
At2g26960	UCGAGUUCCCU~UCAUUCCAAU	Predicted	6
At4g26930	AUGAGCUCUCU~UCAAACCAAA	Predicted	7
At2g26950	UGGAGCUCCCU~UCAUUCCAAG	Predicted	8
At2g32460	UAGAGCUUCCU~UCAAACCAAA	Predicted	9
At3g60460	UGGAGCUCCAU~UCGAUCCAAA	Predicted	10
At5g55020	AGCAGCUCCCU~UCAAACCAAA	Predicted	11
PvMYB	CAGAGCUCCCU~UCACUCCAAU	Predicted	12
VvMYB	UGGAGCUCCCU~UCACUCCAAU	Predicted	13
HvMYB33	UGGAGCUCCCU~UCACUCCAAG	Predicted	14
OsMYB33	UGGAGCUCCCU~UUAAUCCAAU	Predicted	15
miR160 family target sequences – all plants			
At1g77850	UGGCAUGCAGG~GAGCCAGGCA	Validated	16
At2g28350	AGGAAUACAGG~GAGCCAGGCA	Validated	17
At4g30080	GGGUUUACAGG~GAGCCAGGCA	Validated	18
OsARF	AGGCAUACAGG~GAGCCAGGCA	Predicted	19
LjARF	AAGCAUACAGG~GAGCCAGGCA	Predicted	20
miR161 family target sequences – *Arabidopsis*			
At5g41170	ACCUGAUGUAA~UCACUUUCAA	Validated	21
At1g06580	CCCGGAUGUAA~UCACUUUCAG	Validated	22
At1g63150	UUGUUACUUUC~AAUGCAUUGA	Validated	23
At5g16640	CCCUGAUGUAU~UUACUUUCAA	Predicted	24
At1g62590	UAGUCACGUUC~AAUGCAUUGA	Predicted	25
At1g62670	CCCUGAUGUAU~UCACUUUCAG	Predicted	26
At1g62860	CCCUGAUGUUG~UUACUUUCAG	Predicted	27
At1g62910	UAGUCACUUUC~AGCGCAUUGA	Predicted	28
At1g62930	UCCAAAUGUAG~UCACUUUCAG	Predicted	29
At1g63080	UCCAAAUGUAG~UCACUUUCAA	Predicted	30
At1g63130	UCCAAAUGUAG~UCACUUUCAG	Predicted	31
At1g63400	UCCAAAUGUAG~UCACUUUCAA	Predicted	32

At1g63230	UUGUAACUUUC~AGUGCAUUGA	Predicted	33
At1g63330	UAGUCACGUUC~AAUGCAUUGA	Predicted	34
At1g63630	UUGUUACUUUC~AGUGCAUUGA	Predicted	35
At1g64580	CCCUGAUGUUG~UCACUUUCAC	Predicted	36
At2g41720	UUGUUACUUAC~AAUGCAUUGA	Predicted	37
At1g63070	UAGUCUUUUUC~AACGCAUUGA	Predicted	38
miR162 family target sequences – all plants			
At1g01040	CUGGAUGCAGA~GGUAUUAUCGA	Validated	39
PtDCL1	CUGGAUGCAGA~GGUCUUAUCGA	Predicted	40
OsDCL1	CUGGAUGCAGA~GGUUUUAUCGA	Predicted	41
miR163 family target sequences – *Arabidopsis*			
At1g66700	AUCGAGUUCCAAG~UCCUCUUCAA	Validated	42
At1g66720	AUCGAGUUCCAGG~UCCUCUUCAA	Validated	43
At3g44860	AUCGAGUUCCAAG~UUUUCUUCAA	Validated	44
miR164 family target sequences – all plants			
At1g56010	AGCACGUACCC~UGCUUCUCCA	Validated	45
At5g07680	UUUACGUGCCC~UGCUUCUCCA	Validated	46
At5g53950	AGCACGUGUCC~UGUUUCUCCA	Validated	47
At5g61430	UCUACGUGCCC~UGCUUCUCCA	Validated	48
At5g39610	CUCACGUGACC~UGCUUCUCCG	Predicted	49
OsNAC1	CGCACGUGACC~UGCUUCUCCA	Predicted	50
MtNAC	CUUACGUGUCC~UGCUUCUCCA	Predicted	51
GmNAC	CUUACGUGCCC~UGCUUCUCCA	Predicted	52
LeNAC	GCCACGUGCAC~UGCUUCUCCA	Predicted	53
miR165/166 family target sequences – all plants			
At1g30490	UUGGGAUGAAG~CCUGGUCCGG	Validated	54
At5g60690	CUGGGAUGAAG~CCUGGUCCGG	Validated	55
At1g52150	CUGGAAUGAAG~CCUGGUCCGG	Validated	56
PtHDZIPIII	CCGGGAUGAAG~CCUGGUCCGG	Predicted	57
miR167 family target sequences – all plants			
At1g30330	GAGAUCAGGCU~GGCAGCUUGU	Validated	58
At5g37020	UAGAUCAGGCU~GGCAGCUUGU	Validated	59
OsARF6	AAGAUCAGGCU~GGCAGCUUGU	Predicted	60
miR168 family target sequences – all plants			
At1g48410	UUCCCGAGCUG~CAUCAAGCUA	Validated	61
miR169 family target sequences – all plants			
At1g17590	AAGGGAAGUCA~UCCUUGGCUG	Validated	62
At1g54160	ACGGGAAGUCA~UCCUUGGCUA	Validated	63
At1g72830	AGGGGAAGUCA~UCCUUGGCUA	Validated	64
At3g05690	AGGCAAAUCAU~CUUUGGCUCA	Validated	65
At3g20910	GCGGCAAUUCA~UUCUUGGCUU	Validated	66
At5g12840	CCGGCAAAUCA~UUCUUGGCUU	Predicted	67
At3g14020	AAGGGAAGUCA~UCCUUGGCUA	Predicted	68
ZmHAP2	GUGGCAACUCA~UCCUUGGCUC	Predicted	69
VvHAP2	UGGGCAAUUCA~UCCUUGGCUU	Predicted	70
OsHAP2	AUGGCAAAUCA~UCCUUGGCUU	Predicted	71
GmHAP2	UAGGGAAGUCA~UCCUUGGCUC	Predicted	72
GhHAP2	CUGGGAAGUCA~UCCUUGGCUC	Predicted	73
miR170/171 family target sequences – all plants			
At2g45160	GAUAUUGGCGC~GGCUCAAUCA	Validated	74
miR172 family target sequences – all plants			
At4g36920	CUGCAGCAUCA~UCAGGAUUCU	Validated	75
At2g28550	CAGCAGCAUCA~UCAGGAUUCU	Validated	76
At5g60120	AUGCAGCAUCA~UCAGGAUUCU	Validated	77
At5g67180	UGGCAGCAUCA~UCAGGAUUCU	Validated	78

At2g39250	UUGUAGCAUCA~UCAGGAUUCC	Predicted	79
At3g54990	UUGCAGCAUCA~UCAGGAUUCC	Predicted	80
miR319 family target sequences – all plants			
At4g18390	CAGGGGGACCC~UUCAGUCCAA	Validated	81
At1g53230	GAGGGGUCCCC~UUCAGUCCAU	Validated	82
At3g15030	GAGGGGUCCCC~UUCAGUCCAG	Validated	83
At2g31070	AAGGGGUACCC~UUCAGUCCAG	Validated	84
At1g30210	UAGGGGGACCC~UUCAGUCCAA	Validated	85
OsPCF5	GAGGGGACCCC~UUCAGUCCAG	Predicted	86
OsPCF8	UCGGGGCACAC~UUCAGUCCAA	Predicted	87
miR393 family target sequences – all plants			
At1g12820	AAACAAUGCGA~UCCCUUUGGA	Validated	88
At4g03190	AGACCAUGCGA~UCCCUUUGGA	Validated	89
At3g23690	GGUCAGAGCGA~UCCCUUUGGC	Validated	90
At3g62980	AGACAAUGCGA~UCCCUUUGGA	Validated	91
miR394 family target sequences – all plants			
At1g27340	GGAGGUUGACA~GAAUGCCAAA	Validated	92
miR395 family target sequences – all plants			
At5g43780	GAGUUCCUCCA~AACACUUCAU	Validated	93
At3g22890	GAGUUCCUCCA~AACUCUUCAU	Predicted	94
At5g10180	AAGUUCUCCCA~AACACUUCAA	Predicted	95
miR396 family target sequences – all plants			
At2g22840	UCGUUCAAGAA~AGCCUGUGGAA	Validated	96
At2g36400	CCGUUCAAGAA~AGCCUGUGGAA	Validated	97
At4g24150	UCGUUCAAGAA~AGCAUGUGGAA	Validated	98
At2g45480	ACGUUCAAGAA~AGCUUGUGGAA	Validated	99
At3g52910	CCGUUCAAGAA~AGCCUGUGGAA	Predicted	100
miR397 family target sequences – all plants			
At2g29130	AAUCAAUGCUG~CACUCAAUGA	Validated	101
At2g38080	AGUCAACGCUG~CACUUAAUGA	Validated	102
At2g60020	AAUCAAUGCUG~CACUUAAUGA	Validated	103
miR398 family target sequences – all plants			
At1g08830	AAGGGGUUUCC~UGAGAUCACA	Validated	104
At2g28190	UGCGGGUGACC~UGGGAAACAUA	Validated	105
At3g15640	AAGGUGUGACC~UGAGAAUCACA	Validated	106
miR173 family target sequences – *Arabidopsis*			
At1g50055	GUGAUUUUUCUC~AACAAGCGAA	Validated	107
At2g39675	GUGAUUUUUCUC~UACAAGCGAA	Validated	108
At3g39680	GUGAUUUUUCUC~UCCAAGCGAA	Validated	109
miR399 family target sequences – all plants			
At2g33770	UAGGGCAUAUC~UCCUUUGGCA	Validated	110
At2g33770	UUGGGCAAAUC~UCCUUUGGCA	Validated	111
At2g33770	UCGAGCAAAUC~UCCUUUGGCA	Validated	112
At2g33770	UAGAGCAAAUC~UCCUUUGGCA	Validated	113
At2g33770	UAGGGCAAAUC~UUCUUUGGCA	Predicted	114
OsE2UBC	UAGGGCAAAUC~UCCUUUGGCA	Predicted	115
OsE2UBC	CUGGGCAAAUC~UCCUUUGGCA	Predicted	116
OsE2UBC	UCGGGCAAAUC~UCCUUUGGCA	Predicted	117
OsE2UBC	CCGGGCAAAUC~UCCUUUGGCA	Predicted	118
PtE2UBC	GCGGGCAAAUC~UUCUUUGGCA	Predicted	119
MtE2UBC	AAGGGCAAAUC~UCCUUUGGCA	Predicted	120
TaE2UBC	UAGGGCAAAUC~UCCUUUGGCG	Predicted	121
TaE2UBC	CUGGGCAAAUC~UCCUUUGGCG	Predicted	122
TaE2UBC	UUCGGCAAAUC~UCCUUUGGCA	Predicted	123
miR403 family target sequences – dicots			

At1g31280	GGAGUUUGUGC~GUGAAUCUAAU	Validated	124
miR390 family target sequences – all plants			
At3g17185	CUUGUCUAUCCC~UCCUGAGCUA	Validated	125
SbTAS3	UAUGUCUAUCCC~UUCUGAGCUG	Predicted	126
SoTAS3	UAUGUCUAUCCC~UUCUGAGCUA	Predicted	127
ZmTAS3a	UAUGUCUAUCCC~UUCUGAGCUG	Predicted	128
OsTAS3	UCGGUCUAUCCC~UCCUGAGCUG	Predicted	129
PgTAS3	UUAGUCUAUCCC~UCCUGAGCUA	Predicted	130
VvTAS3	AUUGCCUAUCCC~UCCUGAGCUG	Predicted	131
TcTAS3	CCUUGCUAUCCC~UCCUGAGCUG	Predicted	132
LeTAS3	CUUGUCUAUCCC~UCCUGAGCUG	Predicted	133
ZmTAS3b	CCCUUCUAUCCC~UCCUGAGCUA	Predicted	134
PtTAS3	CUUGUCUAUCCC~UCCUGAGCUA	Predicted	135
OsTAS3b	CCCUUCUAUCCC~UCCUGAGCUA	Predicted	136
TaTAS3	CCCUUCUAUCCC~UCCUGAGCUA	Predicted	137
HvTAS3	CCUUUCUAUCCC~UCCUGAGCUA	Predicted	138
PtTAS3b	CCUGUCUAUCCC~UCCUGAGCUA	Predicted	139
McTAS3	UGUGUCUAUCCC~UCCUGAGCUA	Predicted	140
miR447 family target sequences – *Arabidopsis*			
At5g60760	UGACAAACAUC~UCGUCCCCAA	Validated	141
At3g45090	UGACAAACAUC~UCGUUCCUAA	Predicted	142
miR408 family target sequences – all plants			
At2g02850	CCAAGGGAAGA~GGCAGUGCAU	Predicted	281
At2g30210	ACCAGUGAAGA~GGCUGUGCAG	Validated	282
At2g47020	GCCAGGGAAGA~GGCAGUGCAU	Predicted	283
At5g05390	GCCGGUGAAGA~GGCUGUGCAA	Predicted	284
At5g07130	GCCGGUGAAGA~GGCUGUGCAG	Predicted	285

Between January 7, 2005 and January 7, 2006, the following changes were made to nomenclature related to nuclei acid molecules described herein:

Systematic names assigned to TAS loci by The Arabidopsis Information Resource (TAIR)

At2g39680 antisense (*TAS1511*) has become At2g39681 (*TAS2*)
AU235820 (*TAS255a*) has become At1g50055 (*TAS1b*)
CD534192 (*TAS255b*) has become At2g27400 (*TAS1a*)
TAS255c has become At2g39675 (*TAS1c*)
At3g17185 (ASR) has become At3g17185 (*TAS3*)

Official miRNA name assigned by the miRNA Registry (miRBase)

ASRP1890 has become miR447

These nomenclature changes are reflected in this document.

Interfering with or inhibiting (expression of a target sequence): This phrase refers to the ability of a small RNA, such as an siRNA or a miRNA, or other molecule, to measurably reduce the expression and/or stability of molecules carrying the target sequence. A target sequence can include a DNA sequence, such as a gene or the promoter region of a gene, or an RNA sequence, such as an mRNA. "Interfering with or inhibiting" expression contemplates reduction of the end-product of the gene or sequence, *e.g.*, the expression or function of the encoded protein or a protein, nucleic acid, other biomolecule, or biological function influenced by the target sequence, and thus includes reduction in the amount or longevity of the mRNA transcript or other target sequence. In some embodiments, the small RNA or other molecule guides chromatin modifications which inhibit the expression of a target sequence. It is understood that the phrase is relative, and does not require

absolute inhibition (suppression) of the sequence. Thus, in certain embodiments, interfering with or inhibiting expression of a target sequence requires that, following application of the small RNA or other molecule (such as a vector or other construct encoding one or more small RNAs), the sequence is expressed at least 5% less than prior to application, at least 10% less, at least 15% less, at least 20% less, at least 25% less, or even more reduced. Thus, in some particular embodiments, application of a small RNA or other molecule reduces expression of the target sequence by about 30%, about 40%, about 50%, about 60%, or more. In specific examples, where the small RNA or other molecule is particularly effective, expression is reduced by 70%, 80%, 85%, 90%, 95%, or even more.

Isolated: A biological component (such as a nucleic acid molecule, protein or organelle) that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been **isolated** include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

MicroRNA (miRNA): Small, non-coding RNA gene products of approximately 21 nucleotides long and found in diverse organisms, including animals and plants. miRNAs structurally resemble siRNAs except that they arise from structured, foldback-forming precursor transcripts derived from miRNA genes. Primary transcripts of miRNA genes form hairpin structures that are processed by the multidomain RNaseIII-like nuclease DICER and DROSHA (in animals) or DICER-LIKE1 (DCL1; in plants) to yield miRNA duplexes. The mature miRNA is incorporated into RISC complexes after duplex unwinding. Plant miRNAs interact with their RNA targets with perfect or near perfect complementarity.

Mutation: A heritable change in DNA sequence. Mutations include a frame-shift, a point mutation, a missense mutation, a silent mutation, a polymorphism, a nonsense mutation, a deletion, a null mutation, a truncation, an elongation, an amino acid substitution, or an insertion. A mutant is an organism or cell carrying a mutation. The mutant can be genetically engineered or produced naturally.

Nucleotide: "Nucleotide" includes, but is not limited to, a monomer that includes a base linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one monomer in an oligonucleotide/polynucleotide. A nucleotide sequence refers to the sequence of bases in an oligonucleotide/polynucleotide.

The major nucleotides of DNA are deoxyadenosine 5'-triphosphate (dATP or A), deoxyguanosine 5'-triphosphate (dGTP or G), deoxycytidine 5'-triphosphate (dCTP or C) and deoxythymidine 5'-triphosphate (dTTP or T). The major nucleotides of RNA are adenosine 5'-triphosphate (ATP or A), guanosine 5'-triphosphate (GTP or G), cytidine 5'-triphosphate (CTP or C) and uridine 5'-triphosphate (UTP or U). Inosine is also a base that can be integrated into DNA or RNA in a nucleotide (dITP or ITP, respectively).

Oligonucleotide: An oligonucleotide is a plurality of nucleotides joined by phosphodiester bonds, between about 6 and about 300 nucleotides in length. An oligonucleotide analog refers to compounds that function similarly to oligonucleotides but have non-naturally occurring portions. For example, oligonucleotide analogs can contain non-naturally occurring portions, such as altered sugar moieties or inter-sugar linkages, such as a phosphorothioate oligodeoxynucleotide. Functional analogs of naturally occurring polynucleotides can bind to RNA or DNA

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame. In specific embodiments, operably linked nucleic acids as discussed herein are aligned in a linear concatamer capable of being cut into 21-mer fragments, at least one of which is a siRNA.

Ornamental plant: A plant that is grown for visual display. Numerous plants are commonly recognized as ornamental. These include, for example, indoor or outdoor nursery plants, house and garden plants, and florist crops, each of which may include without limitation trees, shrubs, perennials, bulbs, annuals, groundcovers, turf grasses, herbs, or native plants.

Ortholog: Two nucleic acid or amino acid sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

Polymerization: Synthesis of a nucleic acid chain (oligonucleotide or polynucleotide) by adding nucleotides to the hydroxyl group at the 3'-end of a pre-existing RNA or DNA primer using a pre-existing DNA strand as the template. Polymerization usually is mediated by an enzyme such as a DNA or RNA polymerase. Specific examples of polymerases include the large proteolytic fragment of the DNA polymerase I of the bacterium *E. coli* (usually referred to as Kleenex polymerase), *E. coli* DNA polymerase I, and bacteriophage T7 DNA polymerase. Polymerization of a DNA strand complementary to an RNA template (*e.g.*, a cDNA complementary to a mRNA) can be carried out using reverse transcriptase (in a reverse transcription reaction).

For *in vitro* polymerization reactions, it is necessary to provide to the assay mixture an amount of required cofactors such as M^{++}, and dATP, dCTP, dGTP, dTTP, ATP, CTP, GTP, UTP, or other nucleoside triphosphates, in sufficient quantity to support the degree of polymerization desired. The amounts of deoxyribonucleotide triphosphates substrates required for polymerizing reactions are well known to those of ordinary skill in the art. Nucleoside triphosphate analogues or modified nucleoside triphosphates can be substituted or added to those specified above.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. The term polypeptide or protein as used herein encompasses any amino acid sequence and includes modified sequences such

as glycoproteins. The term polypeptide is specifically intended to cover naturally occurring proteins, as well as those that are recombinantly or synthetically produced.

Post-Transcriptional Gene Silencing (PTGS): A form of gene silencing in which the inhibitory mechanism occurs after transcription. This can result in either decreased steady-state level of a specific RNA target or inhibition of translation (Tuschl, *ChemBiochem*, 2:239-245, 2001). In the literature, the terms RNA interference (RNAi) and posttranscriptional cosuppression are often used to indicate posttranscriptional gene silencing.

Primer: Primers are relatively short nucleic acid molecules, usually DNA oligonucleotides six nucleotides or more in length. Primers can be annealed to a complementary target DNA strand ("priming") by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then the primer extended along the target DNA strand by a nucleic acid polymerase enzyme. Pairs of primers can be used for amplification of a nucleic acid sequence, *e.g.*, by nucleic-acid amplification methods known in to those of ordinary skill in the art.

A primer is usually single stranded, which may increase the efficiency of its annealing to a template and subsequent polymerization. However, primers also may be double stranded. A double stranded primer can be treated to separate the two strands, for instance before being used to prime a polymerization reaction (see for example, *Nucleic Acid Hybridization. A Practical Approach*, Hames and Higgins, eds., IRL Press, Washington, 1985). By way of example, a double stranded primer can be heated to about 90°-100° C. for about 1 to 10 minutes.

Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of an RNA polymerase II type promoter, a TATA element. Optionally, a promoter may include an enhancer and/ or a repressor element. Enhancer and repressor elements can be located adjacent to, or distal to the promoter, and can be located as much as several thousand base pairs from the start site of transcription. Representative examples of promoters that can be used in the present disclosure are described herein.

Protein: A biological molecule, for example a polypeptide, expressed by a gene and comprised of amino acids.

Purified: The term **purified** does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified protein preparation is one in which the protein referred to is more pure (has fewer impurities) than the protein in its natural environment within a cell.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques.

Regulatable promoter: A promoter whose activity is regulated by an agent, such as a transcription factor, a chemical compound, or a nucleic acid molecule.

Regulating gene expression: The process of controlling the expression of a gene by increasing or decreasing the expression, production, or activity of an agent that affects gene expression. The agent can be a protein, such as a transcription factor, or a nucleic acid molecule, such as a miRNA or an siRNA molecule, which when in contact with the gene or its upstream regulatory sequences, or a mRNA encoded by the gene, either increases or decreases gene expression.

RNA: A typically linear polymer of ribonucleic acid monomers, linked by phosphodiester bonds. Naturally occurring RNA molecules fall into three general classes, messenger (mRNA, which encodes proteins), ribosomal (rRNA, components of ribosomes), and transfer (tRNA, molecules responsible for transferring amino acid monomers to the ribosome during protein synthesis). Messenger RNA includes heteronuclear (hnRNA) and membrane-associated polysomal RNA (attached to the rough endoplasmic reticulum). Total RNA refers to a heterogeneous mixture of all types of RNA molecules.

RNA-dependent RNA polymerase (RDR): Enzyme that polymerizes formation of RNA using a single-stranded RNA template. This frequently results in formation of a double-stranded RNA molecule. Examples of *Arabidopsis* RDRs include RDR1, RDR2 and RDR6 (Xie *et al.*, *PLoS Biol* 2:642-652, 2004). RDRs required for viral replication are also encoded by many viruses (Kao *et al.*, *Virology* 287:251-260, 2001).

RNA interference (RNAi): Gene silencing mechanisms that involve small RNAs (including miRNA and siRNA) are frequently referred to under the broad term RNAi. Natural functions of RNAi include protection of the genome against invasion by mobile genetic elements such as transposons and viruses, and regulation of gene expression.

RNA interference results in the inactivation or suppression of expression of a gene within an organism. RNAi can be triggered by one of two general routes. First, it can be triggered by direct cellular delivery of short-interfering RNAs (siRNAs, usually ~21 nucleotides in length and delivered in a dsRNA duplex form with two unpaired nucleotides at each 3' end), which have sequence complementarity to a RNA that is the target for suppression. Second, RNAi can be triggered by one of several methods in which siRNAs are formed *in vivo* from various types of designed, expressed genes. These genes typically express RNA molecules that form intra- or inter-molecular duplexes (dsRNA) which are processed by natural enzymes (DICER or DCL) to form siRNAs. In some cases, these genes express "hairpin"-forming RNA transcripts with perfect or near-perfect base-pairing; some of the imperfect hairpin-forming transcripts yield a special type of small RNA, termed microRNA (miRNA). In either general method, it is the siRNAs (or miRNAs) that function as "guide sequences" to direct an RNA-degrading enzyme (termed RISC) to cleave or silence the target RNA. In some cases, it is beneficial to integrate an RNAi-inducing gene into the genome of a transgenic organism. An example would be a plant that is modified to suppress a specific gene by an RNAi-inducing transgene. In most methods that are currently in practice, RNAi is triggered in transgenic plants by transgenes that express a dsRNA (either intramolecular or hairpin, or intermolecular in which two transcripts anneal to form dsRNA).

RNA silencing: A general term that is used to indicate RNA-based gene silencing or RNAi.

Sequence identity: The similarity between two (or more) nucleic acid sequences, or two (or more) amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity or homology. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or orthologs of a specified protein, and the corresponding cDNA sequence, will possess a relatively high degree of sequence identity when aligned using standard methods. This homology will be more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (*e.g.*, different plant sequences), compared to species more distantly related (*e.g.*, human and *Arabidopsis* sequences).

Typically, orthologs are at least 50%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 93%, at least 95%, or at least 98% identical at the nucleotide level and at least 50%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 93%, at least 95%, or at least 98% identical at the amino acid level when comparing a protein to an orthologous protein.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman *Adv. Appl. Math.* 2: 482, 1981; Needleman & Wunsch *J. Mol. Biol.* 48: 443, 1970; Pearson & Lipman *Proc. Natl. Acad. Sci. USA* 85: 2444, 1988; Higgins & Sharp *Gene,* 73: 237-244, 1988; Higgins & Sharp *CABIOS* 5: 151-153, 1989; Corpet *et al. Nuc. Acids Res.* 16:10881-10890, 1988; Huang *et al. Computer Appls. Biosciences* 8:155-165, 1992; and Pearson *et al. Meth. Mol. Bio.* 24:307-331, 1994. Altschul *et al.* (*J. Mol. Biol.* 215:403-410, 1990) present a detailed consideration of sequence alignment methods and homology calculations. Multiple sequences can be aligned, for instance, using programs such as CLUSTAL-W or TCoffee.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al. J. Mol. Biol.* 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at the NCBI website, together with a description of how to determine sequence identity using this program.

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 70%, at least 75%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94% or at least 95% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% or more depending on their similarity to

the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web-site, frequently asked questions (FAQ) page. One of ordinary skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided.

5 An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5° C to 20° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic 10 strength and pH) at which 50% of the target sequence remains hybridized to a perfectly matched probe or complementary strand. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, CSHL, New York and Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* Part I, Chapter 2, Elsevier, New York. Nucleic 15 acid molecules that hybridize under stringent conditions to a human *p28ING5* gene sequence will typically hybridize to a probe based on either an entire human *p28ING5* gene or selected portions of the gene under wash conditions of 2x SSC at 50° C.

Nucleic acid sequences that do not show a high degree of identity can nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that 20 changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein.

Silencing agent or molecule: A specific molecule, which can exert an influence on a cell in a sequence-specific manner to reduce or silence the expression or function of a target, such as a target gene or protein. Examples of silence agents include nucleic acid molecules such as naturally 25 occurring or synthetically generated small interfering RNAs (siRNAs), naturally occurring or synthetically generated microRNAs (miRNAs), naturally occurring or synthetically generated dsRNAs, and antisense sequences (including antisense oligonucleotides, hairpin structures, and antisense expression vectors), as well as constructs that code for any one of such molecules.

Specific binding agent: An agent that binds substantially only to a defined target. Thus a 30 protein-specific binding agent binds substantially only the specified protein.

Small interfering RNA (siRNA): RNA of approximately 21-25 nucleotides that is processed from a dsRNA by a DICER enzyme (in animals) or a DCL enzyme (in plants). The initial DICER or DCL products are double-stranded, in which the two strands are typically 21-25 nucleotides in length and contain two unpaired bases at each 3' end. The individual strands within 35 the double stranded siRNA structure are separated, and typically one of the siRNAs then are associated with a multi-subunit complex, the RNAi-induced silencing complex (RISC). A typical function of the siRNA is to guide RISC to the target based on base-pair complementarity.

Target nucleic acid (to be inhibited): Any nucleic acid containing a sequence that interacts with a miRNA or siRNA, or that has the potential to yield a sequence that interacts with a

miRNA or siRNA (for example, through transcription of a locus). The target can be a cellular nucleic acid, such as a mRNA that encodes an essential or non-essential protein, or a foreign nucleic acid, such as a virus-derived or transgene-derived RNA molecule. The target can be a DNA sequence corresponding to a promoter, or a sequence corresponding to any expressed region of a genome, for instance.

Trans-acting siRNAs: A subclass of siRNAs that function like miRNAs to repress expression of target genes, yet have unique biogenesis requirements. Trans-acting siRNAs form by transcription of ta-siRNA-generating genes, cleavage of the transcript through a guided RISC mechanism, conversion of one of the cleavage products to dsRNA, and processing of the dsRNA by DCL enzymes. ta-siRNAs are unlikely to be predicted by computational methods used to identify miRNA because they fail to form a stable foldback structure. Data provided herein demonstrate that ta-siRNAs are not an *Arabidopsis* oddity, but are conserved among distantly related plant species and have been maintained over a long evolutionary period.

A ta-siRNA precursor is any nucleic acid molecule, including single-stranded or double-stranded DNA or RNA, that can be transcribed and/or processed to release a ta-siRNA.

Transcriptional gene silencing (TGS): A phenomenon that is triggered by the formation of dsRNA that is homologous with gene promoter regions and sometimes coding regions. TGS results in DNA and histone methylation and chromatin remodeling, thereby causing transcriptional inhibition rather than RNA degradation. Both TGS and PTGS depend on dsRNA, which is cleaved into small (21-25 nucleotides) interfering RNAs (Eckhardt, *Plant Cell*, 14:1433-1436, 2002; Aufsatz *et al., Proc. Natl. Acad. Sci. U.S.A.*, 99:16499-16506, 2002).

Transgenic (plant/fungus/cell/other entity): This term refers to a plant/fungus/cell/other entity that contains recombinant genetic material not normally found in entities of this type and which has been introduced into the entity in question (or into progenitors of the entity) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant that contain the introduced transgene (whether produced sexually or asexually).

Triggering RNA: RNA transcript of an siRNA generating locus which is converted into a dsRNA molecule by an RNA-dependent RNA polymerase (RDR) *in vivo*.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All

publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

An siRNA-triggering or RNAi-triggering nucleic acid cassette is provided, which cassette comprises an initiator sequence consisting of about 20 to 25 nucleotides, the initiator sequence having an initiation cleavage site between the tenth and eleventh nucleotide counted from the 3' end of the initiator sequence; and at least one gene suppressing segment in about 21-nucleotide register (or phase) counted either upstream or downstream from the initiation cleavage site, wherein the gene suppressing segment or its complement is substantially complementary to an RNA transcribed from a target gene selected for siRNA inhibition. Also provided are expression vectors which include at least one such nucleic acid cassette operably linked to a promoter.

Specific example initiator sequences are provided herein, for instance, in SEQ ID NOs: 1-142 and 281-285.

Also provided are siRNA- or RNAi-triggering nucleic acids (both cassettes and vectors) that comprise two or more gene suppressing segments. In embodiments having two or more gene suppressing segments, these segments optionally can be directed to (complementary with) two or more different genes or other target sequences selected for siRNA inhibition.

Cells and organisms into which have been introduced a vector or cassette of this disclosure are also provided, as are parts of multicellular organisms that contain such transgenic nucleic acids. Thus, another specific embodiment is a seed for a transgenic plant that expresses RNA for suppressing a target gene, wherein said seed and plant comprise recombinant DNA from which there is transcribed a first RNA comprising an initiator segment consisting of 20-25 nucleotides wherein an initiation cleavage site is located between the tenth and eleventh nucleotide counted from the 3' end of the initiator segment and wherein said initiator segment is linked to or overlaps with at least one gene suppressing segment of 21-nucleotides in precise 21-nucleotide register counted either upstream or downstream from the initiation cleavage site, wherein said gene suppressing segment or it complement is complementary to mRNA transcribed from said target gene.
Yet other embodiments are seed for a transgenic plant further comprising DNA from which there is transcribed a second RNA that hybridizes to said first RNA at said initiation cleavage site. By way of example, the second RNA in some instances is an exogenous miRNA, or a miRNA transcribed from a native plant gene or a heterologous gene or any gene not native to the plant. Seed are also provided for a transgenic plant wherein the first RNA comprises two or more gene suppressing segments.

Optionally, the target gene in any provided organism can be endogenous to that organism. For instance, the target gene may be an endogenous plant gene, an endogenous fungal gene, or an endogenous invertebrate gene, in plant, fungal, or invertebrate embodiments, respectively. There is

also therefore provided a seed for a transgenic plant of the disclosure, wherein the plant is corn and the endogenous plant gene encodes lysine ketoglutarate reductase.

Alternatively, the target gene could be exogenous to the transgenic organism, for instance it could be a gene of a pathogen or a pest, such as a plant pathogen or plant pest. In specific examples, such plant pest is a nematode or insect or such pant pathogen is a virus or fungus. In one particular embodiment, a seed for a transgenic plant is provided wherein said plant is soybean and said plant pest is soybean cyst nematode.

In yet another provided seed for a transgenic plant of the disclosure, the recombinant DNA comprises a promoter functional in said plant and operably linked to DNA coding for the first RNA. Such a promoter in some cases is characterized as being a constitutive promoter, an inducible promoter, a tissue specific promoter, a ubiquitous promoter or a combination thereof.

Also provided are seed for a transgenic plant as described, wherein the plant is a corn, soybean, cotton, canola, wheat or rice plant.

Optionally, in any of the provided embodiments of seed for a transgenic plant, the recombinant DNA further comprises nucleotides for expressing at least one protein.

Also provided herein are methods of inhibiting expression of a target gene in a cell, the method comprising exposing the cell to an effective amount of a RNAi-triggering or siRNA-triggering nucleic acid cassette or a vector as described. The cell can be, for instance, a plant cell, a fungal cell, or an invertebrate cell. It is particularly contemplated that the cell could be *in vitro* or *in vivo*, for instance, contained in a multicellular organism.

Yet another method is provided, which is a method of inducing production of at least one siRNA in a cell. This method involves transforming the cell with a recombinant nucleic acid molecule comprising a nucleic acid cassette as described herein, wherein the recombinant nucleic acid molecule directs expression of a mRNA from the nucleic acid cassette, which mRNA is processed in the cell to produce at least one siRNA, thereby inducing the production of at least one siRNA in the cell.

Another method is provided, which is a method of inhibiting activity of a target gene in a plant cell. This method involves transforming the plant cell with a recombinant nucleic acid molecule comprising a nucleic acid cassette as described herein, wherein at least one gene suppressing segment of the nucleic acid cassette is specific for the target gene; and expressing the nucleic acid molecule, thereby producing in the plant cell at least one siRNA specific for the target gene which inhibits activity of the target gene in the plant cell.

. Another method is a method of inhibiting activity of a target gene in a plant seed, comprising providing in cells of said plant a recombinant nucleic acid molecule comprising a nucleic acid cassette of the disclosure, wherein at least one gene suppressing segment of the nucleic acid cassette is specific for the target gene and wherein said cassette comprises a seed-specific promoter operably linked to said initiator sequence and said at least one gene suppressing segment; a recombinant DNA with a seed specific promoter operably linked to DNA transcribing an miRNA that hybridizes to said initiator sequence at said initiation cleavage site; both.

IV. Methods of Triggering RNA Interference (RNAi)

Plants and animals use small RNAs [microRNAs (miRNAs) and siRNAs] as guides for posttranscriptional and epigenetic regulation of target genes. In plants, miRNAs and *trans*-acting (ta) siRNAs form through distinct biogenesis pathways, although they both interact with target transcripts and guide cleavage. An integrated approach to identify targets of *Arabidopsis thaliana* miRNAs and ta-siRNAs revealed several new classes of small RNA-regulated genes. These included conventional genes, such as the RNAi factor Argonaute2 (miR403), an E2-ubiquitin conjugating enzyme (miR399), and two Auxin Response Factors (TAS3 ta-siRNAs). Five ta-siRNA-generating transcripts were identified as targets of miR173 or miR390. Rather than functioning to negatively regulate these transcripts, miR173- and miR390-guided cleavage was shown to set the 21-nucleotide phasing for ta-siRNA precursor processing. These data support a model in which miRNA-guided formation of a 5' or 3' terminus within pre-ta-siRNA transcripts, followed by RDR6-dependent formation of dsRNA and DCL1-mediated processing, yields phased ta-siRNAs that negatively regulate other genes.

In Example 1, new *Arabidopsis* miRNA and ta-siRNA targets are identified through an integrated strategy that included computational, genome-wide expression profiling and experimental validation components. Through identification of genes significantly upregulated in miRNA or ta-siRNA biogenesis mutants (hyl1-2, hst-15, dcl1-7, hen1-1, and rdr6-2) using microarrays, data is presented herein that demonstrates identification of genes potentially regulated by miRNAs and ta-siRNAs. Two genes, ARF3 and ARF4, were found to contain a duplicated conserved 21 sequence. Analysis of an *Arabidopsis* sequence, conserved across angiosperms, identified small RNAs typical of ta-siRNAs that could target ARF3 and ARF4 mRNAs.

As taught herein, RNAi can be induced using transgenes or other delivered genes or constructs that encode non-dsRNA-forming transcripts. This method exploits the occurrence of natural siRNAs and miRNAs that can: 1) interact with the delivered transcript through base-pairing, 2) engage a natural dsRNA-forming enzyme termed an RNA-dependent RNA polymerase (RDR), and 3) engage natural DICER-LIKE (*e.g.*, DCL1) enzymes to form siRNAs in precise and predictable register. The siRNAs that form under this mechanism can function to suppress target mRNA expression if the target contains a high degree of sequence complementarity to the siRNAs. One advantage of this method is that it circumvents the need to deliver a dsRNA-forming entity or transgene to initiate the RNAi process of gene suppression.

The methods described herein also enable RNAi to target multiple mRNAs or other target RNAs, depending on the specific siRNA units designed into the construct. The method also permits highly specific siRNA formation rather than non-specific siRNA formation (which results in an increased chance of off-target effects) using conventional dsRNA-forming constructs. The method also may take advantage of naturally occurring miRNAs and siRNAs with tissue- or cell-specific expression characteristics to drive tissue – and cell-specificity of RNAi. Alternatively, a heterologous miRNA or siRNA can be added to the cell (for instance by providing an expression

cassette encoding such molecule) in order to provide the receptive element necessary to mediate cleavage and release of siRNAs from a RNAi-triggering nucleic acid cassette.

Also provided herein are nucleic acid constructs that generate, *in vivo*, siRNAs useful for triggering RNAi-like responses. Representative methods for producing such constructs, as well as guidelines for selecting elements included therein, are provided.

V. Initiator Sequences and Identification thereof

When present in an RNA molecule, an initiator sequence serves as a site that interacts with a miRNA or siRNA, which guides cleavage through the activity of RISC. Cleavage at an initiator sequence cleavage site (usually between the tenth and eleventh nucleotide counting from the 3' end of the initiator sequence) sets the 21-nucleotide register within the RNA molecule, resulting in additional cleavages of the RNA molecule by the Dicer or DCL protein at usually 21-nucleotide intervals upstream and/or downstream of the initiator sequence. In an engineered RNAi-triggering nucleic acid cassette as described herein, such additional, in-phase cleavages release siRNAs from RNA molecules that are transcribed from the cassette. Representative initiator sequences in RNA form, also referred to as miRNA target sequences, are shown in SEQ ID NOs: 1-142 and 281-285.

Any sequence in an RNA molecule to which a siRNA or miRNA can bind by complementarity, or any sequence in a DNA molecule that encodes for such a sequence in an RNA molecule, can serve as an initiator sequence. In addition to representative initiator sequences provided herein, methods are provided for identifying additional sequences from other genes, other plant species, or any other organisms. An integrated system is provided herein for identifying new miRNA and ta-siRNA targets. This system involves computational, genome-wide expression profiling and experimental validation components. As demonstrated in Example 1, the system reliably identifies prospective initiator sequences, which are target sites for miRNAs. Representative initiator sequences, including many identified and validated using the computational system provided, are shown in SEQ ID NO: 1-142 and 281-285.

In general, an initial pool of predicted target sites for validated miRNAs was created by FASTA searches using a +15/-10 scoring matrix of the TAIR AGI transcript database, limited to 4 mispairs, 4 G:U pairs, to a total of seven, with 100,000 results obtained for the reverse complement of each small RNA. A single, one nucleotide gap was allowed. In the embodiment described in Example 1, the miRNA target prediction algorithm used to score these sites was developed based on 94 experimentally validated and predicted family members of miRNA-target site duplexes, including 66 targets validated in previous studies plus 28 family members with conserved miRNA target sites (Target Rule Set).

Three filters based on the Target Rule Set were applied sequentially. In each case, base one was considered to be the first nucleotide from the 5' end of the miRNA. First, targets with a mismatch score greater than four were excluded. The Minimum Free Energy (ΔG_{MFE}) of a perfect miRNA-target duplex was determined by computationally attaching a perfectly complementary target sequence to a small RNA using a four base "gap" linker sequence (----). The free energy each

miRNA-predicted target site (ΔG_{target}) was determined by computationally linking the target sequence to the small RNA, from which the MFE ratio was calculated ($\Delta G_{target}/\Delta G_{MFE}$). All thermodynamic values were calculated using RNAFold in the Vienna RNA package. Remaining targets with an MFE ratio less than 0.73 were excluded. Conservation of the target sequence was determined by using the region containing the target sequence in a BLAST search against target transcripts, for instance, the *Arabidopsis* transcript and EST databases, NCBI EST database, and *O. sativa* Unigene database in Example 1, and removing any targets with no matches with less than three base changes in the target sequence. Duplicate target sites (identical genes) for related miRNA family members were combined in the final target gene set.

VI. Selection of Initiation Sequence for RNAi-Triggering Constructs

Any nucleic acid sequence that will serve to mediate cleavage by a miRNA- or siRNA-guidedRISC mechanism may be used as the initiator sequence in constructs provided herein. Examples of such sequences are provided herein, for instance in SEQ ID NOs: 1-142 and 281-285. It is noted that the presented sequences are RNA sequences. It will be apparent to one of ordinary skill in the art that DNA constructs, such as DNA constructs used in transformation of target cells, will contain the DNA equivalent of the listed RNA sequences.

By way of example, SEQ ID NO: 1 is GUGCUCUCUCUCUUCUGUCA (shown 5' to 3'). The corresponding miRNA sequence (also shown 5' to 3') is UGACAGAAGAGAGUGAGCAC (SEQ ID NO: 155); this is the reverse complement of the target/initiation sequence shown in SEQ ID NO: 1. A DNA construct containing an initiator sequence corresponding to SEQ ID NO: 1 would include the following sequence: 5'-GTGCTCTCTCTCTTCTGTCA-3' (SEQ ID NO: 280), which may be generated in double-stranded format depending on the embodiment. In such a DNA construct, the transcription site and strandedness would be designed so the initiator sequence is produced as shown in SEQ ID NO: 1. This enables the native or provided, corresponding miRNA to bind by complementarity to the initiator sequence.

It is noted that, in many embodiments, the initiator sequence and a first gene suppressing element may overlap. This arises because the register that is set by the initiator cleavage site begins at that site. Thus, the nucleotides of the 5' or 3' portion of the initiator sequence will be incorporated into the first 21-mer gene suppressing element (*e.g.*, siRNA) produced. This is illustrated, for instance, in Figures 5A-C, Figure 6A, and Figure 7.

Many miRNAs and their corresponding target sequences (also referred to herein as initiator sequences) are highly conserved among distantly related species. In plants in particular, target sequences that are recognized by related miRNAs in different species differ only by one to three bases, making computational prediction of target sites by similarity searches relatively straightforward (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004). Owing to the high level of conservation of miRNAs, a functional miRNA target site from one plant species is likely to be functional in a species which expresses the targeting miRNA. For example, miRNA target genes from *Arabidopsis* expressed in *Nicotiana* are cleaved by endogenous miRNAs (Llave *et al.*, *Science*

297:2053-2056, 2002). In *Oryza* and *Populus*, for which near-complete genomic sequence information exists, homologous miRNA and/or target genes have been identified for 20 of 25 validated miRNA families in Arabidopsis. For these 20 conserved miRNA families, conserved homologous miRNA and/or target genes have also been found in several other plant species with less complete sequence information.

By way of example, in Table 2, miRNAs are grouped by related families (one to three nucleotide differences), or by targets of the miRNA family. Presence of the miRNA or target in a listed plant genus is indicated by an "X". In generating this table, miRNA genes were considered to be conserved if the homologous sequence was within 1-3 nucleotides of the *Arabidopsis* sequence, formed a stable foldback structure, and did not encode an identifiable protein. Target sites were considered to be conserved if the target gene in the specified genus encodes a protein similar to the *Arabidopsis* target gene.

Table 2: Conservation of miRNAs and target genes in plants.

Genus	miR156/157		miR158		miR159		miR160		miR161		miR162		miR163		miR164		miR165/166		miR167		miR168		miR169		miR170/171		miR172		miR173		miR319		miR390/391		miR393		miR394		miR395		miR396		miR397		miR398		miR399		miR403	
	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target	miRNA	Target
Acorus		X																X																		X				X		X				X				
Aegilops																				X																						X								
Allium		X																						X		X														X		X				X				
Amborella																																										X				X				
Antirrhinum	X	X				X										X		X										X				X	X	X															
Apium																																														X				
Arabidopsis	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Arachis																																														X				
Beta																		X																						X				X		X				
Betula																					X																													
Brassica	X					X										X				X				X				X				X								X	X	X				X				
Brugeria																																		X	X															
Capsicum		X				X												X																		X				X		X				X				
Ceratopteris	X						X										X				X		X		X	X	X						X																
Citrus		X									X									X							X							X		X				X		X			X	X				
Cryptomeria																				X																				X						X				
Cycas																																										X								
Descurainia																								X				X																						
Eschscholzia		X						X																										X				X				X				X				
Eucalyptus																																				X														
Glycine	X	X			X	X	X	X			X					X	X	X	X	X	X		X	X	X	X	X	X			X	X	X	X		X	X	X	X	X	X	X		X	X	X				

Table 2: Conservation of miRNAs and target genes in plants. (Part 2)																																																		
Gossypium		X				X		X								X								X				X				X	X	X		X		X				X		X		X				
Hedyotis																	X				X	X			X															X						X				
Helianthus	X	X																		X																X		X							X	X				
Hordeum		X			X	X		X									X			X				X	X	X		X			X	X	X	X		X				X		X	X			X		X		
Ipomoea		X				X											X			X												X						X		X										
Lactuca		X				X										X		X		X								X				X						X		X		X			X	X				
Linum																																						X												
Liriodendron	X				X		X								X		X		X		X		X		X		X				X		X				X				X	X				X				
Lotus	X							X				X								X						X							X	X		X				X		X		X	X	X		X		
Lupinus											X									X																				X										
Lycopersicon		X				X		X								X		X		X	X						X	X				X	X	X						X		X		X		X			X	
Malus																																		X																
Manihot																																	X	X																
Mesembryan-themum																																	X	X							X									
Medicago	X				X	X	X	X			X					X	X			X			X	X	X	X		X			X	X			X	X			X	X		X		X	X	X	X	X		
Nicotiana	X	X			X	X	X								X		X		X	X	X		X		X		X					X	X				X			X	X		X	X	X	X				
Nuphar		X																										X														X								
Oryza	X	X			X	X	X	X			X	X			X		X	X	X	X	X		X		X	X	X	X			X	X	X	X	X	X		X	X		X	X	X		X	X		X		
Pennisetum						X																																X												
Persea																																	X	X		X														
Phaseolus		X				X													X													X										X				X				
Phycomitrella	X					X																									X		X																	
Picea																																	X	X												X				
Pinus		X						X										X		X														X		X						X		X		X				
Poncirus																		X		X																				X										
Populus	X	X			X	X	X	X			X	X			X	X	X	X	X	X	X		X	X	X		X					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
Prunus		X				X		X												X																X					X					X				

Table 2: Conservation of miRNAs and target genes in plants. (Part 3)

Robinia																																					X													
Rosa																																								X										
Saccharum	X	X			X	X		X										X	X	X	X		X			X		X			X	X	X	X		X		X		X	X	X				X				
Schedonorus					X																																													
Sueada																								X																										
Secale								X																																										
Sesamum	X																																													X				
Solanum	X	X				X		X										X		X	X			X		X	X	X				X				X		X		X	X	X				X				X
Sorghum	X	X			X	X	X				X				X		X	X	X	X	X		X		X	X	X	X			X	X	X	X	X	X	X	X	X	X	X	X				X	X	X		
Stevia																																								X										
Thellungiella																																								X										
Theobroma																												X					X	X		X														
Triphysaria																																				X										X				
Triticum	X	X			X	X	X	X							X		X	X	X	X	X		X	X	X	X		X			X	X	X	X		X	X	X	X	X	X	X	X			X		X		
Vitis	X	X			X	X					X					X				X	X			X		X		X			X	X	X	X		X						X		X		X				
Zea	X	X			X	X	X										X	X	X	X	X		X	X	X	X		X			X	X	X	X	X	X	X	X	X		X	X			X	X	X			
Zinnia																				X						X		X																X		X				

VII. Selection of Gene Suppressing Elements and Targets for RNAi-Triggering Constructs

A gene suppressing element is any nucleotide sequence which leads to the downregulation of the final functional product of a gene, either RNA or protein. For RNAi, this sequence is a 20 to 25 nucleotide RNA with complementarity to the gene to be suppressed.

Beneficial characteristics of a gene suppressing element useful for inclusion "in register" in an RNAi-triggering cassette are those known to produce a functional (measurably effective for reducing expression of a target gene/sequence) siRNA sequence. Empirical studies such as described herein can be used to identify gene suppressing elements. There are also art-recognized guidelines that provide predictive RISC incorporation rules (Khvorova *et al.*, *Cell* 115:209-216, 2003; Schwarz *et al.*, *Cell* 115:199-208, 2003)

Specific gene suppressive elements can be designed depending on the target sequences (*e.g.*, gene(s), regulatory sequence or invasive or pathogenic entities) to be suppressed. Gene suppressive elements (usually about 21-nucleotides in length), complementary to a target (*e.g.*, gene transcript) to be suppressed, are included the RNAi-triggering cassette, in register, in either sense or antisense orientation starting from the initiation cleavage site. At least eight, possibly more, unique (or duplicated) sequences can be included either upstream or downstream of the initiation cleavage site. Beyond the eighth register, processing by DICER or DCL enzymes may become less precise, and the 21 nucleotide register is more likely to be compromised. Even so, gene suppressive elements beyond eight can be optionally included in constructs, including elements that are not in precise 21-nucleotide register.

Gene suppressive elements contained in the RNAi-inducing cassette can be designed to target one or more genes, with one or more unique target sequences. Potential targets might include, but are not limited to, pathogens, toxins, genes that lead to production of undesirable flavors and/or odors, reproductive genes which could facilitate pollination or increase crop yield, color or pigment genes, transcription factors, pathogen response genes, and genes involved in cold/water/drought and other environmental stresses. Related gene families, pathway-related genes, or quantitative trait loci also may be targeted, for instance in a single RNAi-inducing cassette or a set of such cassettes. Such family-directed cassettes are useful in the down-regulation of all (or select) members of a gene family, all (or select) members in a biosynthetic pathway, and so forth, thereby yielding coordinated downregulation of sets of genes.

Additional gene suppressive elements that are contemplated are directed to the genes of pathogens or pests associated with the resultant target organism; endogenous genes of the target organism that are involved in response to such pathogens or pests; and exogenous (heterologous) transgenes provided to the target organism (separately or in a single construct containing the RNAi-triggering cassette) to influence infection or infestation or association of such pathogens or pests.

Gene suppressive elements also can be from any endogenous gene that it is desired to downregulate. Genes that negatively influence a characteristic (that cause an unpleasant flavor, aroma, etc.) of the target organism; genes that lead to production of a toxin, allergen, or other

detrimental component (*e.g.*, erucic acid in an oil seed; hazardous allergens in peanuts; toxic compounds in potatoes, apricots); genes involved in reproduction (where inhibition will result in increased vegetative production in a plant, for instance); genes involved in male fertility in plants (in order to produce male-sterile, non-selfing plants); genes that enhance vegetative growth (where reproductive growth is desired over seed production, such as in leaf crops like lettuce and spinach); genes that govern or influence color (for instance, the color of leaves or bracts, flowers, stems, fruit, and so forth, where it is desired to change the color); genes that govern or influence susceptibility to stress (such as cold stress, water or drought stress, shear stress, and so forth); and transcription factors (where it is desired to influence a downstream gene or set of genes the expression of which is influenced by the transcription factor) are all examples of conceived of targets for suppression using the methods and constructs described herein.

It is further contemplated that transgenic plants produced using methods and cassettes described herein can be further enhanced with stacked traits, *e.g.* a crop having an enhanced agronomic trait resulting from gene suppression from an siRNA-triggering nucleic acid cassette in combination with DNA expressing a protein supplementing the agronomic trait, or conferring another trait such as herbicide and/or pest resistance traits. For example, a trait can be enhanced by simultaneous suppression of one gene and over expression of another gene to provide transgenic corn with an enhance level of the amino acid lysine. Transgenic corn with recombinant DNA for expression of the gene encoding dihydodipicolinate synthase in the lysine synthetic pathway and suppression of the gene encoding lysine ketoglutarate reductase (LKR) in the lysine catabolic pathway has enhanced lysine as compared to control plants. Following the methods of this disclosure, the suppression of LKR can be effected by identifying a 21-nucleotide segment of the gene encoding LKR for insertion into an siRNA-triggering nucleic acid cassette. To effect the enhance lysine trait preferentially in seed tissue, seed specific promoters are used to express the siRNA-triggering nucleic acid cassette and/or to express the RNA that hybridizes to the initiation cleavage site in the initiator segment.

The siRNA-triggering nucleic acid cassettes can also be stacked with DNA imparting other traits of agronomic interest including DNA providing herbicide resistance or insect resistance such as using a gene from *Bacillus thuringensis* to provide resistance against lepidopteran, coliopteran, homopteran, hemiopteran, and other insects. Herbicides for which resistance is useful in a plant include glyphosate herbicides, phosphinothricin herbicides, oxynil herbicides, imidazolinone herbicides, dinitroaniline herbicides, pyridine herbicides, sulfonylurea herbicides, bialaphos herbicides, sulfonamide herbicides and glufosinate herbicides. Persons of ordinary skill in the art are enabled in providing stacked traits by reference to U.S. patent application publications 2003/0106096A1 and 2002/0112260A1 and U.S. Patents 5,034,322; 5,776,760; 6,107,549 and 6,376,754 and to insect/nematode/virus resistance by reference to U.S. Patents 5,250,515; 5,880,275; 6,506,599; 5,986,175 and U.S. Patent Application Publication 2003/0150017 A1, all of which are incorporated herein by reference.

VIII. Constructs for Triggering RNAi

RNAi-inducing constructs contain an initiator (target) sequence and one or more gene-suppressing elements in-phase or near-phase to the initiation cleavage site in the in initiator (target) sequence. These are operably linked to a promoter or other regulatory sequence which governs transcription from the RNAi-triggering cassette (comprising the initiator sequence with an initiator cleavage site and at least one gene suppressing element upstream or downstream of the initiator sequence and that may optionally overlap a portion of the initiator sequence) in order to generate a single-stranded RNA comprising one or more elements that, when cleaved in register or nearly in register from the in initiator cleavage site, yield one of more siRNA.

DNA constructs for plant transformation are assembled using methods well known to persons of ordinary skill in the art, and typically comprise a promoter operably linked to DNA, the expression of which provides an enhanced trait, *e.g.* by gene suppression using an siRNA-triggering (or RNAi-triggering) nucleic acid cassette alone or in combination with a DNA for expressing a protein or another RNA molecule. Other construct components may include additional regulatory elements, such as 5' introns for enhancing transcription, 3' untranslated regions (such as polyadenylation signals and sites), DNA for transit or signal peptides.

Vectors suitable for stable transformation of culturable cells are well known. Typically, such vectors include a multiple-cloning site suitable for inserting a cloned nucleic acid molecule, such that it will be under the transcriptional control of 5' and 3' regulatory sequences. In addition, transformation vectors include one or more selectable markers; for bacterial transformation this is often an antibiotic resistance gene. A number of vectors suitable for stable transformation of plant cells or for the establishment of transgenic plants have been described in, *e.g.*, Pouwels *et al.* (*Cloning Vectors: A Laboratory Manual*, 1985, Suppl., 1987), Weissbach and Weissbach (*Meth. Plant Mol. Bio.*, Academic Press, 1989) and Gelvin *et al.* (*Plant Molecular Biology Manual*, Kluwer Academic Publishers, 1990). In addition, on of ordinary skill in the art is aware of the components useful in a transformation vector, and will be able to select and assemble such components in order to tailor make a vector for their specific use.

Typically, transformation and expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (*e.g.*, a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Also included in most expression vectors will be a promoter, which is an array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of an RNA polymerase II type promoter, a TATA element. Optionally, a promoter may include an enhancer and/ or a repressor element. Enhancer and repressor elements can be located adjacent to, or distal to the

promoter, and can be located as much as several thousand base pairs from the start site of transcription. Examples of promoters that can be used in the present disclosure include, but are not limited to the Cauliflower mosaic virus 35S promoter, SV40 promoter, the CMV enhancer-promoter, the CMV enhancer/β-actin promoter, and the tissue-specific promoter probasin. Other promoter sequences that can be used to construct nucleic acids and practice methods disclosed herein include, but are not limited to: the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors, any retroviral LTR promoter such as the RSV promoter; inducible promoters, such as the MMTV promoter; the metallothionein promoter; heat shock promoters; the albumin promoter; the histone promoter; the α-actin promoter; TK promoters; B19 parvovirus promoters; the SV10 late promoter; the ApoAI promoter and combinations thereof.

In certain embodiments, a promoter is a strong promoter, which promotes transcription of RNA at high levels, for example at levels such that the transcriptional activity of the promoter generally accounts for about 5% or more of the transcriptional activity of all transcription within a cell. The strength of a promoter is often tissue-specific and thus may vary from one cell type to another. Examples of strong promoters include, but are not limited to: viral promoters (such as CaMV 35S or CoYMV), ubiquitin promoter (such as *Ubi-1* from maize), actin promoter (*e.g*, *Act* from rice), nopaline synthase promoter, and the octopine synthase promoter, pEMU promoter, MAS promoter, or a H3 histone promoter.

In another embodiment, a promoter is a tissue-specific, cell-specific, or developmental stage-specific promoter, which promotes transcription in a single cell or tissue type, a narrow range of cells or tissues, or in one or more specific developmental stages, or at least promotes measurable more transcription in such. Examples of such promoters include, but are not limited to: anther-specific, embryo-specific, endosperm-specific, floral-specific, leaf-specific, meristem-specific, nodule-specific, phloem-specific, seed-specific, stem-specific, stomata-specific, trichome-specific, root-specific, tapetum-specific, and xylem-specific promoters. See, for instance, Carpenter *et al.*, *The Plant Cell* 4:557-571, 1992, Denis *et al.*, *Plant Physiol.* 101:1295-1304 1993, Opperman *et al.*, *Science* 263:221-223, 1993, Stockhause *et al.*, *The Plant Cell* 9:479-489, 1997; Roshal *et al.*, *EMBO J.* 6:1155, 1987; Schernthaner *et al.*, *EMBO J.* 7:1249, 1988; and Bustos *et al.*, *Plant Cell* 1:839, 1989.

Inducible promoters or gene-switches are used to both spatially and temporally regulate gene expression. By allowing the time and/or location of gene expression to be precisely regulated, gene-switches or inducible promoters may control deleterious and/or abnormal effects caused by overexpression or non-localized gene expression. Thus, for a typical inducible promoter in the absence of the inducer, there would be little or no gene expression while, in the presence of the inducer, expression should be high (*i.e.*, off/on). Examples of stimulus-responsive promoters include, but are not limited to hormone-responsive promoters (*e.g*, ethanol inducible alcR-encoded

transcriptional activator (ALCR), a promoter derived from *alcA*), light-inducible promoters (such as a rbcS promoter), metal-inducible promoters, heat-shock promoters, wound-inducible and stress-inducible (*e.g.*, drought stress, salt stress, shear stress, nutrient stress) promoters. Others are activated by chemical stimuli, such as IPTG or Tetracycline (Tet), or galactose. Other promoters are responsive to pathogen infection or insect damage.

A number of controllable gene expression systems have been devised, including those regulated by light (*e.g.*, the pea rbcS-3A promoter, Kuhlemeier *et al.*, *The Plant Cell*, 1:471-478, 1989, and the maize rbcS promoter, Schaffner and Sheen, *Plant Cell* 3:997, 1991), heat (Callis *et al.*, *Plant Physiol.* 88:965, 1988; Ainley and Key, *Plant Mol. Biol.*, 14:949-967, 1990; Holtorf *et al.*, *Plant Mol. Biol.* 29:637-646, 1995), pathogens (PR1-a; Williams *et al.*, *Biotechnology* 10:540-543, 1992; Gatz, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:89-108, 1997), herbicide safeners (In2-2, GST-27; De Veylder *et al.*, *Plant Cell Physiol.* 38:568-577, 1997), light (Kuhlemeier *et al.*, *Plant Cell* 1:471-478, 1989), wounding (Firek *et al. Plant Mol. Biol.* 22:129-212, 1993), ethanol (Salter *et al.*, *Plant J.* 16:127-132, 1998), phytohormones (Li *et al.*, *Plant Cell* 3:1167-1175, 1991), steroids (Aoyama and Chua, *Plant J.*, 11:605-612, 1997), wounding (*e.g.*, wunI, Siebertz *et al.*, *Plant Cell* 1:961, 1989), hormones, such as abscisic acid (Marcotte *et al.*, *Plant Cell* 1:969, 1989); chemicals such as methyl jasminate or salicylic acid (see Gatz *et al.*, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 48:89-108 1997), and tetracycline (Gatz *et al.*, *Plant J.* 2:397-404, 1992; Weinmann *et al.*, *Plant J.*, 5:559-569, 1994; Sommer *et al.*, *Plant Cell Rep.* 17:891-896, 1998) (from Granger & Cyr, *Plant Cell Reports* 20:227–234, 2001).

It is specifically contemplated that useful promoters will include promoters present in plant genomes as well as promoters from other sources, including nopaline synthase (nos) promoter and octopine synthase (ocs) promoters carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*, caulimovirus promoters such as the cauliflower mosaic virus or figwort mosaic virus promoters. For instance, see U.S. Patents 5,322,938 and 5,858,742 which disclose versions of the constitutive promoter derived from cauliflower mosaic virus (CaMV35S), US Patent 5,378,619 which discloses a Figwort Mosaic Virus (FMV) 35S promoter, U.S. Patent 5,420,034 which discloses a napin promoter, U.S. Patent 6,437,217 which discloses a maize RS81 promoter, U.S. Patent 5,641,876 which discloses a rice actin promoter, U.S. Patent 6,426,446 which discloses a maize RS324 promoter, U.S. Patent 6,429,362 which discloses a maize PR-1 promoter, U.S. Patent 6,232,526 which discloses a maize A3 promoter, U.S. Patent 6,177,611 which discloses constitutive maize promoters, U.S. Patent 6,433,252 which discloses a maize L3 oleosin promoter, U.S. Patent 6,429,357 which discloses a rice actin 2 promoter and intron, U.S. Patent 5,837,848 which discloses a root specific promoter, U.S. Patent 6,084,089 which discloses cold inducible promoters, U.S. Patent 6,294,714 which discloses light inducible promoters, U.S. Patent 6,140,078 which discloses salt inducible promoters, U.S. Patent 6,252,138 which discloses pathogen inducible promoters, U.S. Patent 6,175,060 which discloses phosphorus deficiency inducible promoters, U.S. Patent 6,635,806 which discloses a coixin promoter, U.S. 2002/0192813 A1 which discloses 5', 3' and intron elements useful in the design of effective plant expression vectors, U.S. 2004/0216189 A1 which discloses a

maize chloroplast aldolase promoter, and U.S. 2004/0123347 A1 which discloses water-deficit inducible promoters, all of which are incorporated herein by reference. These and numerous other promoters that function in plant cells are known to those skilled in the art and available for use in recombinant polynucleotides of the present disclosure to provide for expression of desired genes in transgenic plant cells.

Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Such enhancers are known in the art. By including an enhancer sequence with such constructs, the expression of the selected protein may be enhanced. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted upstream (5') or downstream (3') to the coding sequence. In some instances, these 5' enhancing elements are introns. Particularly useful as enhancers are the 5' introns of the rice actin 1 (see US Patent 5,641,876) and rice actin 2 genes, the maize alcohol dehydrogenase gene intron, the maize heat shock protein 70 gene intron (U.S. Patent 5,593,874) and the maize shrunken 1 gene.

In other aspects, sufficient expression in plant seed tissues is desired to effect improvements in seed composition. Exemplary promoters for use for seed composition modification include promoters from seed genes such as napin (U.S. 5,420,034), maize L3 oleosin (U.S. 6,433,252), zein Z27 (Russell *et al.* (1997) *Transgenic Res.* 6(2):157-166), globulin 1 (Belanger *et al* (1991) Genetics 129:863-872), glutelin 1 (Russell (1997) *supra*), and peroxiredoxin antioxidant (Per1) (Stacy *et al.* (1996) *Plant Mol Biol.* 31(6):1205-1216).

Recombinant DNA constructs prepared in accordance with this disclosure will often include a 3' element that typically contains a polyadenylation signal and site, especially if the recombinant DNA is intended for protein expression as well as gene suppression. Well-known 3' elements include those from *Agrobacterium tumefaciens* genes such as *nos 3'*, *tml 3'*, *tmr 3'*, *tms 3'*, *ocs 3'*, *tr7 3'*, *e.g.* disclosed in U.S. 6,090,627, incorporated herein by reference; 3' elements from plant genes such as wheat (*Triticum aesevitum*) heat shock protein 17 (*Hsp17 3'*), a wheat ubiquitin gene, a wheat fructose-1,6-biphosphatase gene, a rice glutelin gene a rice lactate dehydrogenase gene and a rice beta-tubulin gene, all of which are disclosed in U.S. published patent application 2002/0192813 A1, incorporated herein by reference; and the pea (*Pisum sativum)* ribulose biphosphate carboxylase gene (*rbs 3')*, and 3' elements from the genes within the host plant.

Constructs and vectors may also include a transit peptide for targeting of a gene target to a plant organelle, particularly to a chloroplast, leucoplast or other plastid organelle. For descriptions of the use of chloroplast transit peptides see U.S. Patent 5, 188,642 and U.S. Patent No. 5,728,925, incorporated herein by reference. For description of the transit peptide region of an *Arabidopsis* EPSPS gene useful in the provided constructs; see Klee*et al* (MGG 210:437-442, 1987).

For expression of constructs in fungi such as yeast, there are a variety of promoters to choose from for various purposes. The following are provided by way of example, and are not meant to be in any way limiting:

The Gal 1,10 promoter: This promoter is inducible by galactose. It can be used to turn expression of an associated nucleic acid on and off, for instance in order to follow the time dependent effects of expression. The Gal promoter is slightly leaky, and so is appropriate where it is not essential to have absolutely no expression of the passenger gene in the absence of galactose. The Gal 1 gene and Gal 10 gene are adjacent and transcribed in opposite directions from the same promoter region. The regulatory region containing the UAS sequences can be cut out on a DdeI Sau3A fragment and placed upstream of any other gene to confer galactose inducible expression and glucose repression.

PGK, GPD and ADH1 promoters: These are high expression constitutive promoters. PGK = phosphoglycerate kinase, GPD = glyceraldehyde 3 phosphate dehydrogenase, ADH1 = alcohol dehydrogenase

ADH2 promoter: This gene is glucose repressible and it is strongly transcribed on non-fermentable carbon sources (similar to GAL 1,10, except not inducible by galactose).

CUP1 promoter: This is the metalothionein gene promoter. It is activated by copper or silver ions added to the medium. The CUP1 gene is one of a few yeast genes that is present in yeast in more than one copy. Depending on the strain, there can be up to eight copies of this gene. By way of example, a gene, when placed under CUP1 regulation, should e provided with a degree of control of the level of expression based on the amount of copper (or silver) in the medium. Copper is toxic and any cells should be tested to see how well it tolerates copper before making a CUP1 construct.

PHO5 promoter: This promoter is derived from a gene that encodes an acid phosphatase. It is induced by low or no phosphate in the medium. The phosphatase is secreted in the chance it will be able to free up some phosphate from the surroundings. When phosphate is present, no PHO5 message can be found. When phosphate is absent, the promoter is strongly turned on.

Steroid inducible expression: Keith Yamamoto's lab has developed an inducible system in yeast similar to the ecdysone system for mammalian cells. The rat glucocorticoid receptor gene has been inserted behind the constitutive GPD promoter to express the rat glucocorticoid receptor in yeast. A second vector was made with three glucocorticoid response elements upstream of the CYC1 gene minimal promoter (cytochrome c gene). A cloning site was placed after this so a selected gene or other engineered construct could be placed under control of the 3GRE/CYC1 promoter. Both vectors are high copy vectors. This system works well with dose dependent expression, when steroid hormone is added to the medium. Response time is rapid with $t_{1/2}$ of 7-9 minutes after addition of hormone.

Heat shock expression: By placing the UAS from a heat shock gene in front of the minimal CYC1 promoter, any gene or synthetic construct can be placed under heat shock induction. This is a specialized requirement usually used in studies of heat shock response, or in regulation of RNAi under different temperature regimens.

GAL1-10 promoter: This promoter is highly regulatable by galactose, such that there is a basal level on glucose, but over 100 fold increase when cells are placed in galactose medium.

The yeast *GAL* genes form one of the most intensely studied model systems for eukaryotic gene regulation. The structural genes, *e.g. GAL1* and *GAL10,* are induced to very high level expression in galactose by the action of the activator Gal4p. Gal4p binds to activation sequences (UASG) that lie up stream of *GAL* genes and activates transcription in a process that depends on gene-proximal TATA elements and involves numerous coactivators and general transcription factors including TBP. The activation function of Gal4p is modulated by Gal80p, an inhibitory regulator that binds specifically to the activation domain of Gal4p, thus preventing gene activation in nongalactose carbon sources.

In certain embodiments, the provided constructs or methods are used or carried out in animal cells, particularly cells from the nematode *C. elegans*. In such embodiments, promoters or other regulatory sequences that function in animal cells are useful. Myriad animal promoters are well known to those of ordinary skill in the art, including constitutive promoters and inducible or repressible promoters, as well as promoters that show cell or tissue specificity or other regulated expression. Where a siRNA triggering cassette is expressed in *C. elegans* or a cell from a *C. elegans* organism, optionally a *C. elegans* promoter can be used. See, for instance published U.S. Applications No. 10/239,249 (2003-0177507) and 09/422,569 (2003-0023997), which describe the use of various promoters for construct expression in the invertebrate animal *C. elegans*. Specific examples of *C. elegans* promoters include the following: unc-54, hsp16-2, unc-119, G_{0A1} and sel-12. It is also appropriate to use heterologous promoters in animal cells, including cells from (or in) *C. elegans* organisms.

Additional promoters and/or regulatory sequences are discussed elsewhere in this document.

Plant expression vectors optionally include RNA processing signals, *e.g.*, introns, which may be positioned upstream or downstream of a polypeptide-encoding sequence in the transgene. In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes, *e.g.*, a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions.

Such vectors also generally include one or more dominant selectable marker genes, including genes encoding antibiotic resistance (*e.g.*, resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin, paromomycin, or spectinomycin) and herbicide-resistance genes (*e.g.*, resistance to phosphinothricin acetyltransferase or glyphosate) to facilitate manipulation in bacterial systems and to select for transformed plant cells.

Screenable markers are also used for cell transformation, such as fungus or plant cell transformation, including color markers such as genes encoding ß-glucuronidase (*gus*) or anthocyanin production, or fluorescent markers such as genes encoding luciferase or green fluorescence protein (GFP).

IX. In Vitro *Production of Oligonucleotides*

Though it is often appropriate to produce RNAi triggering constructs through genetic engineering techniques such as those discussed above, in some instances components of such constructs can be advantageously produced using *in vitro* chemical synthesis.

In vitro methods for the synthesis of oligonucleotides are well known to those of ordinary skill in the art; such conventional methods can be used to produce IROs for the disclosed methods. The most common method for *in vitro* oligonucleotide synthesis is the phosphoramidite method, formulated by Letsinger and further developed by Caruthers (Caruthers *et al.*, *Chemical synthesis of deoxyoligonucleotides*, in *Methods Enzymol.* 154:287-313, 1987). This is a non-aqueous, solid phase reaction carried out in a stepwise manner, wherein a single nucleotide (or modified nucleotide) is added to a growing oligonucleotide. The individual nucleotides are added in the form of reactive 3'-phosphoramidite derivatives. See also, Gait (Ed.), *Oligonucleotide Synthesis. A practical approach*, IRL Press, 1984.

In general, the synthesis reactions proceed as follows: First, a dimethoxytrityl or equivalent protecting group at the 5' end of the growing oligonucleotide chain is removed by acid treatment. (The growing chain is anchored by its 3' end to a solid support such as a silicon bead.) The newly liberated 5' end of the oligonucleotide chain is coupled to the 3'-phosphoramidite derivative of the next deoxynucleoside to be added to the chain, using the coupling agent tetrazole. The coupling reaction usually proceeds at an efficiency of approximately 99%; any remaining unreacted 5' ends are capped by acetylation so as to block extension in subsequent couplings. Finally, the phosphite triester group produced by the coupling step is oxidized to the phosphotriester, yielding a chain that has been lengthened by one nucleotide residue. This process is repeated, adding one residue per cycle. See, for instances, U.S. Patent Nos. 4,415,732, 4,458,066, 4,500,707, 4,973,679, and 5,132,418. Oligonucleotide synthesizers that employ this or similar methods are available commercially (*e.g.*, the PolyPlex oligonucleotide synthesizer from Gene Machines, San Carlos, CA). In addition, many companies will perform such synthesis (*e.g.*, Sigma-Genosys, TX; Operon Technologies, CA; Integrated DNA Technologies, IA; and TriLink BioTechnologies, CA).

Oligonucleotides are conveniently available commercially up to approximately 125 nucleotides; beyond this length the efficiency and purification drops. Modified nucleotides can be incorporated into an oligonucleotide essentially as described above for non-modified nucleotides.

Methods described above, or other methods known to those of ordinary skill in the art, can be used to produce oligonucleotides comprising an initiation sequence, a gene suppressing element, or combinations thereof, for instance. Such oligonucleotides can be used to construct RNA-trigger nucleic acid cassettes, for instance.

X. *Plants for production of siRNAs*

The presence of the cellular systems described herein necessary to respond to initiator sequences, and thereby produce siRNAs from the described constructs, appears to be nearly universal within the plant and fungal kingdoms. These systems are also present in some invertebrates, such as

C. elegans. At the molecular level for instance, *DCL* and *RDR* homologs have been found in a variety of plant and fungi species, as well as *C. elegans*. Thus, expression of target genes using the synthetic siRNA-bearing constructs (RNAi-triggering nucleic acid molecules) described herein may be modified, particularly inhibited, in a wide range of target organisms and cells of such organisms. These include plants, including both monocotyledonous and dicotyledonous plants. The described system for inducing RNAi finds equal application in fungal systems, including filamentous (mold-type) and some yeast-type fungi, as well as *C. elegans*, a representative invertebrate animal.

Representative, non-limiting example plants include *Arabidopsis*; field crops (*e.g.* alfalfa, barley, bean, clover, corn, cotton, flax, lentils, maize, pea, rape/canola, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (*e.g.* asparagus, beet, brassica generally, broccoli, Brussels sprouts, cabbage, carrot, cauliflower, celery, cucumber (cucurbits), eggplant, lettuce, mustard, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (*e.g.* almond, apple, apricot, banana, blackberry, blueberry, cacao, cassava, cherry, citrus, coconut, cranberry, date, hazelnut, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); tree woods and ornamentals (*e.g.* alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose and rubber).

XI. Delivery of Constructs to Target Cells

Once a nucleic acid molecule (*e.g.*, synthetic construct) encoding at least one siRNA for use in RNAi is generated, standard techniques may be used to express the encoded siRNA molecule(s) in transgenic plants, yeast, or animals. The basic approach is to clone, for instance, the synthetic siRNA construct into a transformation vector, such that it is operably linked to control sequences (*e.g.*, a promoter) that direct expression of the nucleic acid in target cells. The transformation vector is then introduced into the target cells by one of a number of techniques (*e.g.*, electroporation) and progeny containing the introduced nucleic acid construct are selected. In some embodiments, all or part of the transformation vector will stably integrate into the genome of the target cell. That part of the transformation vector that integrates into the target cell and that contains the introduced synthetic siRNA construct and associated sequences for controlling expression (the introduced "transgene") may be referred to as the recombinant expression cassette.

Selection of progeny, for instance, progeny plants, yeast, or invertebrate cells, containing the introduced transgene may be based upon the detection of an altered phenotype. Such a phenotype may result directly from the synthetic construct cloned into the transformation vector or may be manifested as enhanced (or reduced) resistance to a chemical agent (such as an antibiotic) as a result of the inclusion of a selectable marker gene incorporated into the transformation vector.

Examples of the modification of plant characteristics by transformation with cloned cDNA sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the knowledge in this field of technology, include: U.S. Patent No. 5,451,514; U.S. Patent

No. 5,750,385; U.S. Patent No. 5,583,021; U.S. Patent No. 5,589,615; U.S. Patent No. 5,268,526; U.S. Patent No. 5,741,684; U.S. Patent No. 5,773,692; WO 96/13582; published U.S. Application No. 10/450,412 (2004-0139494), No. 09/850846 (2002-0147168). These examples include descriptions of transformation vector selection, transformation techniques and the assembly of constructs designed to express or over-express the introduced nucleic acid.

In light of the foregoing and the provision herein of methods for producing siRNA-producing synthetic constructs governed by described initiator sequences, one of ordinary skill in the art will be able to introduce such nucleic acid constructs into plants, fungi, and animals (particularly invertebrates) in order to produce specimens exhibiting RNAi of one or more target genes.

XII. Plant Transformation, Regeneration, and Selection

Transformation and regeneration of both monocotyledonous and dicotyledonous plant cells is routine, and the most appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

Following transformation and regeneration of plants with the transformation vector, transformed plants may be selected using a dominant selectable marker incorporated into the transformation vector. Typically, such a marker will confer antibiotic resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic.

After transformed plants are selected and grown to maturity, they can be assayed using the methods described herein, and other methods appropriate to the synthetic construct of the transgene, to determine whether the passenger siRNA(s) are being produced, and/or whether the target gene(s) are measurably inhibited by RNAi as a result of the introduced transgene.

Numerous methods for transforming plant cells with recombinant DNA are known in the art and may be used. Two commonly used methods for plant transformation are *Agrobacterium*-mediated transformation and microprojectile bombardment. Microprojectile bombardment methods are illustrated in U.S. Patents 5,015,580 (soybean); 5,550,318 (corn); 5,538,880 (corn); 5,914,451 (soybean); 6,160,208 (corn); 6,399,861 (corn) and 6,153,812 (wheat) and *Agrobacterium*-mediated transformation is described in U.S. Patents 5,159,135 (cotton); 5,824,877 (soybean); 5,591,616 (corn); and 6,384,301 (soybean), all of which are incorporated herein by reference. For *Agrobacterium tumefaciens* based plant transformation system, additional elements present on transformation constructs will include T-DNA left and right border sequences to facilitate incorporation of the recombinant polynucleotide into the plant genome.

In general it is useful to introduce recombinant DNA randomly, *i.e.* at a non-specific location, in the genome of a target plant line. In special cases it may be useful to target recombinant DNA insertion in order to achieve site-specific integration, *e.g.* to replace an existing gene in the genome, to use an existing promoter in the plant genome, or to insert a recombinant polynucleotide at a predetermined site known to be active for gene expression. Several site specific recombination systems exist which are known to function implants include çre-lox as disclosed in U.S. Patent 4,959,317 and FLP-FRT as disclosed in U.S. Patent 5,527,695, both incorporated herein by reference.

Transformation methods are preferably practiced in tissue culture on media and in a controlled environment. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. Recipient cell targets include, but are not limited to, meristem cells, callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. It is contemplated that any cell from which a fertile plant may be regenerated is useful as a recipient cell. Callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Cells capable of proliferating as callus are also recipient cells for genetic transformation. Practical transformation methods and materials for making transgenic plants, *e.g.* various media and recipient target cells, transformation of immature embryos and subsequent regeneration of fertile transgenic plants are disclosed in U.S. Patents 6,194,636 and 6,232,526, which are incorporated herein by reference.

The seeds of transgenic plants can be harvested from fertile transgenic plants and be used to grow progeny generations of transformed plants including hybrid plants line for screening of plants having an enhanced agronomic trait. In addition to direct transformation of a plant with a recombinant DNA, transgenic plants can be prepared by crossing a first plant having a recombinant DNA with a second plant lacking the DNA. For example, recombinant DNA can be introduced into first plant line that is amenable to transformation to produce a transgenic plant which can be crossed with a second plant line to introgress the recombinant DNA into the second plant line. A transgenic plant with recombinant DNA providing an enhanced agronomic trait, *e.g.* enhanced yield, can be crossed with transgenic plant line having other recombinant DNA that confers another trait, *e.g.* herbicide resistance or pest resistance, to produce progeny plants having recombinant DNA that confers both traits. Typically, in such breeding for combining traits the transgenic plant donating the additional trait is a male line and the transgenic plant carrying the base traits is the female line. The progeny of this cross will segregate such that some of the plants will carry the DNA for both parental traits and some will carry DNA for one parental trait; such plants can be identified by markers associated with parental recombinant DNA Progeny plants carrying DNA for both parental traits can be crossed back into the female parent line multiple times, *e.g.* usually 6 to 8 generations, to produce a progeny plant with substantially the same genotype as one original transgenic parental line but for the recombinant DNA of the other transgenic parental line

In the practice of transformation DNA is typically introduced into only a small percentage of target cells in any one transformation experiment. Marker genes are used to provide an efficient system for identification of those cells that are stably transformed by receiving and integrating a

transgenic DNA construct into their genomes. Preferred marker genes provide selective markers which confer resistance to a selective agent, such as an antibiotic or herbicide. Any of the herbicides to which plants may be resistant are useful agents for selective markers. Potentially transformed cells are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene is integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Commonly used selective marker genes include those conferring resistance to antibiotics such as kanamycin and paromomycin (*nptII*), hygromycin B (*aph IV*) and gentamycin (*aac3* and *aac*C4) or resistance to herbicides such as glufosinate (*bar* or *pat*) and glyphosate (*aroA* or EPSPS). Examples of such selectable are illustrated in U.S. Patents 5,550,318; 5,633,435; 5,780,708 and 6,118,047, all of which are incorporated herein by reference. Screenable markers which provide an ability to visually identify transformants can also be employed, *e.g.*, a gene expressing a colored or fluorescent protein such as a luciferase or green fluorescent protein (GFP) or a gene expressing a *beta*-glucuronidase or *uid*A gene (GUS) for which various chromogenic substrates are known.

Cells that survive exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in regeneration media and allowed to mature into plants. Developing plantlets can be transferred to plant growth mix, and hardened off, *e.g.*, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO_2, and 25-250 microeinsteins m^{-2} s^{-1} of light, prior to transfer to a greenhouse or growth chamber for maturation. Plants are regenerated from about 6 weeks to 10 months after a transformant is identified, depending on the initial tissue. Plants may be pollinated using conventional plant breeding methods known to those of skill in the art and seed produced, *e.g.* self-pollination is commonly used with transgenic corn. The regenerated transformed plant or its progeny seed or plants can be tested for expression of the recombinant DNA and screened for the presence of enhanced agronomic trait.

XIII. Transgenic Plants and Seeds

Transgenic plant seed provided herein are grown to generate transgenic plants having an enhanced trait as compared to a control plant. Such seed for plants with enhanced agronomic trait(s) is identified by screening transformed plants, progeny, or progeny seed for the enhanced trait(s). For efficiency, a screening program is beneficially used to evaluate multiple transgenic plants (events) comprising the recombinant DNA, *e.g.* multiple plants from 2 to 20 or more transgenic events.

Transgenic plants grown from transgenic seed provided herein demonstrate improved agronomic traits that contribute to increased yield or other trait that provides increased plant value, including, for example, improved seed quality. Of particular interest are plants having enhanced yield resulting from improved plant growth and development, stress tolerance, improved seed development, higher light response, improved flower development, or improved carbon and/or nitrogen metabolism

Many transgenic events which survive to fertile transgenic plants that produce seeds and progeny plants will not exhibit an enhanced agronomic trait. Screening is necessary to identify the

transgenic plant having enhanced agronomic traits from populations of plants transformed as described herein by evaluating the trait in a variety of assays to detect an enhanced agronomic trait. These assays also may take many forms, including but not limited to, analyses to detect changes in the chemical composition, biomass, physiological properties, morphology of the plant.

XIV. Targets for RNAi

The target gene can be in any cell derived from or contained in any organism. The organism can be a plant, an animal, or fungus, as described herein. The target gene may be a cellular gene (*i.e.*, derived from a cell, as opposed to a virus or other exogenous source), an endogenous gene (*i.e.*, a cellular gene found in the genome), a transgene (*i.e.*, a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen or invasive entity which is capable of infecting or infesting an organism from which the cell is derived. Specific, non-limiting examples of target genes include genes encoding: structural or regulatory molecules; enzymes; toxins; transcription factors; chromatin factors; metabolic factors: secreted factors; mRNA expressed by pathogens; reproductive factors; pigments; pathogen response factors; environmental stress factors; allergens; and so forth. Also contemplated are target genes that are involved in reproduction, particularly male fertility in plants; genes that enhance vegetative growth. Targets also can be selected from non-coding regions of the genome of the target organism.

In addition to endogenous gene and non-gene targets, it is contemplated that the RNAi-triggering constructs and methods described herein can be used to inhibit expression of pathogen or parasite genes, for instance gene sequences expressed bacterial, viral, other pathogen, animal pest, or plant pest (*e.g.*, nematode) targets. By way of example, such gene inhibition in the context of an organism infected or infested with such pathogenic target could be used to combat the pathogen. Treatment of pathogens using such a system could be preventative, wherein the RNAi-triggering construct(s) are introduced before there is known infection or introduction of the pathogenic organism. In such embodiments, the presence of the RNAi-triggering system is intended to prevent, reduce, or ameliorate a subsequent infection or contamination with the target pathogen or other microorganism. Alternatively, infected or infested organisms could be treated after the microorganism(s) are present. In such embodiments, the RNAi-triggering system is intended to treat or eradicate the infection/infestation.

In yet other embodiments, an RNAi-triggering system is introduced to provide inhibitory control over a transgenic target gene sequence, or set of transgenic sequences, for instance that have been introduced into a transgenic plant, fungus, or other cell. Such targets might include transgenes that confer desirable or undesirable traits to the target organism. Representative non-limiting examples of categories of transgenes are discussed herein; any transgene could serve as a target, and specific targets will be best selected by the practitioner.

Inhibition of target gene expression or activity can be measured by monitoring the levels of target gene mRNA or proteins encoded by the target gene. Examples of known techniques used to measure mRNA levels include RNA solution hybridization, nuclease protection, Northern blot

analysis, and reverse transcription which can be used in combination with polymerase chain reaction. Examples of techniques used to measure target gene protein levels include antibody binding, enzyme linked immunosorbent assay (ELISA), Western blot analysis, immunoassays (*e.g.* radioimmunoassay), and fluorescence activated cell sort (FACS).

Depending on the particular target gene and the level of production of the siRNA, increasing the production of siRNA(s), for example through expression from a transgene described herein, may provide partial or complete loss of expression, or function, of the target gene. The inhibition in target gene expression in different embodiments is at least a 5%, at least a 10%, at least a 20%, at least a 30%, at least a 50%, at least a 75%, at least an 80%, at least an 85%, at least a 90%, at least a 95%, or a 100% inhibition in target gene expression.

XV. Regulated RNAi

The RNAi-triggering systems described herein can further be employed to exploit differentially regulated systems within a target, for instance in order to provide cell-specific, tissue-specific, or developmentally specific RNAi of one or more specific genes. In particular, miRNAs frequently accumulate in specific cell-types or tissues (*e.g.* Palatnik *et al.*, *Nature* 425:257-263, 2003) or are induced under specific conditions, such as nutrient or abiotic stress (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004). Thus, cell-, tissue-, or conditional RNAi may be regulated by cell-, tissue-or condition-specific miRNA or siRNA expression by employing a target sequence (initiator sequence) that interacts with a specific regulated small RNA to guide cleavage of the target sequence in the desired expression pattern. Representative miRNAs and functions associated with their target(s) are listed in Table 4.

Alternatively, or in combination, regulated RNAi can also be achieved using expression cassettes that are only transcribed, or preferentially transcribed, in certain cells, tissues, conditions, and so forth. Represented promoters useful for such regulated expression are discussed herein.

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

Example 1: MiRNA-Directed Phasing During *Trans*-Acting siRNA Biogenesis in Plants.

Small RNA blot analysis

Low molecular weight RNA (5 μg) from *Arabidopsis* inflorescence tissue was used for miRNA and endogenous siRNA analysis as described (Allen *et al.*, *Nat Genet* 36:1282-1290, 2004). Mutant lines for *dcl1-7*, *dcl2-1*, *dcl3-1*, *rdr1-1*, *rdr2-1*, *hen1-1*, *hyl1-2*, *rdr6-11*, *rdr6-15*, and *sgs3-11* were described previously (Allen *et al.*, *Nat Genet* 36:1282-1290, 2004; Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Curr Biol*

14:346-351, 2004a; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). The *hst-15* allele used was the SALK_079290 T-DNA insertion line from ABRC, which contains a T-DNA at position 1584 from the start codon. Probes for miR159, miR167, and *AtSN1*-siRNA blots were described previously (Llave *et al.*, *Plant Cell* 14:1605-1619, 2000a; Zilberman *et al.*, *Science* 299:716-719, 2003). All other miRNAs were detected using end-labeled DNA oligonucleotides. Probes for ta-siRNA loci were PCR amplified from Col-0 genomic DNA, cloned into pGEMT-Easy, and verified by sequencing. Radiolabeled probes incorporating ^{32}P-UTP were made by T7 RNA polymerase transcription, to obtain strand specific small RNA probes. Probes were as follows: *TAS3* locus, Chr3:5862146-5862295; *At3g39680* (*TAS2*) locus, Chr2:16546831-16547300.

Computational prediction of miRNA targets

An initial pool of predicted target sites for validated miRNAs was created by FASTA searches using a +15/-10 scoring matrix of the TAIR AGI transcript database, limited to 4 mispairs, 4 G:U pairs, to a total of seven, with 100,000 results obtained for the reverse complement of each small RNA. A single, one nucleotide gap was allowed. The miRNA target prediction algorithm used to score these sites was developed based on 94 experimentally validated and predicted family members of miRNA-target site duplexes, including 55 targets validated in previous studies, 11 new validated targets, plus 28 family members with conserved miRNA target sites (Target Rule Set, Table 3). Three filters based on the Target Rule Set were applied sequentially. In each case, base one is considered to be the first nucleotide from the 5' end of the miRNA. First, targets with a mismatch score greater than four were excluded. The Minimum Free Energy (ΔG_{MFE}) of a perfect miRNA-target duplex was determined by computationally attaching a perfectly complementary target sequence to a small RNA using a four base "gap" linker sequence (----). The free energy each miRNA-predicted target site (ΔG_{target}) was determined by computationally linking the target sequence to the small RNA, from which the MFE ratio was calculated ($\Delta G_{target}/\Delta G_{MFE}$). All thermodynamic values were calculated using RNAFold in the Vienna RNA package. Remaining targets with an MFE ratio less than 0.73 were excluded. Conservation of the target sequence was determined by using the region containing the target sequence in a BLAST search against the *Arabidopsis* transcript and EST databases, NCBI EST database, and *O. sativa* Unigene database, and removing any targets with no matches with less than three base changes in the target sequence. Duplicate target sites (identical genes) for related miRNA family members were combined in the final target gene set.

Table 3. Summary of miRNA target gene predictions represented in Figure 1

Bin 1. Previously predicted miRNA target genes, experimentally validated

	Systematic name[a]	Common name[a]	Gene family	miRNA family	Rule Development Set	Score[b]	MFE Ratio	Pass/Fail	Original prediction reference
1	At1g27370	*SPL10*	SPL	miR156	yes	3	0.808	Pass	c
2	At5g43270	*SPL2*	SPL	miR156	yes	3	0.842	Pass	c
3	At1g53160	*SPL4*	SPL	miR157	yes	3	0.820	Pass	c
4	At5g06100	*MYB33*	MYB	miR159	yes	3	0.787	Pass	c; d
5	At3g11440	*MYB65*	MYB	miR159	yes	3	0.787	Pass	c; d
6	At1g77850	*ARF17*	ARF	miR160	yes	0.5	0.990	Pass	c
7	At2g28350	*ARF10*	ARF	miR160	yes	2	0.844	Pass	c
8	At4g30080	*ARF16*	ARF	miR160	yes	2.5	0.863	Pass	c
9	At1g06580		PPR	miR161.1	yes	3	0.713	Fail	c
10	At1g63150		PPR	miR161.2	yes	1.5	0.856	Pass	c
11	At5g41170		PPR	miR161.1	yes	1	0.792	Pass	c
12	At1g01040	*DCL1*	DCL	miR162	yes	2	1.000	Pass	e
13	At1g66690		SAMT	miR163	yes	1	0.898	Pass	d
14	At1g66700		SAMT	miR163	yes	1	0.898	Pass	d
15	At1g66720		SAMT	miR163	yes	2	0.886	Pass	f
16	At3g44860		SAMT	miR163	yes	3	0.765	Pass	f
17	At1g56010	*NAC1*	NAC	miR164	yes	2	0.823	Pass	c
18	At3g15170	*CUC1*	NAC	miR164	yes	3	0.856	Pass	c
19	At5g07680		NAC	miR164	yes	2	0.849	Pass	c
20	At5g53950	*CUC2*	NAC	miR164	yes	3	0.856	Pass	c
21	At5g61430		NAC	miR164	yes	2	0.849	Pass	c
22	At1g30490	*PHV*	HD-ZipIII	miR166	yes	3	0.860	Pass	c
23	At1g52150	*AtHB15*	HD-ZipIII	miR166	yes	2.5	0.867	Pass	c

Table 3. Summary of miRNA target gene predictions represented in Figure 1 (Part 2)

24	At2g34710	*PHB*	HD-ZipIII	miR166	yes	3	0.860	Pass	c
25	At5g60690	*REV/IFL1*	HD-ZipIII	miR166	yes	3	0.860	Pass	c
26	At1g30330	*ARF6*	ARF	miR167	yes	3.5	0.844	Pass	c; d
27	At5g37020	*ARF8*	ARF	miR167	yes	4	0.779	Pass	c; d
28	At1g48410	*AGO1*	AGO	miR168	yes	4	0.735	Pass	c
29	At1g17590		HAP2	miR169	yes	2.5	0.866	Pass	c
30	At1g54160		HAP2	miR169	yes	3	0.840	Pass	c
31	At1g72830	*HAP2c*	HAP2	miR169	yes	2.5	0.834	Pass	b
32	At3g05690	*HAP2b*	HAP2	miR169	yes	3	0.746	Pass	b
33	At3g20910		HAP2	miR169	yes	4	0.735	Pass	b
34	At5g06510		HAP2	miR169	yes	3	0.746	Pass	b
35	At2g45160	*SCL6(II)*	SCL	miR171	yes	0	1.000	Pass	g; c
36	At3g60630	*SCL6(III)*	SCL	miR171	yes	0	1.000	Pass	g; c
37	At4g00150	*SCL6(IV)*	SCL	miR171	yes	0	1.000	Pass	g; c
38	At2g28550	*TOE1/RAP2.7*	AP2	miR172	yes	3.5	0.857	Pass	d
39	At4g36920	*AP2*	AP2	miR172	yes	2.5	0.896	Pass	d
40	At5g60120	*TOE2*	AP2	miR172	yes	1.5	0.928	Pass	d
41	At5g67180	*TOE3*	AP2	miR172	yes	3.5	0.896	Pass	d
42	At1g30210	*TCP24*	TCP	miR319	yes	3.5	0.792	Pass	i
43	At1g53230	*TCP3*	TCP	miR319	yes	4	0.751	Pass	i
44	At2g31070	*TCP10*	TCP	miR319	yes	3.5	0.777	Pass	i
45	At3g15030	*TCP4*	TCP	miR319	yes	3.5	0.777	Pass	i
46	At4g18390	*TCP2*	TCP	miR319	yes	3.5	0.792	Pass	i
47	At1g12820		TIR/F-box	miR393	yes	2	0.862	Pass	b
48	At3g23690	*bHLH077*	bHLH	miR393	yes	3	0.871	Pass	b

Table 3. Summary of miRNA target gene predictions represented in Figure 1 (Part 3)

49	At3g26810		TIR/F-box	miR393	yes	2	0.862	Pass	b
50	At3g62980	*TIR1*	TIR/F-box	miR393	yes	2.5	0.876	Pass	b
51	At4g03190		TIR/F-box	miR393	yes	3.5	0.761	Pass	b
52	At1g27340		F-box	miR394	yes	1	0.820	Pass	b
53	At5g43780	*APS4*	ATP sulfurylase	miR395	yes	2	0.792	Pass	b
54	At3g22890	*APS1*	ATP sulfurylase	miR395	yes	3.5	0.744	Pass	b
55	At2g22840	*GRF1*	GRF	miR396	yes	3.5	0.861	Pass	b
56	At2g36400	*GRF3*	GRF	miR396	yes	3	0.861	Pass	b
57	At2g45480	*GRF9*	GRF	miR396	yes	4	0.861	Pass	b
58	At4g24150	*GRF8*	GRF	miR396	yes	3.5	0.861	Pass	b
59	At4g37740	*GRF2*	GRF	miR396	yes	3.5	0.861	Pass	b
60	At5g53660	*GRF7*	GRF	miR396	yes	3.5	0.861	Pass	b
61	At2g29130		Laccase	miR397	yes	3.5	0.755	Pass	b
62	At2g38080		Laccase	miR397	yes	2.5	0.877	Pass	b
63	At5g60020		Laccase	miR397	yes	2.5	0.828	Pass	b
64	At3g15640		Cytochrome C oxidase	miR398	yes	3	0.804	Pass	b
65	At1g08830	*CSD1*	Copper superoxide dismutase	miR398	yes	5	0.712	Fail	b
66	At2g28190	*CSD2*	Copper superoxide dismutase	miR398	yes	6.5	0.761	Fail	b
Bin 2. Previously predicted miRNA target gene, computational prediction only[c]									
	Systematic name[a]	Common name[a]	Gene family	miRNA family	Rule Development Set	Score[b]	MFE Ratio	Pass/Fail	Original prediction reference
1	At1g27360	*SPL11*	SPL	miR156	yes	3	0.808	Pass	c
2	At1g69170	*SPL6*	SPL	miR156	yes	3	0.808	Pass	c
3	At2g33810	*SPL3*	SPL	miR156	yes	3	0.808	Pass	c
4	At2g42200	*SPL9*	SPL	miR156	yes	2	0.832	Pass	c
5	At3g57920	*SPL15*	SPL	miR156	yes	2	0.832	Pass	c

Table 3. Summary of miRNA target gene predictions represented in Figure 1 (Part 4)

6	At5g50570	*SPL13*	SPL	miR156	yes	2	0.832	Pass	c
7	At5g50670		SPL	miR156	yes	2	0.832	Pass	c
8	At3g15270	*SPL5*	SPL	miR157	yes	4	0.778	Pass	c
9	At2g26950	*MYB104*	MYB	miR159	yes	4	0.880	Pass	c; d
10	At2g32460	*MYB101*	MYB	miR159	yes	3.5	0.802	Pass	c
11	At3g60460	*MYB125*	MYB	miR159	yes	3.5	0.786	Pass	c
12	At5g55020	*MYB120*	MYB	miR159	yes	3.5	0.732	Pass	c; d
13	At2g26960	*MYB81*	MYB	miR159	yes	4.5	0.719	Fail	c
14	At4g26930	*MYB97*	MYB	miR159	yes	4	0.729	Fail	c
15	At1g62670		PPR	miR161.1	yes	3	0.765	Pass	c
16	At1g64580		PPR	miR161.1	yes	3.5	0.787	Pass	c
17	At1g62720		PPR	miR161.1	yes	5	0.754	Fail	c
18	At1g63080		PPR	miR161.2	yes	4	0.732	Pass	c
19	At1g63400		PPR	miR161.2	yes	2	0.846	Pass	c
20	At5g16640		PPR	miR161.2	yes	2.5	0.715	Fail	c
21	At3g44870		SAMT	miR163	yes	3	0.765	Pass	f
22	At5g39610		NAC	miR164	yes	3.5	0.763	Pass	b
23	At4g32880	*AtHB8*	HD-Zip	miR166	yes	3	0.860	Pass	c
24	At5g12840	*HAP2a*	HAP2	miR169	yes	3	0.735	Pass	b
25	At2g39250	*SNZ*	AP2	miR172	yes	2.5	0.922	Pass	h
26	At3g54990	*SMZ*	AP2	miR172	yes	1.5	0.954	Pass	h
27	At4g14680	*APS3*	ATP sulfurylase	miR395	yes	3.5	0.744	Pass	b
28	At3g52910	*GRF4*	GRF	miR396	yes	3	0.861	Pass	b
	At3g28460		unclassified	miR173		7	0.760	Fail, not conserved	d

Table 3. Summary of miRNA target gene predictions represented in Figure 1 (Part 5)

	At2g40760		Rhodenase-like	miR396		5.5	0.700	Fail, not conserved	b
	At4g27180	*ATK2*	Kinesin-like protein B	miR396		6.5	0.527	Fail, not conserved	b
	At5g12250		Beta-6 tubulin	miR397		10	0.698	Fail, not conserved	b
	At3g54700		phosphate transporter	miR399		3.5	0.743	Fail, not conserved	b
Bin 3. New predicted miRNA target genes from existing target families									
	Systematic name[a]	Common name[a]	Gene family	miRNA family	Score[b]	MFE Ratio	Pass/Fail		
1	At1g62860		PPR	miR161.1	4	0.749	Pass		
2	At1g63330		PPR	miR161.2	1	0.852	Pass		
3	At1g62590		PPR	miR161.2	1	0.852	Pass		
4	At1g63630		PPR	miR161.2	2.5	0.859	Pass		
5	At1g62930		PPR	miR161.2	3	0.882	Pass		
6	At1g63130		PPR	miR161.2	3	0.882	Pass		
7	At1g62910		PPR	miR161.2	3	0.882	Pass		
8	At1g63230		PPR	miR161.2	3	0.735	Pass		
9	At3g14020		HAP2	miR169	2	0.859	Pass		
Bin 4. Novel miRNA target genes, experimentally validated									
	Systematic name[a]	Common name[a]	Gene family	miRNA family	Score[b]	MFE Ratio	Pass/Fail	Associated ESTs	
1	At5g60760		2PGK	miR447	3.5	0.807	Pass		
2	At5g10180	*AST68*	Sulfate transporter	miR395	3	0.760	Pass		
3	At2g27400	*TAS1a*		miR173	2.5	0.768	Pass	CD534192,CD534180	
4	At1g50055	*TAS1b*		miR173	4.5		Fail		
5	At2g39675	*TAS1c*		miR173	2.5	0.768	Pass		
6	At2g39681	*TAS2*		miR173	2.5	0.768	Pass	BE521498	
7	At3g17185	*TAS3*		miR390	3.5	0.755	Pass	AV534298,AI998599,BX838290,AA651246	
8	At2g33770		E2-UBC	miR399	3.5	0.763	Pass		
8	At1g31280	*AGO2*	AGO	miR403	1	0.948	Pass	BP648434,AU230620	

Table 3. Summary of miRNA target gene predictions represented in Figure 1 (Part 6)

Bin 5. Predicted miRNA target genes tested experiementally but not validated

	Systematic name[a]	Common name[a]	Gene family	miRNA family	Score	MFE Ratio	Pass/Fail	Original prediction reference
1	At1g64100		PPR	miR158	4	0.733	Pass	C
2	At3g03580		PPR	miR158	3.5	0.770	Pass	
3	At2g03210	*FUT2*	FUT	miR158	4	0.731	Pass	
4	At2g03220	*FUT1*	FUT	miR158	4	0.737	Pass	

a, Systematic and common names for genes were from TAIR (www.arabidopsis.org) and AGRIS (http://arabidopsis.med.ohio-state.edu/AtTFDB/index.jsp); b, Score was derived from a modified version of the scoring system developed by Jones-Rhoades et al., 2004.
References: b: Jones-Rhoades et al., 2004; c: Rhoades et al., 2002; d: Park et al., 2002 ; e : Xie et al., 2003; f: Allen et al., 2004; g: Llave et al., 2002 ; h : Schmid et al., 2003; i:

Targets for ARF3 and ARF4 were predicted by aligning nucleotide sequence from orthologs from 17 selected species using TCoffee. Similarity over a 21 nucleotide window (characteristic of a miRNA target site) was plotted using PLOTCON in the EMBOSS software suite. Regions beyond the two predicted target sites showing low nucleotide conservation were removed for clarity. Orthologs of the At3g17185 were identified using BLAST, with ESTs only in the predicted miRNA orientation chosen. All selected ESTs were analyzed for the presence of an ARF gene or other conserved ORF by BLASTX analysis against an *Arabidopsis* protein database, and any match eliminated. ESTs were aligned using TCoffee, and the poorly conserved region surrounding the putative miRNAs removed.

Microarray analysis

Inflorescence tissue (stages 1-12) was collected in triplicate, with three bulked plants for each genotype per replicate. Controls for *dcl1-1* and *hen1-1* were La-*er*, controls for *hyl1-2*, *hst-15*, *dcl2-1*, *dcl3-1*, *rdr1-1* ,*rdr2-1*, and *rdr6-15* were Col-0. RNA was extracted using Trizol, followed by purification using the Plant RNeasy Midi kit (Qiagen). Biotinylated cRNA was synthesized from 5 μg total RNA using the MessageAmp kit (Ambion). Twenty micrograms (20 μg) of concentration-adjusted cRNA were fragmented and hybridized to ATH1 GeneChip arrays according to the manufacturer's protocol (Affymetrix). Samples were normalized using RMA Express (Bolstad *et al.*, *Bioinformatics* 19, 185-193, 2003), and imported into Genespring v7 (Silicon Genetics) for analysis. Hierarchical clustering was performed using the standard clustering algorithm.

5' RACE analysis of miRNA directed cleavage of target genes

Cleavage sites of miRNA target genes were mapped using the Invitrogen GeneRacer 5' RACE procedure as described previously (Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Llave *et al.*, *Science* 297:2053-2056, 2002). Gene specific primers were designed approximately 500 nucleotides downstream of the predicted cleavage site. These primers were used in combination with an adapter specific primer to amplify cleavage products by PCR. Purified PCR products were cloned into pGEM-T Easy.

Phylogeny reconstruction methods

The phylogenetic tree for the ARF family was generated by aligning the conserved ARF domain using TCoffee, followed by Bayesian reconstruction of a consensus family tree (Allen *et al.*, *Nat Genet* 36:1282-1290, 2004).

RESULTS

Computational prediction and validation of new miRNA targets

A rigorous set of computationally predicted and validated targets for most *Arabidopsis* miRNA families has emerged (Table 4 and Table 3) (Aukerman & Sakai, *Plant Cell* 15:2730-2741, 2003; Chen, *Science* 303:2022-2025, 2004; Emery *et al.*, *Curr Biol* 13:1768-1774, 2003; Jones-

Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Llave *et al.*, *Science* 297:2053-2056, 2002b; Mallory *et al.*, *Curr Biol* 14:1035-1046, 2004; Palatnik *et al.*, *Nature* 425:257-263, 2003; Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Rhoades *et al.*, *Cell* 110:513-520, 2002; Tang *et al.*, *Genes & Dev* 17:49-63 2003; Vaucheret *et al.*, *Genes Dev* 18:1187-1197, 2004; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004a; Xie *et al.*, *Curr Biol* 13:784-789, 2003). However, clear targets for several miRNAs (miR158, miR173, miR390/391, miR399, miR403 and miR447) are not yet known.

Table 4. *Arabidopsis* microRNA and ta-siRNA Target Families

	Small RNA family[a]	Target family	Number of targets	Target Function
microRNA				
1	miR156[b]	SBP	11	transcription factor
2	miR158			
3	miR159[b]	MYB	8	transcription factor
	miR319[b]	TCP[g]	5	transcription factor
4	mir160[b]	ARF	3	transcription factor
5	miR161[b]	PPR	17	unknown
6	miR162[b]	DCL	1	miRNA metabolism
7	miR163[b]	SAMT	5	metabolism
8	miR164[b]	NAC	6	transcription factor
9	miR166[b]	HD-ZIPIII	5	transcription factor
10	miR167[b]	ARF	2	transcription factor
11	miR168[b]	AGO1	1	miRNA metabolism
12	miR169[b]	HAP2	8	transcription factor
13	miR171[b]	SCR	3	transcription factor
14	miR172[b]	AP2	6	transcription factor
15	miR173	TAS1, TAS2	4	ta-siRNA biogenesis
16	miR390	TAS3	1	ta-siRNA biogenesis
17	miR393[b]	TIR1/F-box	4	hormone signaling
		bHLH	1	transcription factor
18	miR394[b]	F-box	1	hormone signaling
19	miR395[b]	ATPS	4	metabolism
		AST		metabolism
20	miR396[b]	GRF	7	transcription factor
21	miR397[b]	laccase/Cu oxidase	3	metabolism
22	miR398[b]	CSD	2	stress response
		CytC oxidase	1	metabolism
23	miR399	E2-UBC	1	ubiquitin conjugation
24	miR447	2PGK	1	metabolism
25	miR403	AGO2	1	miRNA metabolism
26	miR408	laccase	1	metabolism
Trans-acting siRNA				
1	TAS1	unclassified[s,t]	5	unknown
2	TAS2	PPR[c]	8	unknown
3	TAS3	ARF[c]	4	transcription factor

a, miRNA families contain at least one member, with related miRNAs with up to five changes grouped into a family; b, miRNAs with targets used in the Rule development set; c, targets families validated in previous

studies are in blue, italics indicated additional family members validated in this study, red indicates gene families validated only in this study.

To further extend and refine the analysis of miRNA targets in plants, we developed a set of computational "rules" for *Arabidopsis* miRNA-target interactions involving 22 miRNA families. These were used to produce a target prediction set that was experimentally tested (Figure 1A). The rule development set included 66 experimentally validated targets and 28 previously predicted targets that are closely related to validated family members. Among the 66 validated targets were 55 previously published targets and 11 new validated targets.

Experimental validation of targets involved 5'RACE assays to detect a cleavage site opposite of position 10 from the 5' end of the miRNA (Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Llave *et al.*, *Science* 297:2053-2056, 2002). Detection of a cleavage product with a 5' terminus corresponding to the predicted miRNA-guided cleavage site is strong evidence in support of target site function. Validated targets included genes from multigene families in which closely related paralogs were shown previously to be miRNA targets (Bins 1 and 3, Figure 2A), and nine novel targets discussed in detail below (Bin 4, Figures 3A and 3B).

Two parameters were analyzed for rule development. First, the occurrence of mispaired bases between miRNAs and targets was analyzed. All miRNA-target duplexes within the rule set contained four or fewer unpaired bases, four or fewer G:U pairs, up to one single-nucleotide bulge, and a total of seven or fewer unpaired plus G:U positions. The positions of mispairs were examined by plotting the percentage of mismatched and G:U pairs at each target nucleotide positions (counting from the 3' end) (Figure 1B). Nucleotide pairs at positions 2-13 formed a core segment with relatively few mismatches relative to positions 1 and 14-21. This core segment is longer than the core segment of animal miRNA-target duplexes (positions 2-8) (Lewis *et al.*, *Cell* 115:787-798, 2003). A mispair scoring system, modified from that used by Jones-Rhoades and Bartel (*Mol Cell* 14:787-799, 2004), was applied to account for the reduced occurrence of mispairs within the core segment. Mismatched pairs or single nucleotide bulges were each scored as 1 and G:U pairs were scored as 0.5. Mismatches and G:U pair scores were doubled within the core segment. A score of ≤ 4 captured 91 of 94 targets in the rule development set for a false negative rate of 0.03.

Second, a relative thermodynamic parameter was investigated. The minimum free energy (MFE) of a hypothetical duplex containing each of the 94 targets paired with a perfectly complementary sequence (ΔG_{MFE}) was calculated and compared to the free energy calculated for the actual miRNA-target duplex (ΔG_{target}). The MFE ratio ($\Delta G_{target}/\Delta G_{MFE}$) was calculated for each duplex in the rule set. Eighty-nine of the duplexes in the rule set had an MFE ratio ≥ 0.73 (Figure 1C), corresponding to a false negative rate of 0.05. Combining the mispair (≤ 4) and MFE ratio (≥ 0.73) limits in a series of filters resulted in capture of 87 targets from the rule set (false negative rate= 0.07). The mispair and MFE ratio limits were applied in searches using all validated miRNAs from the 25 families (Table 4) and the *Arabidopsis* transcript database, resulting in 145 prospective targets (Figure 1D).

Target sequence conservation across species and between closely related paralogs was applied as a final filter. For all miRNAs that were conserved between monocots and dicots (or between dicot families), predicted target sites were required to be similarly conserved (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004). For non-conserved miRNAs, targets sites were required to be present within more than one paralog in *Arabidopsis*. When applied to the rule development set, the respective conservation filters resulted in loss of no genes. Application of the conservation filter to the 145 genes that passed the mispair and MFE ratio filters resulted in 103 genes (Figure 1A).

To further extend the chances for target identification, an miRNA target search was also done using the *Arabidopsis* EST database. The same mispair and conservation filters were used, but the MFE ratio filter limit was lowered to 0.70 to account for known sequencing errors within the EST dataset. A redundancy filter was added to subtract all prospective target genes that also passed the target search using the transcript database. Six new prospective targets were identified in the EST search, resulting in a total of 109 predicted targets. These were assigned to several bins (Figure 1A, Table 3). Bin 1 contained 63 of 66 previously validated targets that contributed to the rule set. Bin 2 contained 24 of the 28 predicted targets from the rule set. Thus, the overall false negative rate was 0.07. Bin 3 contained nine new predicted targets from existing target gene families. These previously nonpredicted targets included eight pentatricopeptide repeat (PPR) genes targeted by miR161.1 and miR161.2, a HAP2a gene (At1g14020) targeted by miR169, and a sulfate transporter (AST68, At5g10180) gene targeted by miR395. Bin 4 contained nine novel targets that were experimentally validated and analyzed in detail (see following sections). Bin 5 contained four genes that were predicted to interact with miR158, but each of these failed the 5'RACE validation assay. If it is assumed that Bin 5 genes represent all incorrect predictions from this search, then the false positive rate was 0.04.

Genes encoding an E2-ubiquitin conjugating enzyme (E2-UBC, At2g33770), Argonaute2 (AGO2, At1g31280), and a 2-phosphoglycerate kinase (2PGK, At5g60760) were validated as targets of miR399, miR403 and miR477, respectively, and represent the only conventional genes in Bin 4 (Figure 3A). Possibly because of computational searches using a transcript database containing a misannotated E2-UBC, miR399 was predicted previously to target a different mRNA encoding a phosphate transporter (At3g54700) (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004). This gene was not predicted in our analysis, and the 5'RACE assay failed to reveal a miR399-guided cleavage product. The E2-UBC target, which was identified here and predicted by Sunkar and Zhu (*Plant Cell* 16:2001-2019, 2004) only using EST databases, contains up to five miR399-interacting sites in the 5' untranslated region (UTR). Cleavage products were detected with 5' termini corresponding to cleavage at four of these sites, most prominently sites 2 and 3 (Figure 3A). Orthologous E2-UBC genes in rice and at least three other plant species each contain 3-5 conserved target sites. This is the only example of both a 5'UTR target position and multiple miRNA-target sites in plant genes. The miR403-target site was identified within the 3'UTR of the AGO2 transcript from *Arabidopsis* and several other dicot families, but not in orthologous AGO2 transcripts from monocots. This is the

second miRNA-targeted AGO family member identified, as AGO1 was shown to be targeted by miR168. Whereas AGO1 is required for miRNA activity (Vaucheret *et al.*, *Genes Dev* 18:1187-1197, 2004), presumably within RISC, a function for AGO2 is currently not known. The 2PGK gene (At5g60760) was validated as an miR447 target (Figure 3A), and joins a growing list of plant miRNA targets that encode proteins with metabolic functions (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004).

The five remaining Bin 4 genes were validated as miR173 and miR390 targets (Figure 3B), and were predicted only from EST database due to their unusual nature. These are discussed in detail below.

Expression profiling of predicted miRNA targets

Most miRNAs of plants direct cleavage of their targets. Loss-of-function mutations in miRNA metabolic or biogenesis genes, therefore, frequently result in elevated target transcript levels (Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Palatnik *et al.*, *Nature* 425:257-263, 2003; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004a; Xie *et al.*, *Curr Biol* 13:784-789, 2003). To systematically analyze the effects of miRNA and endogenous siRNA defects on validated and predicted miRNA target genes in *Arabidopsis*, expression profiling was done using nine mutant (condition) plants and two control plants. The mutants included miRNA-defective *dcl1-7*, *hen1-1* and *hyl1-2* (Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Schauer *et al.*, *Trends Plant Sci* 7:487-491, 2002; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004a), which were shown to reduce or eliminate accumulation of miRNAs. A new insertion mutant, *hst-15*, with predicted defects in nucleocytoplasmic transport of miRNA and ta-siRNA precursors (Bollman *et al.*, *Development* 130:1493-1504,2003) was used. Using inflorescence tissue, *hst-15* had only modest or no effects on miRNA accumulation. However, as shown using the *hst-1* mutant (Bollman *et al.*, *Development* 130:1493-1504,2003; Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004), *hst-15* had several developmental abnormalities, including a more rapid juvenile to adult phase change, leaf curling and epinasty, altered silique phyllotaxy and small flowers (Figure 4A). The *hst-15* transcript accumulated to low levels specifically in the *hst-15* mutant plant; this was in contrast to the *dcl1-7* transcript, which was upregulated in each of the miRNA-defective mutants due to loss of miR162-mediated feedback regulation (Xie *et al.*, *Curr Biol* 13:784-789, 2003).

The mutant series also included five siRNA-defective mutants. The *dcl3-1* and *rdr2-1* mutants lack chromatin RNAi-associated, 24-nucleotide siRNAs, *dcl2-1* and *rdr1-1* mutants have defects in antiviral siRNA biogenesis, and the *rdr6-15* mutant is defective in ta-siRNA biogenesis (Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004b; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). The *rdr6-15* mutant contains a new insertion allele, but displays most of the same properties of previously characterized *rdr6* mutants (Allen *et al.*, *Nat Genet* 36:1282-1290, 2004). Specifically, *rdr6-15* plants displays rapid juvenile-to-adult phase change and accompanying morphological defects (Figure 4A), and accumulates low levels of *rdr6-15* transcript.

Expression profiling was done with triplicate biological samples on Affymetrix ATH1 arrays. Because DCL1, HEN1, HYL1, and likely HST, are required for miRNA biogenesis or function, we predicted that miRNA target genes would be upregulated coordinately in the corresponding mutants and largely unaffected in the siRNA biogenesis mutants. As a group, previously validated and predicted target genes (Bin 1+2 genes) generally behaved as anticipated, although clearly not all genes were upregulated in the miRNA mutants (Figure 4B). Of the 81 genes present on the ATH1 array, 27 were significantly ($P<0.01$, ANOVA) upregulated in two or more of the miRNA mutants, although only 16 genes were significantly upregulated in all four miRNA mutants. Transcripts for MYB101 (miR159 target At2g32460) and a NAC domain gene (miR164 target At5g61430) were significantly ($P<0.01$, ANOVA) downregulated in the miRNA mutants, suggesting they may be negatively regulated by a factor that is under miRNA control. Targets from Bins 3+4, of which only 12 were represented on the array, were generally upregulated in the miRNA mutants but unaffected by the siRNA mutants, although the At2g39680 transcript (antisense to validated miR173 target) was significantly upregulated in *rdr6-15* as well as in miRNA-defective mutant plants (Figure 4C). In addition, a list of genes that were affected ($P<0.01$, ANOVA) in each of the *dcl1-7, hen1-1* and *rdr6-15* mutants was generated. This list contained five genes [At4g29770, At2g39680, At5g60450 (Auxin Response Factor4, ARF4), At2g33860 (ARF3) and At1g12770], all of which were up-regulated in the three mutants (Figure 4D). These genes were predicted to be either miRNA targets that were also subject to a RDR6-dependent RNAi pathway, or ta-siRNA targets. Three of these genes were shown to yield transcripts that function as ta-siRNA targets (At4g29770, ARF3 and ARF4), one a predicted ta-siRNA target (At1g12770), and one a novel type of miRNA target (At2g39680).

To analyze the variation patterns among all predicted and validated miRNA targets, two analyses were done. First, a Principal Components Analysis (PCA) was done using expression data from Bins 1-4. An eigenvector that accounted for 65% of the variation among conditions revealed that the miRNA mutants were unified as having target-upregulation effects, and the siRNA mutants were unified as having no effects (Figure 4E). No other eigenvector accounted for more than 9% of the variation. Among 30 genes highly correlated to the primary eigenvector ($r>0.95$), 6 were validated targets, plus one 2PGK gene (At3g45090) closely related to the validated miR477 target. The predicted miR477 target site in At3g45090 failed the MFE ratio (0.69), although the expression profile suggests that At3g45090 is a miRNA target. Second, an unsupervised hierarchical clustering analysis was done, and correlated conditions were displayed as an expression tree. The four miRNA-defective mutants grouped within one clade, with *dcl1-7* and *hen1-1* forming a subclade distinct from an *hst-15/hyl1-2* subclade (Figure 4F). The *dcl1-1, dcl2-1, rdr1-1* and *rdr2-1* mutants formed a distinct expression clade.

To compare more broadly the effects of miRNA and siRNA defects on the *Arabidopsis* transcriptome, condition pairs were analyzed using scatterplots. Also, a similar clustering analysis was done as for targets, using all genes. Expression values (fold-change relative to controls) for genes that are coordinately affected in two mutants should remain on the diagonal, whereas genes that

are differentially in two mutants affected fall above or below the diagonal. Based on this approach, the effects of *hyl1-2* were most similar to the effects of *hst-15*, and the effects of *dcl1-7* were most similar to the effects of *hen1-1* (Figure 4G). In contrast, there was little similarity between transcriptome-wide effects of any of the miRNA mutants and siRNA mutants, as exemplified by the *hyl1-2/dcl3-1* comparisons (Figure 4G). Among all conditions, the miRNA-defective mutants grouped within one clade, and the siRNA mutants formed a distinct clade (Figure 4F). With all genes considered, the *rdr6-15* mutant did not group with either miRNA- or siRNA-defective mutants. Thus, with the major exceptions described below, the expression profiling data indicate that miRNA-mediated regulation of targets and downstream genes is largely independent of the siRNA pathways.

miR173 guides in-phase processing of precursor transcripts for ta-siRNAs at several loci

Four miR173 targets were predicted based on the EST database but not the annotated transcript database. One of these predicted targets was antisense relative to the annotated gene At2g39680. Two other miR173 target sites were predicted based on ESTs AU235820 and CD534192 from paralogous loci; a third paralogous locus also contained the conserved miR173 site. miR173 target validation data for transcripts deriving from each of these four loci were obtained (Figure 3B). None of the miR173 target transcripts contained extended, conserved protein-coding sequences.

Inspection and analysis of the four loci yielding miR173-targeted transcripts revealed that each was a confirmed or predicted ta-siRNA-generating locus (Figure 5). The three paralogous loci, termed *TAS1a, TAS1b* and *TAS1c* yielded siR255 and several similar sequences (siR289, siR752, and siR850, also referred to as siR289, siR752 and siR850, respectively) in tandem, 21-nucleotide arrays. These ta-siRNAs were characterized previously and shown to require DCL1, RDR6, SGS3, and AGO1 (Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004). siR255 (formally *TAS1a* 3'D6(+), *TAS1b* 3'D6(+), *TAS1c* 3'D3(+)) was shown to target transcripts from the related genes At4g29760, At4g29770, and At5g18040 (functions unknown) for degradation in a manner similar to plant miRNAs. This was consistent with the expression profiling data, in which At4g29770 was one of five genes up-regulated in *dcl1-7, hen1-1,* and *rdr6-15* plants (Figure 4D). The fourth miR173 target locus, *TAS2* (which was antisense to annotated At2g39680), possessed the hallmarks of a ta-siRNA-generating site, including the derivation of five cloned small RNAs representing both polarities in accurate, 21-nucleotide register (Figure 5C) and up-regulation in *dcl1-7, hen1-1,* and *rdr6-15* plants (Figure 4D). The *TAS2* (*At3g39680)* locus mapped approximately 2 kb away from, and in the same orientation as, *TAS1c At2g39675*, raising the possibility that both ta-siRNA sets arise from the same precursor transcript (Figure 5C). Relative to miRNAs, siR255 and siR1511 small RNAs were relatively abundant as they corresponded to the 19th and 10th most frequently cloned sequences, respectively, from the small RNA libraries in the ASRP database (Table 5).

Table 5. Highly represented small RNAs in the ASRP database

Rank	Small RNA Family	ASRP no.	Total sequences	
1	miR169	1430	25570	
2	miR156	1423	14029	
3	miR169	1751	6491	
4	miR161.2	563	6227	
5	miR160	1426	4752	
6	miR159	1425	4567	
7	miR169	1514	3944	
8	miR166	934	3482	
9	miR167	5	2893	
10	siR1511 ta-siRNA	1511	1901	
11	miR390	754	1373	
12	miR169	1802	874	
13	miR169	1749	685	
14	miR169	1761	660	
15	miR168	1429	642	
16	miR390	1703	589	
17	miR169	276	457	
18	miR169	1757	405	
19	siR255 ta-siRNA	255	321	
20	miR169	1775	299	

To confirm that *TAS2* is a ta-siRNA-generating locus, and to extend the analysis of biogenesis requirements of this class of small RNA, *TAS2*-derived small RNAs and siR255 from the miRNA- and siRNA-defective mutants were analyzed in blot assays. Small RNAs from the opposite strand at the *TAS2* locus were also analyzed. Accumulation of each small RNA was lost or diminished in *dcl1-7, hen1-1, hyl1-2, rdr6-11* and *sgs3-11*, but not in *hst-15* (Figure 5D). Accumulation levels were unaffected in *dcl2-1, dcl3-1, rdr1-1* and *rdr2-1* mutants (Figure 5D). These data confirm that *TAS2* is a ta-siRNA-generating locus.

The biogenesis data were consistent with a model in which ta-siRNA precursor transcripts are recognized by RDR6/SGS3 and converted (at least partially) to dsRNA forms, which are then processed by DCL1 in phased, 21-nucleotide intervals to form ta-siRNA duplexes. Setting the correct register must be a critical step in this pathway, as out-of-register processing would yield small RNAs with insufficient complementarity to their targets. We hypothesized that miR173-guided cleavage of precursor transcripts generates a terminus that, after RDR6/SGS3-dependent conversion to dsRNA, functions as a start point for successive DCL1-mediated cleavage events in 21-nucleotide intervals. This hypothesis predicts that the predominant ta-siRNAs will form with a 21-nucleotide phase starting at the miR173 cleavage site. A systematic coding system, in which hypothetical DCL1 cleavage products from the miR173-targeted strand [3'D1(+), 3'D2(+), 3'D3(+), etc.] and opposite strand [3'D1(-), 3'D2(-), 3'D3(-), etc.] were assigned a strict phasing relative to miR173 target sites, was devised (Figure 5A, B, C).

Each of the nine cloned ta-siRNAs identified collectively at the four miR173-targeted loci mapped precisely to the phasing interval set by miR173-guided cleavage (Figure 5A ,B ,C). As

predicted from the known properties of Dicer-like enzymes, small RNAs from the non-targeted strand (for example, siR143 and siR1946) were offset by two nucleotides relative to the complementary sequence on the target strand. The register was maintained at each locus through at least the 3'D6 position, and at *TAS1a* through the 3'D8 position. A total of 19 unique small RNAs, from positions 3'D1 to 3'D8, had 5' ends formed by accurate in-phase cleavage but 3' ends offset by one or two nucleotides. Slight variation of this nature was expected, as *Arabidopsis* miRNA populations frequently contain processing variants that differ by one or a few nucleotides. In addition to *TAS1*-derived siRNAs (e.g. siR255), which were confirmed to guide cleavage of mRNA targets (Figure 5E), a hypothetical ta-siRNA from the 3'D6(-) position at the *TAS2* locus was predicted to interact with at least two PPR gene transcripts (At1g12770 and At1g63130, Figure 5E). At1g12770 was one of the five *dcl1-1, hen1-1 and rdr6-15*-upregulated genes (Figure 4D), which was consistent with identity as a ta-siRNA target, although we were unable to validate a cleavage site at the predicted position within the transcript (Figure 5E).

miR390 guides in-phase processing of ta-siRNAs regulating ARF3 and ARF4

The predicted target of miR390 was a transcript from the annotated gene At3g17185 (Figure 6A), for which no function was assigned previously. The hypothetical protein encoded by this gene is small (50 residues) and contains no recognizable motifs, raising the possibility that At3g17185 is a misannotated, protein-noncoding locus. The miR390 target site was validated by 5'RACE analysis (9/22 PCR products sequenced), although a second cleavage site 33 nucleotides away was detected at approximately the same rate (11/22 PCR products).

The hypothesis that At3g17185 is a ta-siRNA-generating locus targeted by miR390 was tested by analysis of small RNAs from the locus, and prediction and validation of putative ta-siRNA target genes. Two low-abundance, cloned small RNAs from sequences to the 5' side of the miR390 cleavage site were identified (Figure 6A). siR1769 derived precisely from the 5'D1(+) position, whereas siR1778 was out-of-register (relative to the miR390-guided cleavage site) between the – 5'D7 and 5'D8 positions. Blot assays using strand- or sequence-specific radiolabeled probes to detect small RNAs arising from between the 5'D5 to the 5'D11 positions revealed that DCL1-, HEN1- and RDR6- and SGS3-dependent, 21-nucleotide small RNAs arose from both strands (Figure 6B). Thus, the At3g17185 locus forms transcripts that yield small RNAs with biogenesis requirements consistent with other ta-siRNAs. In addition to 21-nucleotide RNAs, this locus also yielded detectable 24-nucleotide RNAs, which were clearly DCL3- and RDR2-dependent and RDR6- and SGS3-independent (Figure 6B).

Potential targets of sequenced and hypothetical ta-siRNAs from the At3g17185 locus were identified through several computational and experimental validation steps. First, phylogenetic conservation of the miR390 target site, which was predicted to set the phasing for ta-siRNA precursor processing, was analyzed. Transcripts and ESTs from each of 17 species of monocot and dicot plants contained a miR390 target site, which was uniquely conserved relative to immediate flanking sequence in each case (Figure 6C). Second, functional ta-siRNAs and their targets were predicted to

be phylogenetically conserved across an equivalent evolutionary distance. In *Arabidopsis*, two highly conserved, tandem 21-nucleotide sequences were detected at positions that nearly co-aligned with the hypothetical 5'D7(+) and 5'D8(+) positions relative to the miR390 cleavage site (Figure 6C). These two intervals contained near-identical copies of the same sequence, which was conserved among all transcripts that contained a miR390 target site (Figure 6C). The spacing between the conserved, tandem sequences and the miR390 target site varied between the 5'D7(+) and 5'D8(+) positions in different species. In all plants, however, the tandem sequences and the miR390 target site varied between the 5'D7(+)/5'D8(+) and the 5'D3(+))/5'D4(+) positions in different species. In all plants, however, the tandem sequences started in either perfect 21-nucleotide register (5/19 species) or one-nucleotide offset (14/19 species) relative to the miR390 cleavage site.

Third, using the rules developed for miRNA target prediction, four genes (ARF1, ARF2, ARF3, and ARF4) were predicted to be targets of these conserved ta-siRNAs. Both ARF3 and ARF4 genes behaved as ta-siRNA targets, as each was up-regulated in *dcl1-7, hen1-1* and *rdr6-15* mutant plants (Figure 4D). Both ARF3 and ARF4 genes from 16 species contained two regions ('A' and 'B') of complementarity to the predicted ta-siRNAs (Figure 6D); the 'A' site was also conserved in ARF1 and ARF2 genes across all plant species tested. And fourth, the 'A' site in both ARF3 and ARF4 was validated as a ta-siRNA target site by 5'RACE. In contrast to most miRNA target sites, the ARF3 and ARF4 'A' site contained several minor cleavage products in addition to the product formed by cleavage at the canonical target position (Figure 6D). Evidence supporting ta-siRNA targeting at the 'B' site within the ARF4 transcript was also obtained (Figure 6D). Thus, the ta-siRNA-generating locus was named *TAS3*.

Although a small RNA from the *TAS3* 5'D2(-) position was not cloned, a hypothetical ta-siRNA from this position may account for the second *TAS3* transcript cleavage site mapped by 5'RACE (Figure 6A). This cleavage site occurs precisely at the position predicted if *TAS3* 5'D2(-) guided cleavage by a RISC-like mechanism. This cleavage site would also set the phase for ta-siRNA precursor processing to generate siR1778. This suggests that ta-siRNAs have the potential to interact with transcripts from which they originate as well as mRNA targets.

DISCUSSION

Combined with previous data, most notably from Jones-Rhoades *et al.*, we are now aware of 25 validated miRNA families, 53 unique miRNA sequences and 99 potential *MIRNA* loci in *A. thaliana*. Seventy-three genes have now been validated experimentally as targets for miRNAs in 24 families. Fifty-three targets were validated in previous studies. Twenty predicted targets of eleven miRNAs were validated or confirmed in this study (Figure 5, Table 3). These included mRNAs for SBP4 (miR156), Auxin Response Factor 16 (ARF16; miR160), two NAC domain proteins (miR164), AtHB15 (miR165/166), ARF6 (miR167), six HAP2 family proteins (miR169), E2-UBC (miR399), AGO2 (miR403), 2PGK (miR447), and five non-coding genes (miR173 and miR390).

miRNAs are processed from genes that produce a primary transcript that forms a stable foldback structure, processed by DCL1, and therefore requires no polymerase and produces no

antisense small RNAs. *Trans*-acting siRNAs have similar biogenesis requirements as miRNAs, but lack a stable foldback structure (Peragine *et al.*, *Genes & Dev* 18:2369-2379, 2004; Vazquez *et al.*, *Mol Cell* 16:69-79, 2004b). As a result, they require a polymerase, most likely RDR6, for second strand generation. Two defining characteristics of ta-siRNAs are the presence of antisense 21-nucleotide small RNAs, and a linear, in-phase processing of both sense and small RNAs. Unlike other classes of siRNAs, ta-siRNAs can be incorporated into RISC and trigger site-specific cleavage of target genes, similar to miRNAs. Both miRNAs and ta-siRNAs are uniquely insensitive to *DCL2*, *DCL3*, *RDR1*, and *RDR2*. In the absence of a comprehensive profile of biogenesis mutants, it is impossible to properly catalog small RNA function. Using this strict set of criteria, we characterized four miRNA families, two of which were previously identified.

Our target prediction algorithm confirmed the robust predictions for the majority of validated miRNAs. Additional targets were validated within this group, including eight targets residing in the untranslated region of the target messenger RNA, including SPL4, an E2-UBC gene At2g33770, and six HAP2 transcripts. Notably, most miR156 targets are located in the coding region of SPL transcripts, whereas two reside immediately downstream of the stop codon in the 3' UTR, SPL3 and SPL4 (Rhoades *et al.*, *Cell* 110:513-520, 2002). Interestingly, two splicing variants of SPL4 exist, one with the miR156 target site (AU227430, BP595743) and one that lacks the target site (BX814070.1), although the coding sequence is unchanged. Potentially the alternately spliced variant of SPL4 would allow an additional level of miRNA-mediated control. The E2-UBC gene is unique in that it contains five miR399 targets in its 5' UTR. The multiple miR399 target sites are conserved among distantly related plant species. The multiple sites might be necessary for miRNA targeting in the 5' UTR to increase the chance of cleavage before ribosomes could clear the miRNA from the mRNA, although the nature of multi-site regulation remains to be determined.

We identified six novel miRNA target loci in the *Arabidopsis* EST database using a computational prediction algorithm developed based on validated miRNA-target characteristics. Previous computational searches for miRNA targets in plants have only used transcript databases, as a result missing these target genes (Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004). The miR403 target, Ago2, is the second Argonaute family gene to be miRNA regulated. *Arabidopsis* Ago2 does not have a close ortholog in mammals, and its role in small RNA function is unknown (Carmell *et al.*, *Genes Dev* 16:2733-2742, 2002; Mochizuki *et al.*, *Cell* 110:689-699, 2002). The remaining five miRNA targets from the EST database search are non-protein coding loci, all of which produce 21-nucleotide small RNAs, in phase with the miRNA cleavage site. Four loci were validated to generate functional ta-siRNAs, including a family of unclassified genes, as well as ARF3 and ARF4. The ta-siRNA target genes were upregulated in *dcl1-7*, *hen1-1*, and *rdr6-15*, which could provide a diagnostic test for ta-siRNA target genes. Both miR390 and the *TAS3* locus are conserved among distantly related plants. A complete profile of small RNA coding-genes will require thorough complementary molecular and computational approaches, perhaps with consideration of conserved 21-nucleotide regions in annotated intergenic regions. Potentially, identification of non-protein

coding genes will be facilitated by genome tiling data (Yamada *et al.*, *Science* 302:842-846, 2003) in combination with small RNA cloning and biogenesis profiling.

We propose a model in which miRNA cleavage initiates the starting phase for ta-siRNA production (Figure 7). The primary miRNA targeted cleavage of an RNA Polymerase II transcript (step 1) recruits a RISC complex to the RNA. In addition, RDR6 and SGS3 could be recruited by the RISC:miRNA:target complex. Cleavage by the miRNA at a specific position creates a unique initiation position. Following cleavage, RDR6/SGS3 polymerize a second strand (step 2), creating a double stranded RNA (dsRNA Either the 5' (*e.g. TAS3*) or 3' (*e.g. TAS1 and TAS2*) cleavage product can be utilized as the RDR6 template. In either case, DCL processing of 21-nucleotide siRNA duplexes (step 3) proceeds in-phase from the primary miRNA cleavage site. Dicer in animals is known to catalyze cleavage from a free end (Zhang *et al.*, *Cell* 118:57-68, 2004). We did not identify any in-phase small RNAs beyond nine phases from the miRNA cleavage initiation site, suggesting either the RDR6/SGS3 complex or the DCL1 complex is not highly processive. One strand of the siRNA duplex is loaded back into a RISC complex, following the known siRNA incorporation rules (Khvorova *et al.*, *Cell* 115:209-216, 2003; Schwarz *et al.*, *Cell* 115:199-208, 2003). Following RISC incorporation of the ta-siRNA (step 4), ta-siRNAs function like miRNAs to facilitate cleavage or target genes in *trans* (step 5).

The regulatory role of miRNAs for all target genes previously identify is to repress target gene expression, through either cleavage or by blocking translation. Our results suggest that miRNAs also act as a positive regulator of ta-siRNA biogenesis through recruitment of RISC and initiation of unique and highly specific phasing for DCL1-mediated processing. Although we have only found evidence for a single active ta-siRNA (or highly similar tandem sequence repeat), multiple, phased ta-siRNAs could provide an advantage through generation of multiple, independent regulatory (ta-siRNA-forming) units from a single locus. The discovery that a miRNA:ta-siRNA:target regulon is conserved among distantly related plants shows that this type of regulation is not specific to *Arabidopsis*, opening the possibility of an entirely new class of small RNA mediated gene regulation.

Example 2: MiRNA-directed biogenesis of ta-siRNAs *in vivo*

To experimentally test the hypothesis that ta-siRNA biogenesis is initiated by miRNA-guided cleavage of primary transcripts, *TAS1* and *TAS2* were co-expressed transiently with *MIR173* in *Nicotiana benthamiana*. If miR173 is required for siR255 production, as predicted herein, then siR255 should be formed only in the presence of miR173. At least some of the the material in this example was published in Allen *et al.* (*Cell* 121:207-221, 2005), which is incorporated herein by reference in its entirety.

Expression cassettes containing the *TAS1a*, *TAS1b*, *TAS1c* and *TAS2* loci (which all include both an initiator sequence, containing an initiator cleavage site, and a gene suppressing element) were delivered into *Nicotiana benthamiana* plant cells (Llave *et al.*, *Plant Cell* 14:1605-1619, 2002; Palatnik *et al.*, *Nature* 425:257-263, 2003) in the presence or absence of an expression cassette containing miR173, and ta-siRNA accumulation was scored. Expression of full-length *TAS1b*

[35S:TAS1b(+)], a short version of *TAS1b* [35S:TAS1b(+)sh], and full-length *TAS1a* [35S:TAS1a(+)] resulted in siR255 accumulation only in the presence of a construct (35S:miR173) expressing miR173 (Figure 14A, lanes 7, 8, 13, 14, 17, 18). Likewise, siR255 from the *TAS1c* construct [35S:TAS1c(+)], and siR1511 from the *TAS2* construct [35S:TAS2(+)], both accumulated only in the presence of the miR173 construct (Figure 14B, lanes 7, 8, 11, 12). Ta-siRNAs were not detected after expression of any of the *TAS1* or *TAS2* constructs alone (Figure 14A, lanes 3, 4, 11, 12, 15, 16; Figure 14B, lanes 5, 6, 9, 10), or after expression of the miR173-non-targeted strand of the short version of *TAS1b* [35S:TAS1b(-)sh] in either the presence or absence of miR173 (Figure 14A, lanes 5, 6, 9, 10). In the presence of miR173, siR255 accumulated to levels up to 7.6 fold higher using the TAS1a(+) and TAS1c(+) constructs compared to the TAS1b(+) constructs. This may reflect a relatively poor miR173-TAS1b interaction, which involves two mismatched positions near the target cleavage site (Figure 5B).

To confirm that ta-siRNA biogenesis requires miRNA-directed targeting of primary transcripts, a *TAS1b* mutant construct [35S:TAS1b(+)shmut1] with a disrupted miR173 target site was expressed in the presence of miR173. The *TAS1b* mutant was also expressed in the presence of a modified miR173 construct (35S:miR173res1) containing base substitutions to restore interaction with the *TAS1b* mutant (Figure 14C, top). Mutations affecting the *TAS1b* target site or miR173 resulted in the loss of siR255 biogenesis (Figure 14C, lanes 7, 8, 11, 12). In contrast, siR255 accumulation was restored when the *TAS1b* mutant was co-expressed with the miR173res1 construct (Figure 14C, lanes 13, 14).

Thus, in each independent experiment, siRNAs from each locus were detected (by RNA blot assay) only in the presence of a construct that formed miR173 (Figure 14). Mutations that disrupted the miR173 target site in the TAS1b construct eliminated siRNA (siR255) formation. However, mutations in the miR173 sequence to restore complementarity with the mutated target sequence restored the formation of siR255 (Figure 14). These data support the model that states ta-siRNA biogenesis requires a miRNA-guided initiation cleavage. It also demonstrates that an expression cassette containing an initiator sequence and a gene suppressing element can direct production of a siRNA in the presence of an expression cassette containing a miRNA. Stated another way, these data show that a functional miRNA target site in the ta-siRNA primary transcript is required to trigger ta-siRNA formation.

See also Example 6, below, for additional details.

Example 3: Plant Transformation Vectors/Plasmids

This example illustrates the construction of plasmids for transferring recombinant DNA into plant cells which can be regenerated into transgenic plants, *e.g.*, expressing in a plant siRNA for suppression of an endogenous gene. See also Example 6, below.

A recombinant DNA construct for plant transformation construct 1A is fabricated for use in preparing recombinant DNA for transformation into corn tissue comprising the a selectable marker expression cassette, a siRNA-triggering cassette and a cleavage initiating cassette. The marker

expression cassette comprises a rice actin 1 promoter element(s) operably linked to sequence(s) encoding a chloroplast transit peptide from *Arabidopsis thaliana* ShkG gene and an aroA protein from *Agrobacterium tumefaciens*, strain CP4, followed by a 3' region of an *Agrobacterium tumefaciens* nopaline synthase gene (*nos*). The siRNA-triggering cassette is positioned tail to tail with the marker expression cassette and comprises 5' regulatory DNA from a maize seed specific promoter L3 (as disclosed in U.S. 6,433,252) operably linked to DNA encoding RNA comprising an initiator sequence that is highly complementary to a microRNA such as miR173 (or any microRNA or siRNA, including any listed herein) and at least one 21-nucleotide segment from LKR. An initiation cleavage cassette is positioned head to head with the marker expression cassette and comprises a maize seed specific promoter L3 and DNA expressing a microRNA (*e.g.*, miR173) that guides cleavage of the initiation cleavage site in the siRNA-triggering cassette. Construct 1A is useful for plant transformation, *e.g.* by microprojectile bombardment. Transgenic corn callus is produced by microprojectile bombardment of construct 1A using methods disclosed in U.S. Patent 6,399,861.

A plasmid vector 1B for use in *Agrobacterium*-mediated methods of plant transformation is prepared by inserting construct 1A into a plasmid between left and right T-DNA border sequences from *Agrobacterium*. Outside of the T-DNA borders the plasmid also contains origin of replication DNA to facilitate replication of the plasmid in both *E. coli* and *Agrobacterium tumefaciens* and a spectinomycin/stretptomycin resistance gene for selection in both *E. coli* and *Agrobacterium*. Transgenic corn callus is produced by *Agrobacterium*-mediated transformation of plasmid vector 1B using methods disclosed in U.S. Patent 5,591,616.

Transgenic corn plants are regenerated from transgenic callus produced by microprojectile bombardment and *Agrobacterium*-mediated transformation; callus is placed on media to initiate shoot development in plantlets which are transferred to potting soil for initial growth in a growth chamber at 26 °C followed by growth on a mist bench before transplanting to 5 inch pots where plants are grown to maturity. The plants are self fertilized and seed is harvested for screening as seed, seedlings or progeny R2 plants or hybrids, *e.g.* for yield trials in the screens indicated above. Transgenic plants with higher levels of lysine resulting from suppressed levels of LKR and which are homozygous for the recombinant DNA are identified. The homozygous plants are self pollinated to produce transgenic seed with the recombinant DNA comprising siRNA-triggering cassettes.

Example 4: Inhibition of Plant Pest Genes

This example illustrates the construction of plasmids for transferring recombinant DNA into plant cells which can be regenerated into transgenic described herein, particularly expressing in a plant siRNA for suppression of genes in a plant pest.

Recombinant DNA constructs 2A, 2B and 2C are fabricated for soybean transformation by microprojectile bombardment essentially like construct 1A except that the promoter used in the siRNA-triggering cassette and the initiation cleavage cassette is a root tissue-expressing promoter and the 21-nucleotide segment is derived from DNA encoding soybean cyst nematode proteins as

disclosed in US Patent Application Publication 2004/0098761 A1. In construct 2A the 21-nucleotide segment is from a major sperm protein; in construct 2B the 21-nucleotide segment is from a chitin synthase; and in construct 2C the 21-nucleotide segment is from an RNA polymerase II. Soybean is transformed by microprojectile bombardment using constructs 2A, 2B and 2C using methods as disclosed in U.S. Patent 5,914,451 and transgenic soybean plants are regenerated which exhibit resistance to soybean cyst nematode infestation as compared to control plants.

Plasmid vectors 2D, 2E and 2F for use in *Agrobacterium*-mediated methods of plant transformation are prepared by inserting constructs 2A, 2B and 2C, respectively, into plasmids with T-DNA borders similar to plasmid vector. Soybean is transformed by *Agrobacterium*-mediated transformation of plasmid vectors 2D, 2E and 2F using methods disclosed in U.S. Patent 6,384,301 and transgenic soybean plants are regenerated which exhibit resistance to soybean cyst nematode infestation as compared to control plants.

Example 5: Expression of *Arabidopsis thaliana MIRNA* Genes

Recent molecular cloning and computational analyses have identified nearly one-hundred potential genetic loci for *MIRNA* genes in the *Arabidopsis thaliana* genome. However, information about the structure and expression of these genes is generally lacking. The transcriptional start site for each of 63 miRNA precursor transcripts from 52 *MIRNA* (99 total loci tested) was mapped. A portion of the loci yielded multiple transcripts from alternative start sites, and some contained introns between the foldback structure and the 5' end. Analysis of a representative set of transcripts revealed characteristics consistent with transcription by Pol II. A canonical TATA box motif was identified computationally upstream of the start site(s) at some *MIRNA* loci. The 5' mapping data were combined with miRNA cloning and 3'-PCR data to definitively validate expression some of known *MIRNA* genes. These data provide a molecular basis to explore regulatory mechanisms of miRNA expression in plants.

Material from this example was published as Xie *et al.*, *Plant Physiol.* 138(4):2145-2154, 2005; Epub 2005 July 22, which is incorporated herein by reference in its entirety.

MicroRNAs (miRNAs) are ~21-nucleotide noncoding RNAs that post-transcriptionally regulate expression of target genes in multicellular plants and animals (Bartel, *Cell* 116:281-297, 2004). Mature miRNAs are generated through multiple processing steps from longer precursor transcripts that contain imperfect foldback structures. In animals, *MIRNA* genes are transcribed by RNA polymerase II (pol II) (Bracht *et al.*, *RNA* 10:1586-1594, 2004; Cai *et al.*, *RNA* 10:1957-1966, 2004; Lee *et al.*, *EMBO* J 23:4051-4060, 2004), yielding a primary transcript (pri-miRNA) that is processed initially by nuclear RNaseIII-like Drosha (Lee *et al.*, *Nature* 425:415-419, 2003). The resulting pre-miRNA transcripts are transported to the cytoplasm and processed by Dicer to yield mature-size miRNAs (Lee *et al.*, *EMBO* J 21:4663-4670, 2002). Less is known about the miRNA biogenesis pathway in plants, although most or all miRNAs require Dicer-like1 (DCL1) (Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002). The lack of a Drosha ortholog in plants, and the finding that DCL1 functions at multiple steps during biogenesis of

miR163, suggest that the plant miRNA pathway may differ from the animal pathway (Kurihara & Watanabe, *Proc Natl Acad Sci U S A* 101:12753-12758, 2004). MiRNAs in both animals and plants incorporate into an effector complex known as RNA-induced Silencing Complex (RISC) and guide either translation-associated repression or cleavage of target mRNAs (Bartel, *Cell* 116:281-297, 2004).

Computational and molecular cloning strategies revealed over 100 potential *MIRNA* genes belonging to at least 27 families in the *Arabidopsis* genome (Llave *et al.*, *Plant Cell* 14:1605-1619, 2002; Mette *et al.*, *Plant Physiol* 130:6-9, 2002; Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002; Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004; Wang *et al.*, *Genome Biol* 5:R65, 2004). These miRNA families target mRNAs encoding proteins that include a variety of transcription factors involved in development, DCL1 and the RISC factor ARGONAUTE1(AGO1), components of the SCF complex involved in ubiquitin-mediated protein degradation, and several other classes of metabolic and stress-related factors (Rhoades *et al.*, *Cell* 110:513-520, 2002; Xie *et al.*, *Curr Biol* 13:784-789, 2003; Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004; Vaucheret *et al.*, *Genes Dev* 18:1187-1197, 2004) (see also Example 1). Based on tissue distribution and limited *in situ* expression data, most plant miRNAs are likely regulated at spatial and/or temporal levels during development (Chen, *Science* 303:2022-2025, 2004; Juarez *et al.*, *Nature* 428:84-88, 2004; Kidner & Martienssen, *Nature* 428:81-84, 2004). Overexpression or knockout of *MIRNA* genes, or expression of *MIRNA* genes outside of their normal expression domains, can lead to severe developmental defects (Aukerman & Sakai, *Plant Cell* 15:2730-2741, 2003; Palatnik *et al.*, *Nature* 425:257-263, 2003; Achard *et al.*, *Development* 131:3357-3365, 2004; Chen, *Science* 303:2022-2025, 2004; Juarez *et al.*, *Nature* 428:84-88, 2004; Kidner & Martienssen, *Nature* 428:81-84, 2004; Laufs *et al.*, *Development* 131:4311-4322, 2004; Mallory *et al.*, *Curr Biol* 14:1035-1046, 2004a; Mallory *et al.*, *EMBO* J 23:3356-3364, 2004; McHale & Koning, *Plant Cell* 16:1730-1740, 2004; Emery *et al.*,*Curr Biol* 13:1768-1774, 2003; Zhong & Ye, *Plant Cell Physiol* 45:369-385, 2004). Understanding the mechanisms governing *MIRNA* gene expression patterns and integration into regulatory networks will be necessary for a clear understanding of the biological function of miRNAs.

In this example, several new *Arabidopsis* miRNAs were identified by a computationally assisted cloning approach and the use of mutants that contained miRNA-enriched pools of small RNAs. Expression of 99 *MIRNA* genes in *Arabidopsis* was examined experimentally. First, features associated with transcription initiation of *MIRNA* genes were analyzed, revealing core promoter, start sites and other properties that were consistent with a pol II mechanism of transcription. And second, a survey of expression of each known *MIRNA* locus was done to identify functional *MIRNA* genes.

MATERIALS AND METHODS

Cloning of *A. thaliana* small RNAs and miRNA prediction.

Extraction of low molecular weight RNA and library construction was done as described (Llave *et al.*, *Plant Cell* 14:1605-1619, 2000; Lau, *Science* 294:858-862,2001). RNA was extracted from three-day post germination seedlings, embryos from developing siliques, aerial tissues including rosette leaves and apical meristems, or stage 1 to 12 enriched inflorescence from wildtype Columbia-0, and *jaw-D*, *rdr2-1* and *dcl3-1* mutants described previously (Palatnik *et al.*, *Nature* 425:257-263, 2003; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). Seedling libraries were constructed for Col-0, *rdr2-1*, and *dcl3-1*, embryo libraries for *rdr2-1*, aerial libraries for *jaw-D*, and inflorescence libraries for Col-0 and *rdr2-1*. Sequences were filtered to remove organellar, rRNA, and those not present in *A. thaliana*. Remaining small RNAs between 18 and 26 nucleotides were deposited in the ASRP database (available on-line at asrp.cgrb.oregonstate.edu/). Candidate miRNA prediction used a set of six filters. First, structural RNAs were filtered before entry into the ASRP database by manual scoring of BLAST hits to known rRNA, tRNA, and organellar RNA. Second, small RNAs from repeats identified using RepeatMasker (Jurka, *Trends Genet* 16:418-420, 2000) or from predicted protein-coding genes and psuedogenes only were removed. Third, a small RNA cluster filter was applied to remove small RNAs within 500 nt of another small RNA in the opposite orientation. The fourth filter removed any small RNA outside the typical size (20-22 nucleotides). Fifth, characteristics including the minimum paired bases of the miRNA:miRNA* duplex in the reference set (≥16), maximum foldback size (350 nucleotides), and a requirement for the miRNA and its duplex to be on a single stem were determined. Foldbacks in which the miRNA:miRNA duplex contained more than three contiguous unpaired bases were excluded. The RNAFold in the Vienna RNA Package was used to predict potential duplexes containing the small RNA, and those with duplexes not meeting the above criteria were excluded (Hofacker, *Nucleic Acids Res* 31:3429-3431, 2003). Sixth, validated miRNAs and closely related family members, as well as small RNAs processed from a miRNA locus (including miRNA*) were identified by FASTA and comparison of small RNA loci on the ASRP genome browser. These small RNAs were annotated as family members of validated miRNAs, and removed from the predicted miRNA pool.

Small RNA blot analysis.

Low molecular weight RNA (5 μg) from *A. thaliana* inflorescence tissue was used for miRNA and endogenous siRNA analysis. Mutant lines for *dcl1-7*, *dcl2-1*, *dcl3-1*, *rdr1-1*, *rdr2-1*, *hen1-1*, *hyl1-2*, *rdr6-11*, *rdr6-15*, and *sgs3-11* were described previously (Park *et al.*, *Curr Biol* 12:1484-1495, 2002; Allen *et al.*, *Nat Genet* 36:1282-1290, 2004; Peragine *et al.*, *Genes & Dev* 18, 2368-2379, 2004; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). The *hst-15* allele used was the SALK_079290 T-DNA insertion line from ABRC, which contains a T-DNA at position 1584 from the start codon. Probes for miR159, miR167, and *AtSN1*-siRNA blots were described previously (Llave *et al.*, *Plant Cell* 14:1605-1619, 2002; Zilberman *et al.*, *Science* 299:716-719, 2003). All other miRNAs were detected using end-labeled DNA

oligonucleotides. Probes for ta-siRNA loci were PCR amplified from Col-0 genomic DNA, cloned into pGEMT-Easy, and verified by sequencing. Radiolabeled probes incorporating ^{32}P-UTP were made by T7 RNA polymerase transcription, to obtain strand specific small RNA probes. Probes were as follows: *At1g17185* locus, Chr3:5862146-5862295; *At2g39680* locus, Chr2:16546831-16547300.

5'RACE mapping of *MIRNA* transcripts

Two *Arabidopsis thaliana* (Col-0) sample preparations were used for RNA isolation: inflorescence tissues from 4-week old plants grown under greenhouse condition and 4-day old seedlings grown on MS media in a growth chamber. Total RNA was extracted using TRIzol reagent (Invitrogen) followed by column purification using a RNA/DNA midi kit (Qiagen). The extracts were subjected to two rounds of purification using Oligotex (Qiagen) for the enrichment of poly(A)$^+$ RNA. The 5'ends of *MIRNA* transcripts were mapped by a RNA ligase-mediated 5'RACE (RLM-5'RACE, Invitrogen). Complementary DNA (cDNA) was synthesized with poly(A)+-enriched RNA (125 ng/reaction), which was first treated with calf intestine phosphatase and tobacco acid pyrophosphatase (CIP+TAP), using random oligonucleotide hexamers as primers. A cDNA pool containing equal amounts of cDNA from each tissue was used as template in 5'RACE PCR with a primer (Invitrogen) specific to the RNA adaptor sequence and a locus-specific reverse primer. In cases where no product was detected, a second-round PCR was done using a 5' nested primer and a locus-specific nested primer. The default annealing temperature in the touchdown PCR reaction was 65°C. For a *MIRNA* locus with a negative 5'RACE result after the second-round PCR, two additional PCR reactions with the nested primers were done with altered annealing temperatures. The PCR products from a positive 5'RACE were gel-purified and cloned into pGEM-Teasy vector. A minimum of 6 clones were sequenced for each PCR product.

The RLM-5'RACE procedure was used to analyze the presence or absence of a cap structure on several miRNA transcripts. A capped mRNA [*Scarecrow-like6-IV (SCL6-IV)*] and a non-capped RNA (miR171-guided cleavage product of *SCL6-IV* mRNA) were used as control RNAs. Parallel RLM-5'RACE reactions were done using poly(A)+-enriched RNA that was CIP+TAP treated and non-treated, which was selective for amplification of 5' ends that contained or lacked a cap structure, respectively.

For some miRNA transcripts, 3'RACE was done using poly(A)+-enriched RNA. cDNA was synthesized using an adaptor-tagged oligo(dT) primer. Two gene-specific forward primers were designed for each locus tested. The identity of the 3'RACE products were confirmed by sequencing. The sequences of the locus-specific primers are provided in SEQ ID NOs: 349 to 614, and were published in Supplementary Table 2 in Xie *et al.*, *Plant Physiol.* 138(4):2145-2154, 2005; Epub 2005 July 22.

Computational identification of conserved upstream sequence motifs

A 60-bp (-50 to +10) genomic sequence flanking the start site for 63 transcripts from 47 *MIRNA* loci was analyzed using BioProspector, a Gibbs sampling-based motif-finding program (Liu

et al., 2004). Searches with a motif width of 6-8 nucleotides were done. In all cases, TATA-like sequences were identified as the only conserved motif. A second search (8-nucleotide width) was done using an extended *MIRNA* upstream region (-200 to +50) to analyze the distribution of the putative TATA motif using MotifMatcher, with the 8-nucleotide motif matrix generated by BioProspector as a sample motif (Ao *et al.*, *Science* 305:1743-1746, 2004). Up to three matches to the TATA motif were allowed.

RESULTS AND DISCUSSION

Identification and validation of *Arabidopsis* miRNAs

Several small RNA libraries were constructed from wild-type (Col-0) *A. thaliana* seedling and inflorescence tissues, and from aerial tissues of *jaw-D* plants that over express miR-JAW (miR319) (Palatnik *et al.*, *Nature* 425:257-263, 2003). Among all 2357 sequences analyzed collectively from these libraries, only 32.7% corresponded to known or subsequently validated miRNA families. Most of the remaining small RNAs corresponded to diverse sets of endogenous small RNAs arising from repeated sequences such as transposons, retroelements, simple sequence repeats, inverted duplications, rDNA genes and other genic and intergenic sequences (Llave *et al.*, *Plant Cell* 14:1605-1619, 2002; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). To genetically enrich for miRNAs, small RNA libraries were constructed from embryo, seedling, and inflorescence tissues of *rdr2-1* mutant plants, and from seedlings of *dcl3-1* mutant plants. These plants contain relatively low levels of ~24-nucleotide siRNAs from repeated sequences, but maintain normal levels of miRNAs (Xie *et al.*, *PLoS Biol* 2:642-652, 2004). Among 3164 sequences analyzed collectively from the *rdr2-1* and *dcl3-1* libraries, 70.5% corresponded to previously characterized miRNAs, representing a 2.2-fold overall enrichment relative to the wild-type libraries. Endogenous siRNAs from known repeat families (identified from RepBase) were reduced 43.9-fold in the mutant libraries. The majority of the remaining small RNAs corresponded to sequences from two *rdr2*-independent small RNA-generating loci, or from rRNA genes. Unique miRNA and endogenous siRNA sequences from all libraries are available in the *Arabidopsis* Small RNA Project (ASRP) database (available on-line at asrp.cgrb.oregonstate.edu).

To identify new miRNAs in the cloned libraries, the small RNA sequences were subjected to a series of five computational filters (Figure 8A). The filters were designed using the properties of a founder set of published, validated Arabidopsis miRNAs with codes within the range of miR156-miR399 (excluding miR390 and miR391; RFAM). Among the 48 unique miRNA sequences from 92 loci (22 validated miRNA families) in the founder set, 34 miRNA sequences from 71 loci (19 families) were in the cloned database. The initial filters eliminated all small RNA sequences deriving from structural RNA genes, other annotated genes and repetitive loci identified by RepeatMasker (Figure 8A). Sequences originating from loci that yielded multidirectional clusters of small RNAs, which is a hallmark of many siRNA-generating loci, were eliminated. Small RNAs that were not 20-22 nucleotides in length, based on the cloned sequence, were also removed. Small RNAs originating from loci that lacked the potential to form a miRNA precursor-like foldback structure, consisting of a

stem in which 16 or more positions within the putative miRNA-miRNA* duplex region were paired, were excluded. To test the sensitivity of these filters, the complete founder set of miRNAs was processed through the five filters. All but three passed, corresponding to a false negative rate of 0.032. MiR163 failed because it is 24 nucleotides long, and miR166 from two loci failed because of 6 mispaired miRNA positions within the foldback stem. From the cloned dataset, a total of 103 small RNAs from passed the five filters (Figure 8A). These did not correspond to 103 unique loci, however, as many miRNA-generating loci yield multiple processed forms that are offset by one or a few nucleotides. Elimination of all sequences corresponding to founder miRNAs yielded a set of 18 small RNAs, corresponding to 13 genetic loci, as candidate new miRNAs (Figure 8A). This set included miR390, miR391, miR403 and miR447 (Figure 8B). Six of the 18 small RNAs corresponded to a cluster of processing variants from the two miR390 loci.

Given the high sensitivity of the computational filters using the founder set, a second set of published *Arabidopsis* sequences with miRNA designations were analyzed. These have not been subjected to extensive experimental validation as miRNAs. This set includes all sequences with codes between miR400-miR420 (Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004; Wang *et al.*, *Genome Biol* 5:R65, 2004), except miR403. In contrast to the founder set, most of the small RNAs in the second set failed at one or more steps. Six small RNAs (miR401, 405a-d, 407, 416) were identified as transposon-derived, two (miR402, 408) were from annotated genes, and ten (miR401, 404,406, 408, 413, 414, 417-420) failed the foldback prediction criteria. Given the high computational failure rate (0.84) of this set, which was 26-fold higher than the false negative rate of the founder set, it is likely that many or most of these are endogenous siRNAs and not bona fide miRNAs.

Candidate miRNAs from each of the 13 loci identified in the computational analysis was subjected to validation blot assays using a series of *Arabidopsis* miRNA-defective (*dcl1, hyl1, hen1, and hst*) and siRNA-defective (*dcl2, dcl3, rdr1, rdr2, rdr6* or *sgs3*) mutants (Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002; Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Vazquez *et al.*, *Curr Biol* 14:346-351, 2004; Xie *et al.*, *PLoS Biol* 2:642-652, 2004). In addition, small RNAs were analyzed in transgenic plants expressing three viral RNAi suppressors (P1/HC-Pro, p19 and p21), which frequently enhance the level of miRNA accumulation (Mallory *et al.*, *Proc Natl Acad Sci USA* 99:15228-15233, 2002; Kasschau *et al.*, *Dev Cell* 4:205-217, 2003; Papp *et al.*, *Plant Physiol* 132:1382-1390, 2003; Chapman *et al.*, *Genes Dev.* 18:1179-86, 2004) but decrease the level of ta-siRNA accumulation. Previously validated miR159, miR167 and miR173, and *AtSN1*-derived siRNAs were analyzed in parallel as controls. Reproducible signals were detected in Col-0 and La-*er* control plants only using probes for miR390, miR391, miR403 and miR447 (Figure 8C). Each of these accumulated to relatively low levels in the *dcl1-7*, *hen1-1* and *hyl1-2* mutants, but accumulated to normal or near-normal levels in the *dcl2-1*, *dcl3-1*, *rdr1-1*, *rdr2-1*, *rdr6-11* and *sgs3-11* mutants (Figure 8C,D). The *hst-15* mutant accumulated nearly normal amounts of the four candidates as well as the three miRNA controls (Figure 8C), indicating that miRNA accumulation in the tissues tested was relatively insensitive to loss of HST function. MiR390,

miR391, miR403 and miR447 were either up-regulated or unaffected by each of the three viral suppressor proteins (Figure 8D). Based on structural and biogenesis criteria, we conclude that miR390, miR391, miR403 and miR447 are *bona fide* miRNAs. Small RNAs from the remaining eight loci (Table 6) were not detected in blot assays and were not characterized further.

Table 6. Predicted miRNA candidates tested experimentally

Locus	ASRP no.	Sequence	miRNA validation	miRNA name, notes
1,2	754[a]	AAGCUCAGGAGGGAUAGCGCC	yes	miR390
		SEQ ID NO: 143		
3	1728	UUCGCAGGAGAGAUAGCGCCA	yes	miR391
		SEQ ID NO: 144		
4	359	AUUAGAUUCACGCACAAACUCG	yes	miR403
		SEQ ID NO: 145		
5	1890	UUGGGGACGAGAUGUUUUGUUG	yes	miR447
		SEQ ID NO: 146		
6	382	GAGCCGACAUGUUGUGCAACUU	no	not detected
		SEQ ID NO: 147		
7	991	AAUGGAAGCCUUGUCAGCUUAU	no	not detected
		SEQ ID NO: 148		
8	1072	UAAAGUCAAUAAUACCUUGAAG	no	not detected
		SEQ ID NO: 149		
9	1345	UAUAAGCCAUCUUACUAGUU	no	not detected
		SEQ ID NO: 150		
10	1744	UUCUGCUAUGUUGCUGCUCAUU	no	not detected
		SEQ ID NO: 151		
11	1928	UCUAAGUCUUCUAUUGAUGUUC	no	not detected
		SEQ ID NO: 152		
12	1943	CUGUCUUCUCAACUUCAUGUGA	no	not detected
		SEQ ID NO: 153		
13	2028	CGGCUCUGAUACCAAUUGAUG	no	not detected
		SEQ ID NO: 154		

a, Four processing variants from the two miR390 loci were cloned.

MiR390 and miR391 are related miRNAs that differ by five nucleotides, whereas miR403 and miR447 are distinct from all other known miRNAs. If miR390 and miR391 are assigned to the same family, then Arabidopsis contains 25 experimentally validated families of miRNAs encoded by up to 99 genes (Table 7). Among these families, 19 are conserved between dicots and monocots. One family (miR403) is conserved among families within dicots, and five families (miR158, miR161, miR163, miR173 and miR447) have been identified only in *Arabidopsis*.

Table 7. *Arabidopsis* miRNA families

				ASRP library[b]				
miRNA families	miRNA family	Locus	Sequence[a]	Col-0	*rdr2/dcl 3*	Plant species[c]	Target family	SEQ ID NO.

1	miR156	a-f	UGACAGAAGAGAGUGAGCAC	+	+	At,Bn,Gm,Ha,Hv,Lj,Mt,Nt,Os,Pta,Ptr,Sb,Si,So,St,Vv,Zm	SBP	155
	miR156	g	CGACAGAAGAGAGUGAGCACA	-	-	At		156
	miR156	h	UUGACAGAAGAAAGAGAGCAC	-	-	At		157
	miR157	a-d	UUGACAGAAGAUAGAGAGCAC	-	+	At,Ptr		158
2	miR158	a	UCCCAAAUGUAGACAAAGCA	+	-	At	PPR	159
		b	CCCCAAAUGUAGACAAAGCA	-	-	At		160
3	miR159	a	UUUGGAUUGAAGGGAGCUCUA	+	+	At,Gm,Hv*,Lj,Mt,Os,Pg*,Ptr,So*,Sb*,Ta*,Vv,Zm	MYB	161
	miR159	b	UUUGGAUUGAAGGGAGCUCUU	-	+	At		162
	miR159	c	UUUGGAUUGAAGGGAGCUCCU	-	-	At		163
	miR319	a-b	UUGGACUGAAGGGAGCUCCCU	+	+	At,Bo,Gm,Lt,Os,Ptr,Ta	TCP	164
	miR319	c	UUGGACUGAAGGGAGCUCCUU	-	-	At,Os		165
4	mir160	a-c	UGCCUGGCUCCCUGUAUGCCA	+	+	At,Gm,Os,Ptr,Tt,Zm	ARF	166
5	miR161.1	a	UUGAAAGUGACUACAUCGGGG	+	+	At	PPR	167
	miR161.2	a	UCAAUGCAUUGAAAGUGACUA	+	+	At		168
6	miR162	a-b	UCGAUAAACCUCUGCAUCCAG	+	+	At,Gm,Ll,Mt,Os,Ptr,Vv	DCL	169
7	miR163	a	UUGAAGAGGACUUGGAACUUCGAU	+	-	At	SAMT	170
8	miR164	a-b	UGGAGAAGCAGGGCACGUGCA	-	+	At,Pb,Ta	NAC	171
	miR164	c	UGGAGAAGCAGGGCACGUGCG	+	+	At		172
9	miR165	a-b	UCGGACCAGGCUUCAUCCCCC	-	+	At,Hc,Ptr	HD-ZIPIII	173
	miR166	a-g	UCGGACCAGGCUUCAUUCCCC	+	+	At,Gm,Hv,In*,Mt,Os,Ptr,Sb,Zm		174
10	miR167	a-b	UGAAGCUGCCAGCAUGAUCUA	+	+	At,Gm,Os,Pc*,Ptr,Zm	ARF	175
	miR167	c	UUAAGCUGCCAGCAUGAUCUU	-	-	At		176
	miR167	d	UGAAGCUGCCAGCAUGAUCUGG	+	+	At,Gm,In,Ptr,So		177
11	miR168	a-b	UCGCUUGGUGCAGGUCGGGAA	+	+	At,Bp,Gm,Ht,Hv,Le,Os,Ptr,Sb,So,St,Vv,Zm	AGO1	178
12	miR169	a	CAGCCAAGGAUGACUUGCCGA	+	+	At,Gm,Os,Ptr,Ptr	HAP2	179
	miR169	b-c	CAGCCAAGGAUGACUUGCCGG	+	+	At,Gm,Os,Ptr,Zm		180
	miR169	d-g	UGAGCCAAGGAUGACUUGCCG	+	+	At,Ptr		181
	miR169	h-n	UAGCCAAGGAUGACUUGCCUG	+	+	At,Ls,Os,Pb,Ptr,Sb,So,Ta		182
13	miR170	a	UGAUUGAGCCGUGUCAAUAUC	-	+	At	SCR	183
	miR171	a	UGAUUGAGCCGCGCCAAUAUC	+	+	At,Os,Ptr,Ta,Zm		184

	miR171.2	b-c	UUGAGCCGUGCCAAUAUCACG	+	-	At,Os,Ptr,**Ta,Zm**		185
	miR171.1	c	UGAUUGAGCCGUGCCAAUAUC	-	+	At,**Gm,Hc,Hv**,Os,Ptr,**Ta,Zm**		186
14	miR172	a-b	AGAAUCUUGAUGAUGCUGCAU	-	+	At,**Gm,Le**,Os,Ptr,**St**	AP2	187
	miR172	c-d	AGAAUCUUGAUGAUGCUGCAG	+	-	At,**Cs**		188
	miR172	e	GGAAUCUUGAUGAUGCUGCAU	-	+	At,Os,Ptr		189
15	miR173	a	UUCGCUUGCAGAGAGAAAUCAC	-	+	At	*TAS1, TAS2*	190
16	miR390	a-b	AAGCUCAGGAGGGAUAGCGCC	+	+	At,**Os**,Ptr,**St,Zm**	*TAS3*	143
	miR391	a	UUCGCAGGAGAGAUAGCGCCA	-	+	At		144
17	miR393	a-b	UCCAAAGGGAUCGCAUUGAUC	-	-	At,**Os,Ptr**	TIR1/ F-box	191
							bHLH	
18	miR394	a-b	UUUGGCAUUCUGUCCACCUCC	-	-	At,**Gm**,Os, Ptr,**Rp**	F-box	192
19	miR395	a,d-e	CUGAAGUGUUUGGGGGAACUC	-	-	At,**Gm,Os**, Ptr,**Ta**	ATPS	193
	miR395	b-c,f	CUGAAGUGUUUGGGGGGACUC	-	-	At		194
20	miR396	a	UUCCACAGCUUUCUUGAACUG	-	+	**At,Bv,Gm, Mc,Os**,Ptr, **Ppe,Ptr,So ,St,Zm**	GRF	195
	miR396	b	UUCCACAGCUUUCUUGAACUU	-	-	At,**Bn,Gm, Mc**,Os,Ptr, **St**		196
21	miR397	a	UCAUUGAGUGCAGCGUUGAUG	-	+	At,**Hv**,Os,Ptr	laccase	197
	miR397	b	UCAUUGAGUGCAUCGUUGAUG	-	-	At		198
22	miR398	a	UGUGUUCUCAGGUCACCCCUU	-	-	At,**Cs,Gm, Lj,Mt**,Os,Ptr	CSD	199
	miR398	b-c	UGUGUUCUCAGGUCACCCCUG	-	+	At,**Gm,Ha, Ls,Mt,Nb, Os,Zm***	CytC	200
23	miR399	a	UGCCAAAGGAGAUUUGCCCUG	-	-	At	E2-UBC	201
	miR399	b,c	UGCCAAAGGAGAGUUGCCCUG	-	+	At,**Mt**,Os,**Ptr,Sb**		202
	miR399	d	UGCCAAAGGAGAUUUGCCCCG	-	-	At,Os		203
	miR399	e	UGCCAAAGGAGAUUUGCCUCG	-	-	At		204
	miR399	f	UGCCAAAGGAGAUUUGCCCGG	-	-	At,Os		205
24	miR403	a	aUUAGAUUCACGCACAAACUCG	+	-	At,**Ptr**	AGO2	145
25	miR447	a-b	UUGGGGACGAGAUGUUUUGUUG	-	+	At	2PGK	146
	miR447	c	UUGGGGACGACAUCUUUUGUUG	-	-			206

a, miRNAs are grouped by related families, with differences among families underlined;

b, Col-0 libraries included Col-0 seedling, aerial, and inflorescence tissues, plus jaw-d sequences, rdr2/dcl3 contained seedling libraries from both mutants, and inflorescence tissues of rdr2;

c, Presence of miRNA in genomic sequence is indicated in regular text, EST sequences are in bold, see information available on the World Wide Web at sanger.ac.uk/Software/Rfam/mirna/index.shtml for primary stem sequences; sequences with 1-2 base changes from the Arabidopsis sequence are indicated by an asterisk.

***Arabidopsis* miRNA precursors exhibit characteristics of pol II transcripts**

To determine if a reference set of *Arabidopsis thaliana MIRNA* gene transcripts contain 5' cap structures typical of RNA pol II transcripts, a series of RNA ligase-mediated 5'RACE reactions were done using poly(A)$^+$-selected RNA that was pretreated with either calf intestine phosphatase plus tobacco acid pyrophosphatase (CIP+TAP) or buffer alone. Only transcripts containing a 5' cap should ligate to adapters, and subsequently amplify by PCR, following CIP+TAP treatment. Transcripts lacking a cap should ligate and amplify only from the sample treated with buffer alone. As controls, capped *Scarecrow-like6-IV* (*SCL6-IV,* At4g00150) transcript and miR171-guided 3' cleavage product from *SCL6-IV* (containing a 5' monophosphate) were analyzed using gene specific primer sets (Figure 9A) (Llave *et al.*, *Science* 297:2053-2056, 2002). CIP+TAP-dependent 5'RACE products of the predicted size, ~400 and ~1,110 bp, were detected using 5'-proximal and cleavage site-proximal primer sets, respectively (Figure 9B, lanes 2 and 4). Buffer-dependent 5'RACE product was detected only using the cleavage site-proximal primer set (Figure 9B, lanes 1 and 3). Using locus-specific primer sets for *MIR163*, *MIR397b* and *MIR398c*, CIP+TAP-dependent products but not buffer-dependent products were detected (Figure 9B, lanes 5-10), indicating that the 5' end of each miRNA transcript was capped. For 47 out of the 92 *Arabidopsis MIRNA* loci tested, 5'RACE products from poly(A)$^+$-selected and 5'capped RNA were detected (see below and Table 8). Combined with previous data for *MIR172b* and *MIR163*, and the evidence for a poly(A) tail on miRNA precursor transcripts, plant *MIRNA* genes are likely transcribed by an RNA pol II mechanism. These data are also consistent with recent analyses of *MIRNA* gene transcripts from animals (Bracht *et al.*, *RNA* 10:1586-1594, 2004; Cai *et al.*, *RNA* 10:1957-1966, 2004; Lee *et al.*, *EMBO* J 23:4051-4060, 2004).

Identification of a core promoter element for *Arabidopsis MIRNA* genes

Products of 5'RACE reactions were detected using locus-specific primers for 52 of 99 *MIRNA* genes tested. Transcription start sites were inferred by sequence analysis of the cloned PCR products. At several loci, such as *MIR171a*, *MIR172b*, and *MIR172e*, multiple 5'RACE products were detected and up to three clusters of alternative transcription start sites were identified (Table 8).

Table 8A. Validated miRNA sequences cloned from *Arabidopsis* small RNA libraries

					Position		
miRNA	Times isolated	ASRP database No	Locus	Chrom.	Start	End	Sequence
miR156	233	1423	a	2	10683613	106683632	UGACAGAAGAGAGUGAGCAC SEQ ID NO: 155
			b	4	15074951	15074970	
			c	4	15415497	15415516	
			d	5	3456714	3456733	
			e	5	3867214	3867233	
			f	5	9136129	9136148	

miR156	3	1662	d	5	3456714	3456734	UUGACAGAAGAGAGUGAGCAC SEQ ID NO: 207
miR156	1	1783	e	5	3867213	3867233	GUGACAGAAGAGAGUGAGCAC SEQ ID NO: 208
			f	5	9136128	9136148	
miR156	1	1950	a	2	10683612	106683632	UGACAGAAGAGAGUGAGCAC SEQ ID NO: 155
			b	4	15074951	15074971	
			c	4	15415496	15415516	
			d	5	3456713	3456733	
			e	5	3867214	3867234	
			f	5	9136129	9136149	
miR157	1	1424	a	1	24916958	24916939	UGACAGAAGAUAGAGAGCAC SEQ ID NO: 209
			b	1	24924768	24924787	
			c	3	6244698	6244679	
			d	1	18030676	18030657	
miR157	6	1770	a	1	24916959	24916939	UUGACAGAAGAUAGAGAGCAC SEQ ID NO: 158
			b	1	24924767	24924787	
			c	3	6244699	6244679	
miR157	2	1952	d	1	18030677	18030657	CUGACAGAAGAUAGAGAGCAC SEQ ID NO: 210
miR157*	1	1782	a	1	24916888	24916868	GCUCUCUAGCCUUCUGUCAUC SEQ ID NO: 211
			b	1	24924838	24924858	
miR158	18	142	a	3	3366373	3366354	UCCCAAAUGUAGACAAAGCA SEQ ID NO: 159
miR158*	1	1727	a	3	3366396	3366416	CUUUGUCUACAAUUUUGGAAA SEQ ID NO: 212
miR158*	1	1735	a	3	3366397	3366416	CUUUGUCUACAAUUUUGGAA SEQ ID NO: 213
miR158*	1	2007	a	3	3366395	3366416	CUUUGUCUACAAUUUUGGAAAA SEQ ID NO: 214
miR159	224	1425	a	1	27716915	27716895	UUUGGAUUGAAGGGAGCUCUA SEQ ID NO: 161
miR159	7	1747	b	1	6220806	6220826	UUUGGAUUGAAGGGAGCUCUU SEQ ID NO: 162
miR159	1	1756	b	1	6220804	6220824	UCUUUGGAUUGAAGGGAGCUC SEQ ID NO: 215
			a	1	27716917	27716897	
miR159	2	1800	a	1	27716915	27716896	UUUGGAUUGAAGGGAGCUCU SEQ ID NO: 216
			b	1	6220806	6220825	
miR159	1	2011	a	1	27716914	27716895	UUGGAUUGAAGGGAGCUCUA SEQ ID NO: 217
miR319	5	1665	a	4	12353119	12353139	UUGGACUGAAGGGAGCUCCCU SEQ ID NO: 164
			b	5	16677717	16677697	
miR160	101	1426	a	2	16347360	16347380	UGCCUGGCUCCCUGUAUGCCA SEQ ID NO: 166
			b	4	9888999	98889019	
			c	5	19026405	19026385	
miR160	1	1752	a	2	16347360	16347381	UGCCUGGCUCCCUGUAUGCCAU SEQ ID NO: 218
miR160	1	1754	a	2	16347360	16347381	GCCUGGCUCCCUGUAUGCCA SEQ ID NO: 219
			b	4	9888999	98889019	
			c	5	19026404	19026385	

miR160*	1	1941	c	5	19026322	19026342	CGUACAAGGAGUCAAGCAUGA SEQ ID NO: 20
miR161.1	4	111	a	1	17829398	17829418	UUGAAAGUGACUACAUCGGGG SEQ ID NO: 167
miR161.1	1	497	a	1	17829399	17829418	UGAAAGUGACUACAUCGGGG SEQ ID NO: 221
miR161.1	10	1746	a	1	17829399	17829419	UGAAAGUGACUACAUCGGGGU SEQ ID NO: 222
miR161.2	307	563	a	1	17829390	17829410	UCAAUGCAUUGAAAGUGACUA SEQ ID NO: 168
miR161.2	6	1707	a	1	17829390	17829411	UCAAUGCAUUGAAAGUGACUAC SEQ ID NO: 223
miR161.2	5	1712	a	1	17829390	17829409	UCAAUGCAUUGAAAGUGACU SEQ ID NO: 224
miR161.2	1	213	a	1	17829391	17829410	CAAUGCAUUGAAAGUGACUA SEQ ID NO: 225
miR162	4	395	a	5	2634957	2634937	UCGAUAAACCUCUGCAUCCAG SEQ ID NO: 169
			b	5	7740613	7740633	
miR163	1	1390	a	1	24888022	24888045	UUGAAGAGGACUUGGAACUUCG AU SEQ ID NO: 170
miR164	2	1427	a	2	19527840	19527860	UGGAGAAGCAGGGCACGUGCA SEQ ID NO: 171
			b	5	287583	287603	
miR164*	2	1812	c	5	9852751	9852771	CACGUGUUCUACUACUCCAAC SEQ ID NO: 226
miR165	30	1428	a	1	78952	78932	UCGGACCAGGCUUCAUCCCCC SEQ ID NO: 173
			b	4	368876	368856	
miR166	299	934	a	2	19183311	19183331	UCGGACCAGGCUUCAUUCCCC SEQ ID NO: 174
			b	3	22933276	22933296	
			c	5	2838738	2838758	
			d	5	2840709	2840729	
			e	5	16792772	16792752	
			f	5	17533605	17533625	
			g	5	25522108	25522128	
miR166	5	1743	a	2	19183311	19183332	UCGGACCAGGCUUCAUUCCCCC SEQ ID NO: 227
			b	3	22933276	22933297	
			c	5	2838738	2838759	
			d	5	2840709	2840730	
miR166	2	1764	a	2	19183310	19183331	UUCGGACCAGGCUUCAUUCCCC SEQ ID NO: 228
miR166	1	1779	a	2	19183310	19183330	UUCGGACCAGGCUUCAUUCCC SEQ ID NO: 229
miR166*	1	1955	a	2	19183198	19183218	GGACUGUUGUCUGGCUCGAGG SEQ ID NO: 230
			b	3	22933187	22933207	
miR167	160	5	a	3	8108097	8108117	UGAAGCUGCCAGCAUGAUCUA SEQ ID NO: 175
			b	3	23417152	23417172	
miR167	3	35	a	3	8108097	8108116	UGAAGCUGCCAGCAUGAUCU SEQ ID NO:231
			b	3	23417152	23417171	
			c	1	11137537	11137556	
miR167	2	447	a	3	8108098	8108117	GAAGCUGCCAGCAUGAUCUA SEQ ID NO: 232
			b	3	23417153	23417172	

miR167	2	697	a	3	8108096	8108117	AUGAAGCUGCCAGCAUGAUCUA SEQ ID NO: 233
miR167	5	557	b	3	23417152	23417173	UGAAGCUGCCAGCAUGAUCUAU SEQ ID NO: 234
miR167	1	790	b	3	23417151	23417172	GUGAAGCUGCCAGCAUGAUCUA SEQ ID NO:235
miR167	1	281	c	1	11137537	11137557	UGAAGCUGCCAGCAUGAUCUG SEQ ID NO: 236
miR167	6	535	c	1	11137537	11137558	UGAAGCUGCCAGCAUGAUCUGG SEQ ID NO: 177
miR168	22	1429	a	4	10578663	10578683	UCGCUUGGUGCAGGUCGGGAA SEQ ID NO: 178
			b	5	18376120	18376100	
miR168*	5	489	a	4	10578748	10578768	CCCGCCUUGCAUCAACUGAAU SEQ ID NO: 237
miR168*	1	1970	a	4	10578748	10578767	CCCGCCUUGCAUCAACUGAA SEQ ID NO: 238
miR168*	1	2076	a	4	10578747	10578767	UCCCGCCUUGCAUCAACUGAA SEQ ID NO: 239
miR169	614	1430	a	3	4359209	4359189	CAGCCAAGGAUGACUUGCCGA SEQ ID NO: 179
miR169	26	1749	a	3	4359209	4359190	CAGCCAAGGAUGACUUGCCG SEQ ID NO: 240
			b	5	8527514	8527533	
			c	5	15888116	15888097	
miR169	119	1751	b	5	8527514	8527534	CAGCCAAGGAUGACUUGCCGG SEQ ID NO: 180
			c	5	15888116	15888096	
miR169	12	1757	a	3	4359211	4359191	UGCAGCCAAGGAUGACUUGCC SEQ ID NO: 241
			b	5	8527512	8527532	
miR169	4	1762		3	4805824	4805805	AGCCAAGGAUGACUUGCCGG SEQ ID NO: 242
				4	11483124	11483105	
			b	5	8527515	8527534	
			c	5	15888115	15888096	
miR169	5	1766	a	3	4359209	4359188	CAGCCAAGGAUGACUUGCCGAU SEQ ID NO: 243
miR169	1	1768	a	3	4359210	4359190	GCAGCCAAGGAUGACUUGCCG SEQ ID NO: 244
			b	5	8527513	8527533	
miR169	13	1775		1	20043242	20043223	AGCCAAGGAUGACUUGCCGA SEQ ID NO: 245
				1	20045256	20045275	
			a	3	4359208	4359189	
miR169	1	1787		1	20043242	20043222	AGCCAAGGAUGACUUGCCGAU SEQ ID NO: 246
				1	20045256	20045276	
			a	3	4359208	4359188	
miR169	5	1802	c	5	15888116	15888095	CAGCCAAGGAUGACUUGCCGGU SEQ ID NO: 247
miR169	3	1813	b	5	8527515	8527535	AGCCAAGGAUGACUUGCCGGA SEQ ID NO: 248
miR169	1	1817		3	4805804	4805824	AGCCAAGGAUGACUUGCCGGU SEQ ID NO: 249
			c	5	15888115	15888095	
miR169	2	1820		3	4805803	4805824	AGCCAAGGAUGACUUGCCGGUU SEQ ID NO: 250
miR169	1	1824	b	5	8527514	8527535	CAGCCAAGGAUGACUUGCCGGA SEQ ID NO: 251

miR169*	1	1772	a	3	4359018	4359037	GGCAAGUUGUCCUUGGCUAC SEQ ID NO: 252
miR169*	1	1773	b	5	8527595	8527616	GGCAAGUUGUCCUUCGGCUACA SEQ ID NO: 253
miR169	22	276	d	1	20043244	20043224	UGAGCCAAGGAUGACUUGCCG SEQ ID NO: 181
			e	1	20045254	20045274	
			f	3	4805826	4805806	
			g	4	11483126	11483106	
miR169	402	1514	h	1	6695555	6695535	UAGCCAAGGAUGACUUGCCUG SEQ ID NO: 182
			i	3	9873362	9873343	
			j	3	9873739	9873720	
			k	3	9876931	9876912	
			l	3	9877296	9877277	
			m	3	9879575	9879555	
			n	3	9879947	9879927	
miR169	1	1760	h	1	6695554	6695535	AGCCAAGGAUGACUUGCCUG SEQ ID NO: 254
			i	3	9873362	9873343	
			j	3	9873739	9873720	
			k	3	9876931	9876912	
			l	3	9877296	9877277	
			m	3	9879574	9879555	
			n	3	9879946	9879927	
miR169	48	1761	i	3	9873363	9873342	UAGCCAAGGAUGACUUGCCUGA SEQ ID NO: 255
			j	3	9873740	9873719	
			l	3	9877297	9877276	
			n	3	9879947	9879926	
miR169	1	1765	i	3	9873362	9873342	AGCCAAGGAUGACUUGCCUGA SEQ ID NO: 256
			j	3	9873739	9873719	
			l	3	9877296	9877276	
			n	3	9879946	9879926	
miR169	3	1771	m	3	9879575	9879554	UAGCCAAGGAUGACUUGCCUGU SEQ ID NO: 257
miR169	1	1774	h	1	6695556	6695535	GUAGCCAAGGAUGACUUGCCUG SEQ ID NO: 258
			i	3	9873364	9873343	
			j	3	9873741	9873720	
			k	3	9876933	9876912	
			m	3	9879576	9879555	
			n	3	9879948	9879927	
miR169	1	1776	i	3	9873363	9873341	UAGCCAAGGAUGACUUGCCUGA C SEQ ID NO: 259
miR169	1	1815		3	4644341	4644361	UAGCCAAGGAUGACUUCCCUU SEQ ID NO: 260
miR169	1	1990	h	1	6695555	6695536	UAGCCAAGGAUGACUUGCCU SEQ ID NO: 261
			i	3	9873363	9873344	
			j	3	9873740	9873721	
			k	3	9876932	9876913	
			l	3	9877297	9877278	
			m	3	9879575	9879556	
			n	3	9879947	9879928	

miR170	1	1431	a	5	26428840	26428820	UGAUUGAGCCGUGUCAAUAUC SEQ ID NO: 183
miR171	34	39	a	3	19084500	19084520	UGAUUGAGCCGCGCCAAUAUC SEQ ID NO: 184
miR171	1	638	a	3	19084500	19084519	UGAUUGAGCCGCGCCAAUAU SEQ ID NO: 262
miR171.2	1	444	b	1	3961387	3961367	UUGAGCCGUGCCAAUAUCACG SEQ ID NO: 185
			c	1	22933780	22933760	
miR171.1	1	1876	c	1	22933783	22933763	UGAUUGAGCCGUGCCAAUAUC SEQ ID NO: 186
miR172	1	811	c	3	3599817	3599797	AGAAUCUUGAUGAUGCUGCAG SEQ ID NO: 188
			d	3	20598970	20598990	
miR172*	1	1854	e	5	24005710	24005729	GCAGCACCAUUAAGAUUCAC SEQ ID NO: 263
			a	5	1188298	1188279	
miR172*	1	2019	a	5	1188298	1188278	GCAGCACCAUUAAGAUUCACA SEQ ID NO: 264
			e	5	24005710	24005730	
miR173	1	886	a	3	8236168	8236189	UUCGCUUGCAGAGAGAAAUCAC SEQ ID NO: 190
miR173*	1	2033	a	3	8236234	8236254	UGAUUCUCUGUGUAAGCGAAA SEQ ID NO: 265
miR390	89	754	a	2	16069049	16069069	AAGCUCAGGAGGGAUAGCGCC SEQ ID NO: 143
			b	5	23654187	23654207	
miR390	25	1703	a	2	16069050	16069069	AGCUCAGGAGGGAUAGCGCC SEQ ID NO: 266
			b	5	23654188	23654207	
miR390	3	1784	a	2	16069049	16069068	AAGCUCAGGAGGGAUAGCGC SEQ ID NO: 267
			b	5	23654187	23654206	
miR390	3	1758	a	2	16069051	16069069	GCUCAGGAGGGAUAGCGCC SEQ ID NO: 268
			b	5	23654189	23654207	
miR390	2	1972	a	2	16069050	16069070	AGCUCAGGAGGGAUAGCGCCA SEQ ID NO:269
			b	5	23654188	23654208	
miR390*	1	206	b	5	23654260	23654279	CGCUAUCCAUCCUGAGUUCC SEQ ID NO: 270
miR390*	1	2051	b	5	23654260	23654280	CGCUAUCCAUCCUGAGUUCCA SEQ ID NO: 271
miR391	7	1728	a	5	24310386	24310406	UUCGCAGGAGAGAUAGCGCCA SEQ ID NO: 144
miR391*	1	1991	a	5	24310737	24310457	ACGGUAUCUCUCCUACGUAGC SEQ ID NO: 272
miR396*	1	1724	a	2	4149413	4149434	GGUUCAAUAAAGCUGUGGGAAG SEQ ID NO: 273
miR397	1	1794	a	4	2625958	2625979	UCAUUGAGUGCAGCGUUGAUGU SEQ ID NO: 274
miR398	4	1994	b	5	4691110	4391130	UGUGUUCUCAGGUCACCCCUG SEQ ID NO: 200
			c	5	4394781	4694801	
miR399	1	1867	b	1	23349074	23349054	CCUGCCAAAGGAGAGUUGCCC SEQ ID NO: 275
ASRP1839	3	1839	a	1	29427439	29427458	UUCGAUGUCUAGCAGUGCCA SEQ ID NO: 276
miR447	1	1890	a	4	1528188	1528209	UUGGGGACGAGAUGUUUUGUUG SEQ ID NO: 146
			b	4	1535480	1535501	

	0		c	4	1523381	1523360	UUGGGGACGACAUCUUUUGUUG SEQ ID NO: 206
miR403	1	359	a	2	19422223	19422244	AUUAGAUUCACGCACAAACUCG SEQ ID NO: 145

Table 8B provides another summary of locus-specific expression of 99 predicted miRNA genes coding for validated *Arabidopsis* microRNAs. Expression of a specific locus was considered definitive (dark shading) if a primary transcript was detected by 5' or 3'RACE, or a unique miRNA sequence was cloned or amplified from the ASRP library described here (gray shading with total clones sequenced) or from another published library (Other Refs.). The number of clones corresponding to a specific miRNA or miRNA*(in parentheses) sequence in the ASRP database is shown. Sequences that were detected only in other studies are indicated by orange in the 3'RACE and references columns. Loci for which data support expression from more than one possible gene are indicated by light shading.

Table 8B. Locus specific expression of 99 predicted MIRNA genes coding for validated *Arabidopsis* microRNAs

Locus	5' RACE	3' RACE	Cloned in ASRP	Other Refs.
MIR156a	Yes	nt	228	[11]
MIR156b	No	No		
MIR156c	Yes	nt		
MIR156d	No	nt		
MIR156e	Yes	nt		
MIR156f	Yes	nt		
MIR156g	No	No		
MIR156h	No	No		
MIR157a	No	Yes	9(1)	
MIR157b	No	Yes		
MIR157c	Yes	nt		
MIR157d	Yes	nt		
MIR158a	No	nt	18(3)	[11]
MIR158b	No	No		
MIR159a	Yes	nt	225	[8-12]
MIR159b	Yes	nt	7	
MIR159c	No	No		
MIR319a	Yes	nt	5	
MIR319b	Yes	nt		
MIR319c	No	nt		
MIR160a	Yes	nt	103(1)	[11]
MIR160b	Yes	nt		
MIR160c	Yes	nt		
MIR161	Yes	nt	334	[1,6,11]
MIR162a	Yes	Yes	4	[11]
MIR162b	Yes	Yes		
MIR163	Yes	nt	1	[1,5,10,11]
MIR164a	Yes	nt	2	[11]
MIR164b	Yes	nt		
MIR164c	No	nt	0(2)	
MIR165a	Yes	nt	30	[11]
MIR165b	No	No		
MIR166a	Yes	nt	307(1)	
MIR166b	Yes	nt		
MIR166c	Yes	nt		
MIR166d	Yes	nt		
MIR166e	No	Yes		
MIR166f	No	Yes		
MIR166g	No	No		
MIR167a	Yes	nt	173	[6,10, 11,13]
MIR167b	Yes	nt		
MIR167c	No	No		
MIR167d	No	nt	7	
MIR168a	No	nt	22(7)	[11,13]
MIR168b	No	No		
MIR169a	Yes	nt	619(1)	[11]
MIR169b	No	nt	128(1)	
MIR169c	Yes	nt		
MIR169d	No	No	22	
MIR169e	No	No		
MIR169f	No	No	22	[11]
MIR169g	No	No		
MIR169h	Yes	nt	458	
MIR169i	No	nt		
MIR169j	No	No		
MIR169k	No	No		
MIR169l	Yes	nt		
MIR169m	No	nt		
MIR169n	Yes	nt		
MIR170	Yes	nt	1	[6,7, 11,12]
MIR171a	Yes	nt	35	
MIR171b	Yes	nt	2	
MIR171c	Yes	nt		
MIR172a	Yes	nt	0(2)	[2,3,10]
MIR172e	Yes	nt		
MIR172b	Yes	nt		
MIR172c	Yes	nt	1	
MIR172d	No	No		
MIR173	No	nt	1(1)	[10]
MIR390a	No	nt	122(2)	[12]
MIR390b	No	nt		
MIR391	No	nt	7(1)	
MIR393a	No	No		[4,12]
MIR393b	No	No		
MIR394a	Yes	nt		[4]
MIR394b	No	No		
MIR395a	No	No		[4]
MIR395b	No	No		
MIR395c	Yes	nt		
MIR395d	No	No		
MIR395e	Yes	nt		
MIR395f	No	No		
MIR396a	Yes	nt	0(1)	[4]
MIR396b	No	No		
MIR397a	No	nt	1	[4,12]
MIR397b	Yes	nt		
MIR398a	No	nt	4	[4,12]
MIR398b	No	Yes		
MIR398c	Yes	nt		
MIR399a	No	No	1	[4,12]
MIR399b	Yes	nt		
MIR399c	Yes	nt		
MIR399d	Yes	nt		
MIR399e	No	No		
MIR399f	No	nt		
MIR403	Yes	Yes	1	[12]
MIR447a	Yes	nt	1	
MIR447b	Yes	nt		
MIR447c	Yes	nt		

nt, not tested. References cited are: 1. Allen *et al.*, *Nat Genet* 36:1282-1290, 2004; 2. Aukerman & Sakai, *Plant Cell* 15:2730-2741, 2003; 3. Chen, *Science* 303:2022-2025, 2004; 4. Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; 5. Kurihara & Watanabe, *Proc Natl Acad Sci U S A* 101:12753-12758, 2004; 6. Llave *et al.*, *Plant Cell* 14:1605-1619, 2002; 7. Llave *et al.*, *Science* 297:2053-2056, 2002; 8. Mette *et al.*, *Plant Physiol* 130:6-9, 2002; 9. Palatnik *et al.*, *Nature* 425:257-263, 2003; 10. Park *et al.*, *Curr Biol* 12:1484-1495, 2002; 11. Reinhardt *et al.*, Genes Dev. 16:1616-1626, 2002; 12. Sunkar and Zhu *Plant Cell* 16:2001-2019, 2004; 13. *Arabidopsis* EST clones were identified for *MIR167d* (GenBank accession AU239920) and *MIR168a* (H77158).

For each 5'RACE product detected, the transcription start site was assigned to the most highly represented sequence among six randomly selected clones. In cases where two clustered sequences were equally represented, the extreme 5' sequence was assigned as the start site. Following this procedure, the 5'ends representing 63 transcripts from the 52 *MIRNA* loci were identified (Figure 10 and Table 8). The vast majority of transcripts initiated with an adenosine (83%) that was preceded by a pyrimidine residue (Figure 10A). Twelve loci yielded multiple transcripts that were consistent with alternative start sites. Three transcripts (one from *MIR156a* and two from *MIR172b*) contained introns between the 5' end and foldback sequence. Each of these characteristics is consistent with transcription by RNA pol II.

To identify conserved motifs flanking the initiation sites at each mapped locus, a 60-bp genomic segment (-50 to +10 relative to the start site) was computationally analyzed using BioProspector. An 8-nucleotide TATA box-like sequence was detected upstream from 83% of transcription start sites (Figure 10B). Using MotifMatcher to scan a broader segment (-200 to +50), the TATA-like sequence was shown to be centered at position removed from the start site (Figure 10C). The TATA motif at position -30 is entirely consistent with TATA motifs for protein-coding genes (Patikoglou *et al.*, *Genes Dev* 13:3217-3230, 1999; Shahmuradov *et al.*, *Nucleic Acids Res* 31:114-117, 2003). We conclude, therefore, that these are authentic TATA box sequences within core promoter elements of *MIRNA* genes.

Expression of *Arabidopsis MIRNA* genes

Despite repeated attempts with multiple primer sets, 5' start sites were mapped for only about one-half of predicted *MIRNA* genes (Table 8B). This may have been due to either less-than-optimal 5'RACE procedures and low expression levels (false negative results) or lack of expression of some loci predicted to be *MIRNA* genes. It is also possible that some primer sets were designed within intron sequences. To develop a more comprehensive account of *Arabidopsis MIRNA* genes with validated expression data, informatic and experimental approaches were taken. In the informatic strategy, the ASRP database was scanned for locus-specific miRNA or miRNA* (miRNA-complementary species within miRNA duplexes) sequences (Gustafson *et al.*, *Nucleic Acid Research* 33:D637-D640, 2005). Unique miRNA or miRNA* sequences specific to *MIR158a*, *MIR167d*, *MIR173*, *MIR391*, *MIR397a* and *MIR164c* loci were each represented in the database (Figure 10). In addition, unique miRNA sequences specific to *MIR319c*, *MIR398a*, and *MIR399f* were represented in an independent *Arabidopsis* small RNA library (Table 8B) (Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004). For each of three families (*MIR390/391*, *MIR393*, and *MIR168*) in which negative 5'RACE data were obtained, multiple predicted loci encode an identical miRNA that was detected in at least one small RNA library (Reinhart *et al.*, *Genes Dev* 16:1616-1626, 2002; Jones-Rhoades & Bartel, *Mol Cell* 14:787-799, 2004; Sunkar & Zhu, *Plant Cell* 16:2001-2019, 2004; Gustafson *et al.*, *Nucleic Acid Research* 33:D637-D640, 2005). For *MIR168a*, a locus-specific EST clone (GenBank accession H77158) exists to confirm expression. For two miRNAs that are represented by a single locus

(miR173 and miR391), expression was inferred by cloning or detection of the miRNA sequence. Thus, 5'RACE and unambiguous miRNA cloning/detection support expression of 68 of 99 predicted *Arabidopsis MIRNA* loci.

For the remaining 31 predicted *MIRNA* genes, locus-specific primers were designed to amplify sequences immediately downstream of the precursor foldback sequence through a 3' RACE procedure. Positive results were obtained for five loci.

Example 6: Small RNA Formation in Plants

This example provides a demonstration of the ability to produce novel siRNAs using engineered ta-siRNA-generating loci. This demonstration includes miRNA-dependent formation of novel siRNAs for RNAi against exogenous and enogenous RNA sequences, and phenotypes associated with silencing of an endogenous gene (phytoene desaturase, or PDS) using the artificial, engineered cassettes.

Development of constructs for wild-type and artificial ta-siRNA biogenesis assays in *N. benthamiana*

The following artificial ta-siRNAs targeting *Arabidopsis* genes encoding phytoene desaturase (PDS) and PINOID (PID), as well as GFP, were designed and expressed using an *Arabidopsis thaliana TAS1c*-based construct: 35S:TAS1c; 35S:TAS1cGFPd3d4 (SEQ ID NO: 277); 35S:TAS1cPDSd3d4 (SEQ ID NO: 278); and 35S:TAS1cPIDd3d4 (SEQ ID NO: 279). The ta-siRNA constructs were made in the *TAS1c* context, as shown in Figure 11.

The artificial ta-siRNAs were expressed in place of the normal 3'D3(+) and 3'D4(+) positions of TAS1c (positional nomenclature as in Allen *et al.*, *Cell* 121:207-221, 2005). Artificial ta-siRNA sequences were chosen based on the principles of the asymmetry rules and presented by Schwarz *et al.* (*Cell* 115:199-208, 2003) and Khvorova *et al.* (*Cell* 115:209-216, 2003). The artificial siRNAs chosen were designed as perfect complementary matches to their corresponding target genes, although it is assumed that artificial siRNAs may contain mismatches similar to those in known miRNA:target duplexes (see Allen *et al.*, *Cell* 121:207-221, 2005, for examples). Each of these constructs contained two 21-nt siRNA modules, with the siRNAs designed to target mRNAs for GFP, PDS and PID.

Engineered TAS1c loci were expressed using the CaMV 35S promoter and the nos terminator as regulatory elements. The expressed sequence was inserted betweens between att sites (positions 55 to 79 and 1106 to 1130 in each) for recombination into a "Gateway" vector. The two consecutive, 21-nucleotide engineered ta-siRNAs correspond to nucleotide positions 520 to 561 in each of SEQ ID NOs: 277, 278, and 279. Vector sequence is shown in postions 1 to 99 and 1090 to 1185 of each of these sequences; primers used to amplify the cassettes hybridize at positions 96 to 123 and 1069 to1089.

Demonstration of artificial ta-siRNA biogenesis and activity in *N. benthamiana*

Transient ta-siRNA expression assays in *Nicotiana benthamiana* were done as in Allen *et al.* (*Cell* 121:207-221, 2005). Stable *Arabidopsis thaliana* transgenic lines were created using the *Agrobacterium* mediated floral dip method. Transgenic seed from transformed plants was plated on Murashige-Skoog media containing kanamycin (50 μg/ml), and blot assays were done as described in Allen *et al.* (*Cell* 121:207-221, 2005).

Introduction of each construct (35S:TAS1c [which forms wild-type TAS1c ta-siRNAs], 35S:TAS1cGFPd3d4, 35S:TAS1cPDSd3d4, and 35S:TAS1cPIDd3d4) into *N. benthamiana* in a transient assay resulted in miR173-dependent formation of ta-siRNAs (Figure 12). In the case of *35S:TAS1cGFPd3d4*, the artificial ta-siRNA construct was co-expressed with a functional GFP gene. Expression of at least one artificial ta-siRNA was detected in a miR173-dependent manner, by blot assay using each construct (Figure 12). The GFP gene was silenced by the artificial GFP ta-siRNAs in a miR173-dependent manner (Figure 12).

A PDS artificial ta-siRNA-generating construct was introduced into wild-type *Arabidopsis* and rdr6- 15 and dcl4-2 (Xie *et al.*, *Proc Natl Acad Sci U S A*. 102(36):12984-12989, 2005; Epub 2005 Aug 29) mutant plants. Both strong and weak loss-of-function PDS phenotypes were detected, but only in wildtype plants and not in rdr6-15 or dcl4-2 plants (Table 9 and Figure 13). This indicates that functional artificial ta-siRNAs were formed through the activity of the normal ta-siRNA pathway.

Table 9. Observed phenotype classe of Arabidopsis stable transgenic lines expressing engineered TAS1cPDSd3d4

	No Phenotype	Weak Phenotype	Strong Phenotype
Col-0 (n=102)	8/102	36/102	58/102
rdr6-15 (n=291)	291/291	-	-
dcl4-2 (n=15)	15/15	-	-

This disclosure describes the discovery of a new system for RNAi *in vivo*, and provides methods, constructs, and compositions useful for exploiting this discovery. The disclosure further provides myriad initiator sequences and methods for identifying additional initiator sequences that are useful in directing *in vivo* generation of predictable 21-mer siRNAs, as well as methods of using constructs containing such an initiator sequence to mediate RNAi. It will be apparent that the precise details of the methods described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the disclosure and the claims below.

CLAIMS

1. An siRNA-triggering or RNAi-triggering nucleic acid cassette, comprising:

an initiator sequence consisting of about 20 to 25 nucleotides, and having an initiation cleavage site between about the tenth and eleventh nucleotide counted from the 3' end of the initiator sequence; and

at least one gene suppressing segment in about 21-nucleotide register counted either upstream or downstream from the initiation cleavage site, wherein the gene suppressing segment or its complement is substantially complementary to mRNA transcribed from a target gene selected for siRNA inhibition.

2. An expression vector, comprising the nucleic acid cassette of claim 1 operably linked to a promoter.

3. The nucleic acid cassette of claim 1, wherein the at least one initiator sequence is selected from any one of SEQ ID NO: 1-142 and 281-285.

4. The nucleic acid cassette of claim 1, comprising two or more gene suppressing segments, at least two of which are specific for different target genes selected for siRNA inhibition.

5. A transgenic plant cell that expresses RNA for suppressing a target gene, wherein said plant cell comprise recombinant DNA from which there is transcribed a first RNA comprising:

an initiator segment consisting of about 20-25 nucleotides wherein an initiation cleavage site is located between about the tenth and eleventh nucleotide counted from the 3' end of the initiator segment and wherein said initiator segment is linked to or overlaps with

at least one gene suppressing segment of 21-nucleotides in 21-nucleotide register counted either upstream or downstream from the initiation cleavage site, wherein said gene suppressing segment or it complement is complementary to RNA transcribed from said target gene.

6. A transgenic plant cell of claim 5 further comprising DNA from which there is transcribed a second RNA that anneals to said first RNA at said initiation cleavage site.

7. A transgenic plant cell of claim 6 wherein said second RNA is an exogenous miRNA or siRNA.

8. A transgenic plant cell of claim 6 wherein said second RNA is a miRNA or siRNA transcribed from a native plant gene.

9. A transgenic plant cell of claim 5 wherein said first RNA comprises two or more of said gene suppressing segments.

10. A transgenic plant cell of claim 5 wherein said target gene is an endogenous plant gene.

11. A transgenic plant cell of claim 10 wherein said plant cell is a corn cell and said endogenous plant gene encodes lysine ketoglutarate reductase.

12. A transgenic plant cell of claim 5 wherein said target gene is a gene of a plant pathogen or plant pest.

13 A transgenic plant cell of claim 12 wherein said plant pest is a nematode or insect and said pant pathogen is a virus or fungus.

14. A transgenic plant cell of claim 13 wherein said plant cell is a soybean cell and said plant pest is soybean cyst nematode.

15. A transgenic plant cell of claim 5 wherein said recombinant DNA comprises a promoter functional in said plant operably linked to DNA coding for said first RNA.

16. A transgenic plant cell of claim 15 wherein said promoter is characterized as being a constitutive promoter, an inducible promoter, a tissue specific promoter, a ubiquitous promoter or a combination thereof.

17. A transgenic plant cell of claim 5 wherein said plant cell is a corn, soybean, cotton, canola, wheat or rice cell.

18. A transgenic plant cell of claim 5 wherein said recombinant DNA further comprises nucleotides for expressing at least one protein.

19. A method of inhibiting expression of a target gene or sequence in a cell, the method comprising exposing the cell to an effective amount of the nucleic acid cassette of claim 1 or the vector of claim 2.

20. The method of claim 19, wherein the cell is a plant cell, a fungal cell, or an invertebrate cell.

21. The method of claim 19, wherein the cell is in a multicellular organism.

22. A method of inducing production of at least one siRNA in a cell, comprising:

transforming the cell with a recombinant nucleic acid molecule comprising a nucleic acid cassette of claim 1, wherein the recombinant nucleic acid molecule directs expression of a RNA from the nucleic acid cassette, which RNA is processed in the cell to produce at least one siRNA, thereby inducing the production of at least one siRNA in the cell.

23. A method of inhibiting activity of a target gene in a plant cell, comprising:

transforming the plant cell with a recombinant nucleic acid molecule comprising a nucleic acid cassette of claim 1, wherein at least one gene suppressing segment of the nucleic acid cassette is specific for the target gene; and

expressing the nucleic acid molecule, thereby producing in the plant cell at least one siRNA specific for the target gene which inhibits activity of the target gene in the plant cell.

24. A method of inhibiting activity of a target gene in a plant seed cell comprising providing in one of more said plant cells:

(a) a recombinant nucleic acid molecule comprising a nucleic acid cassette of claim 1, wherein at least one gene suppressing segment of the nucleic acid cassette is specific for the target gene and wherein said cassette comprises a seed-specific promoter operably linked to said initiator sequence and said at least one gene suppressing segment;

(b) a recombinant DNA with a seed specific promoter operably linked to DNA transcribing an miRNA or siRNA that anneals to said initiator sequence at said initiation cleavage site; or

(c) both (a) and (b).

FIGURE 1A

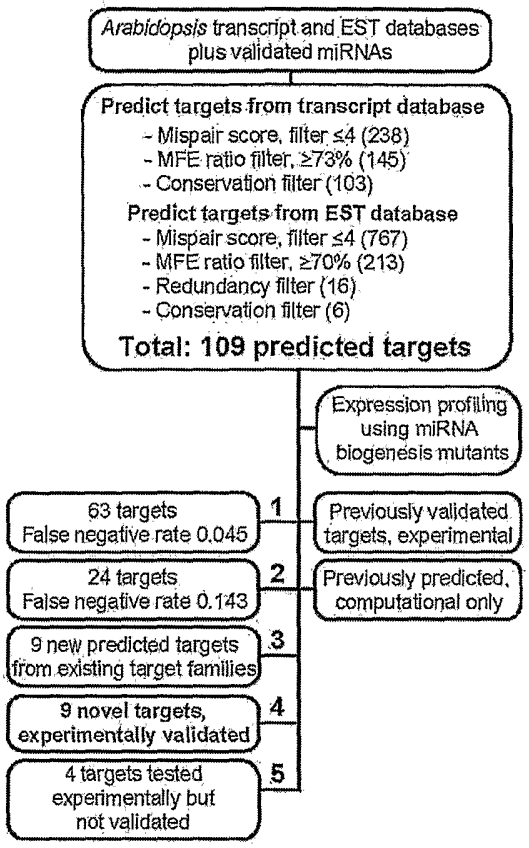


FIGURE 1B

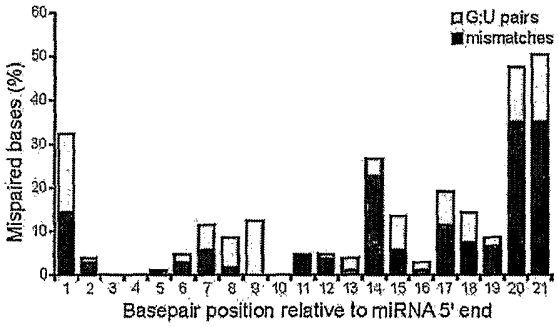


FIGURE 1C

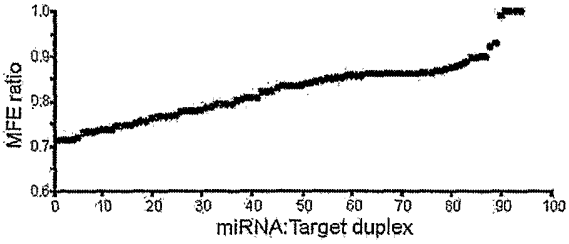


FIGURE 1D

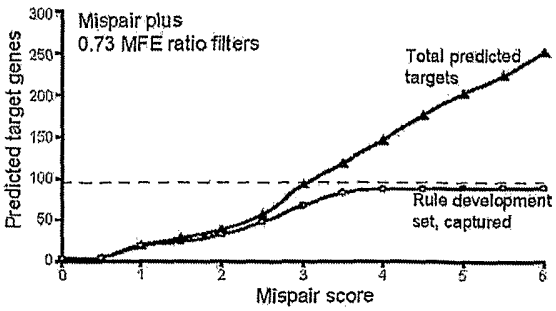


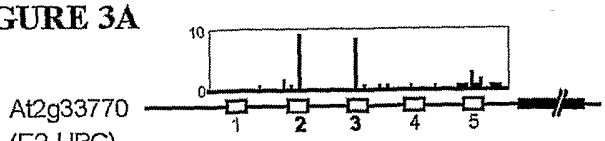
FIGURE 2A

```
                                19/24
At1g53160   5' ...AAGCUGUCUGCUCUCUCUCUUCUGUCAUCUAAA... 3'
(SBP4)                  |||||| |||||||||||||||
                      3' CACGAGUGAGAGAAGACAGU 5'  miR156

                                18/18
At4g30080   5' ...UCCCGUGGGUUUACAGGGAGCCAGGCAUAAUGC... 3'
(ARF16)                 ||. |||||||||||||||||
                      3' ACCGUAUGUCCCUCGGUCCGU 5'  miR160

                                10/10
At1g56010   5'  ...CAAUGAGCACGUACCCUGCUUCUCCAAUUUGU... 3'
(NAC1)                  |||||| |||||||||||||
                      3' ACGUGCACGGGACGAAGAGGU 5'  miR164

                                9/11
At5g61430   5' ...AACCGGUCUACGUGCCCUGCUUCUCCAACCAAA...  3'
(NAC family)            | .||||||||||||||||||
                      3' ACGUGCACGGGACGAAGAGGU 5'  miR164

                        1/25 18/25 1/25
At1g52150   5'   ...AUGCCUGGAAUGAAGCCUGGUCCGGAUUCCAU... 3'
(AtHB15)                ||||||||||||||||||||.
                      3' CCCCUUACUUCGGACCAGGCU 5'  miR166

                                4/4
At1g30330   5' ...CCCUGUGAGAUCAGGCUGGCAGCUUGUAUUUG... 3'
(ARF6)                  |||||| |||||||||||
                      3' AUCUAGUACGACCGUCGAAGU 5'  miR167

                                11/14 1/14
At1g17590   5' ...CAAAAAAAGGGAAGUCAUCCUUGGCUGUACUUC... 3'
(HAP2 family)           | || ||||||||||||||||-
                      3' UGCCGUUCAGUAGGAACCGAU 5'  miR169h

                                11/16 1/16 1/16 2/16
At1g72830   5' ...CAAGAAGGGGAAGUCAUCCUUGGCUACUACUA... 3'
(HAP2c)                 | || ||||||||||||||||
                      3' UGCCGUUCAGUAGGAACCGAU 5'  miR169h

                                4/5
At1g54160   5' ...ACAUGACGGGAAGUCAUCCUUGGCUACUUAUG... 3'
(HAP2 family)           |||| ||||||||||||||||||
                      3' UGCCGUUCAGUAGGAACCGAU 5'  miR169h

                        1/8      6/8        1/8
At3g20910   5' ...CACAAGCGGCAAUUCAUUCUUGGCUUUCUCUUU... 3'
(HAP2 family)           -||||||| |||||-|||||||
                      3' UGCCGUUCAGUAGGAACCGAU 5'  miR169h

                                11/13 1/13
At3g05690   5' ...GUGAACAGGCAAAUCAUCUUUGGCUCAUUCUCU... 3'
(HAP2b)                 | ||||| |||||-||||||
                      3' GACCGUUCAGUAGGAACCGAC 5'  miR169a

                                9/10 1/10
At5g06510   5' ...GUAAACAGGCAAAUCAUCUUUGGCUCAUGCUUC... 3'
(HAP2 family)           | ||||| |||||-||||||
                      3' GACCGUUCAGUAGGAACCGAC 5'  miR169a

                        1/9        3/9  1/9  2/9
At3g22890   5' ...AAUCCGAGUUCCUCCAAACUCUUCAUUUUAACU... 3'
(APS1)                  |||||||-||||||| |||||
                      3' CUCAAGGGGGUUUGUGAAGUc 5'  miR395
```

FIGURE 2B

```
At1g62860   5' ...AUCGACCCUGAUGUUGUUACUUUCAGCGCAU... 3'
(PPR family)            |||-||||| ||-||||||||.
                      3' GGGGCUACAUCAGUGAAAGUU 5'  miR161.1

At1g63330   5' ...UAAUUUAGUCACGUUCAAUGCAUUGAUCGUA... 3'
(PPR family)            ||||||| ||||||||||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g62590   5' ...UAAUUUAGUCACGUUCAAUGCAUUGAUCGUA... 3'
(PPR family)            ||||||| ||||||||||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g63630   5' ...UGAUAUUGUUACUUUCAGUGCAUUGAUCAAU...  3'
(PPR family)            | ||-||||||||-||||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g62930   5' ...AAAUGUAGUCACUUUCAGCGCAUUGAUUGAU... 3'
(PPR family)            ||||||||||||-  |||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g63130   5' ...AAAUGUAGUCACUUUCAGCGCAUUGAUUGAU... 3'
(PPR family)            ||||||||||||-  |||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g62910   5' ...AAAUGUAGUCACUUUCAGCGCAUUGAUUGAU... 3'
(PPR family)            ||||||||||||-  |||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g63230   5' ...UGAUGUUGUAACUUUCAGUGCAUUGAUCAAU... 3'
(PPR family)            | || |||||||-||||||||
                      3' AUCAGUGAAAGUUACGUAACU 5'  miR161.2

At1g14020   5' ...UAAUGAAGGGAAGUCAUCCUUGGCUAAUUUA... 3'
(HAP2 family)           | || ||||||||||||||||||
                      3' UGCCGUUCAGUAGGAACCGAU 5'  miR169h
```

FIGURE 3A



At2g33770 (E2-UBC)

```
                2   1        10
(2)  5' CGUAGGGCAUAUCUCCUUUGGCAAU 3'
          ·|||||||  |||||||||||||
     3' GUCCCGUUUAGAGGAAACCGU 5'   miR399

                         9   1
(3)  5' CGUUGGGCAAAUCUCCUUUGGCAUU 3'
                         1
(4)  5' GUUCGAGCAAAUCUCCUUUGGCAUU 3'
            111        13       1    1 2
(5)  5' CGUAGAGCAAAUCUCCUUUGGCAUUAUC 3'
                         13/16          1/16 2/16
At1g31280 5' AAGGAGUUUGUGCGUGAAUCUAAUUGGGUU 3'
(AGO2)          |||||||||||||||||||||||
     3' GCUCAAACACGCACUUAGAUUA 5' miR403

                         25/27  1/27
At5g10180 5' UCAAGUUCUCCCAAACACUUCAAAU 3'
(AST68)         |||||·|||||||||||||
     3' CUCAAGGGGGUUUGUGAAGUc 5'   miR395

                         1/7 6/7
At5g60760 5' ACUGAC-AAACAUCUCGUCCCCAAGA 3'
(2PGK)          ··|| |||||||||||||||||
     3' GUUGUUUUGUAGAGCAGGGGUU 5' miR447
```

FIGURE 3B

```
                         17/17
At2g27400 5' UUGUGAUUUUUCUCUACAAGCGAAUG 3'
(TAS1a)         |||||||·||||| ||||||||
     3' CACUAAAGAGAGACGUUCGCUU 5' miR173

                         11/11
At1g50055 5' UUGUGAUUUUUCUCAACAAGCGAAUG 3'
(TAS1b)         |||||||·||||  ||||||||
     3' CACUAAAGAGAGACGUUCGCUU 5' miR173

                         23/23
At2g39675 5' UUGUGAUUUUUCUCUACAAGCGAAUA 3'
(TAS1c)         |||||||·||||| ||||||||
     3' CACUAAAGAGAGACGUUCGCUU 5' miR173

                         24/24
At2g39681 5' UUGUGAUUUUUCUCUCCAAGCGAAUG 3'
(TAS2)          |||||||·||||| ||||||||
     3' CACUAAAGAGAGACGUUCGCUU 5' miR173

                         9/22
At3g17185 5' ACCUUGUCUAUCCCUCCUGAGCUAAU 3'
(TAS3)          ·| ||||||||||||||||
     3' CCGC-GAUAGGGAGGACUCGAA 5' miR390
```

FIGURE 4A

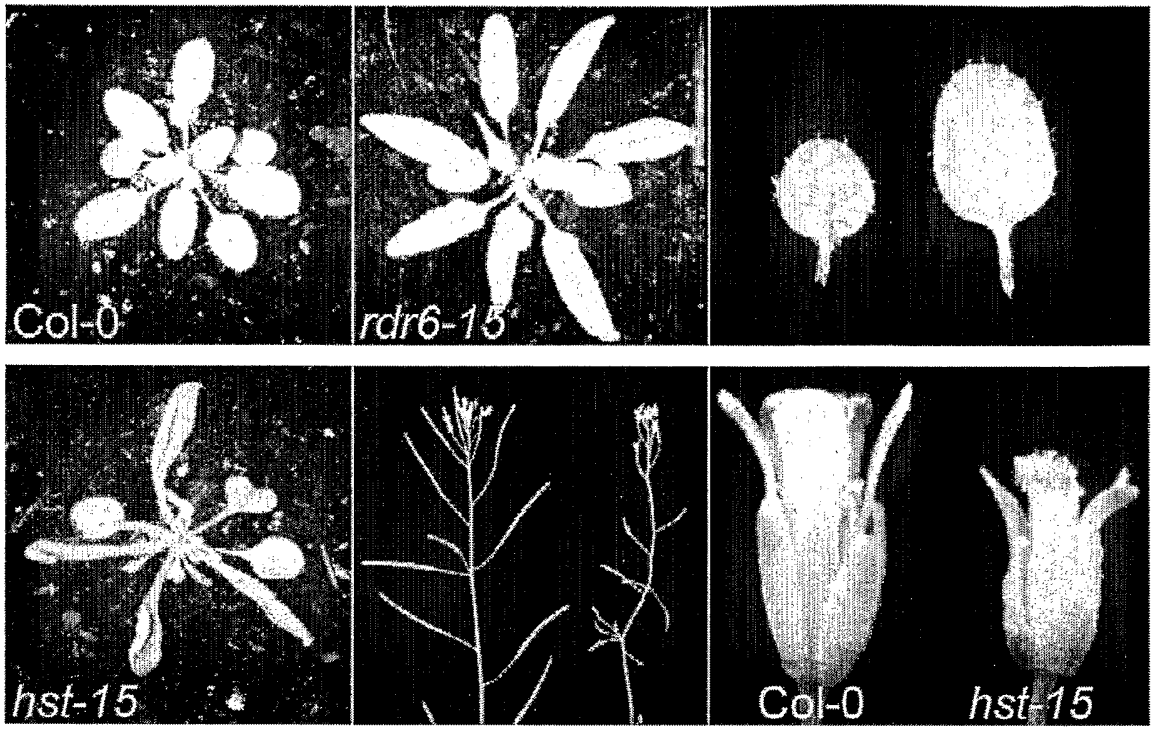


FIGURE 4B

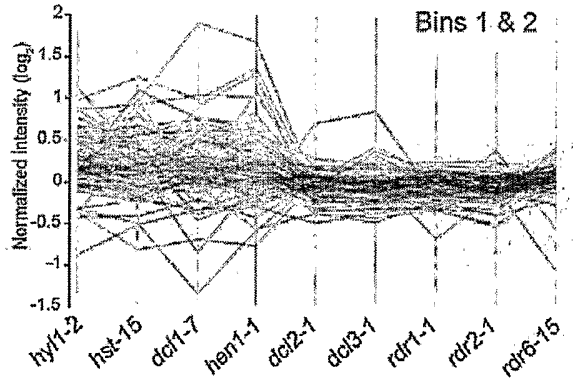


FIGURE 4C

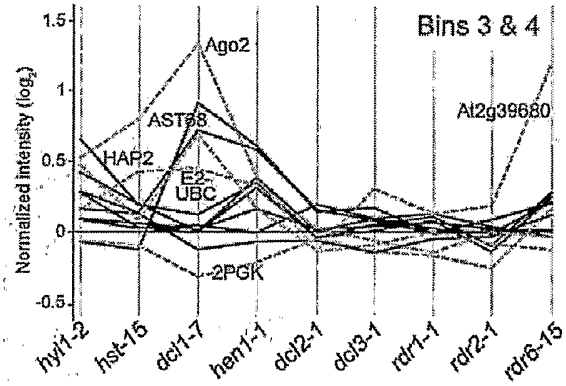


FIGURE 4D

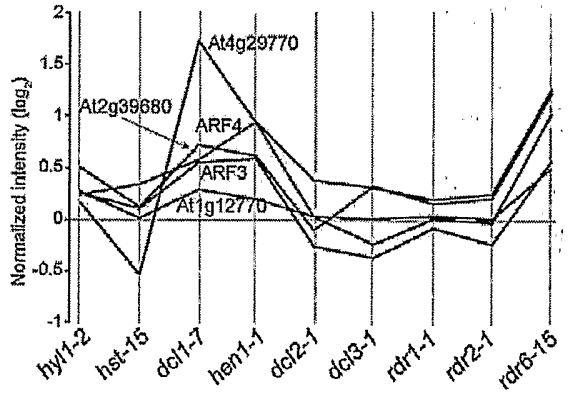


FIGURE 4E

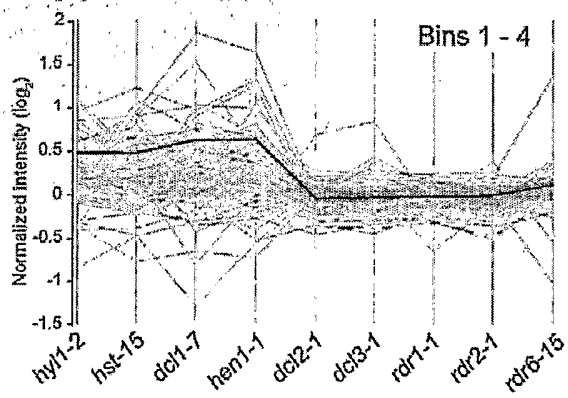


FIGURE 4F

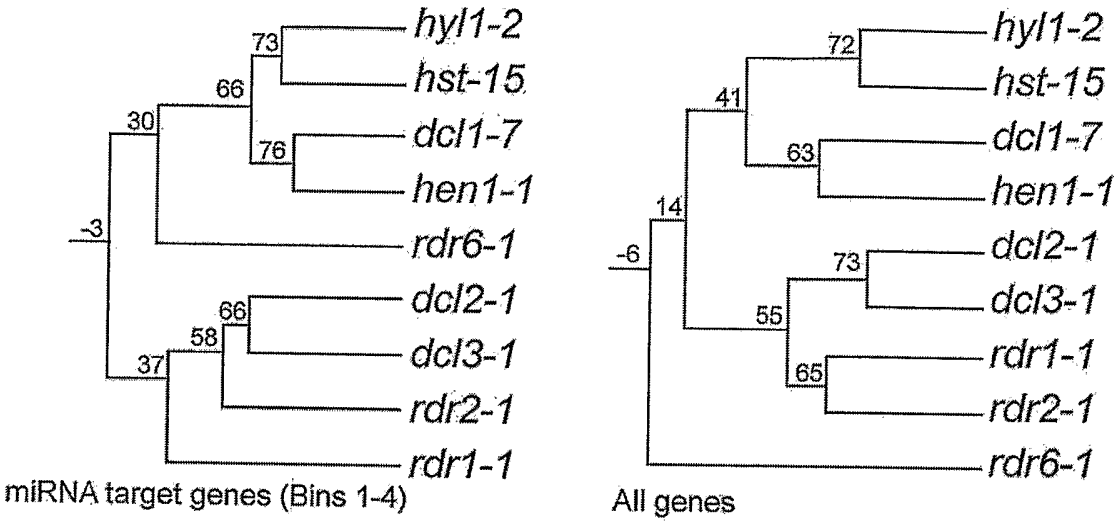


FIGURE 4G

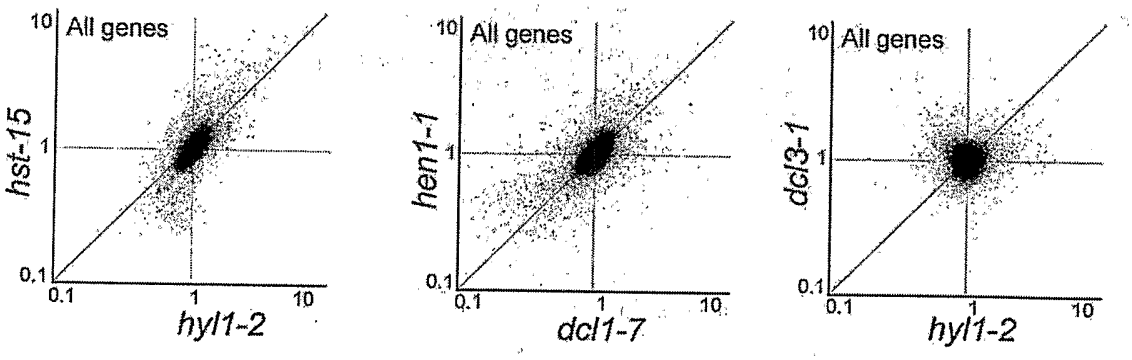


FIGURE 5A

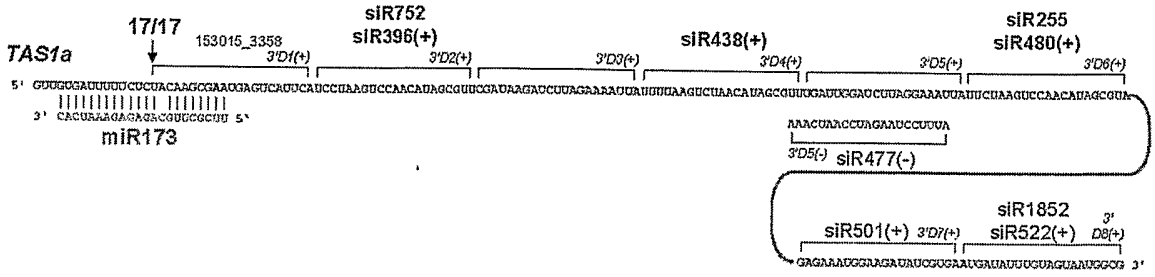


FIGURE 5B

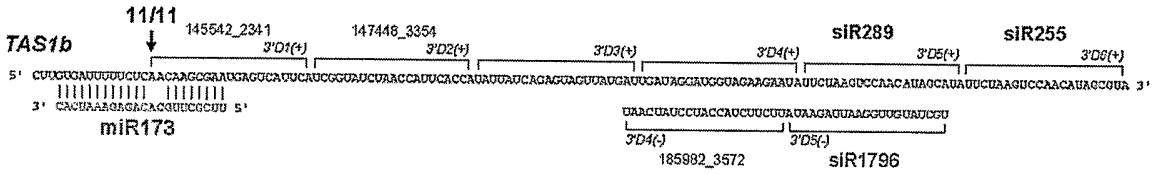


FIGURE 5C

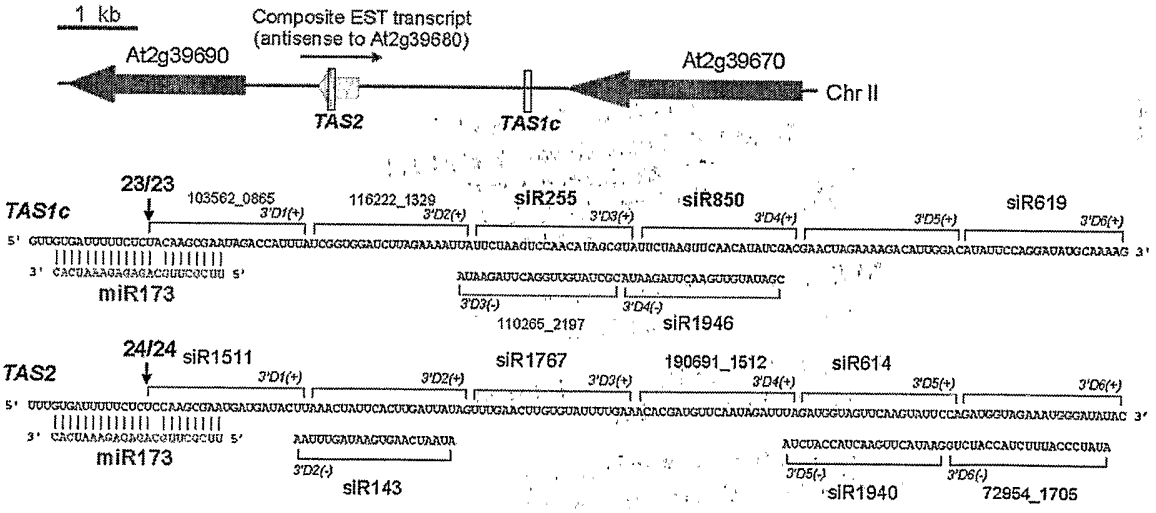


FIGURE 5D

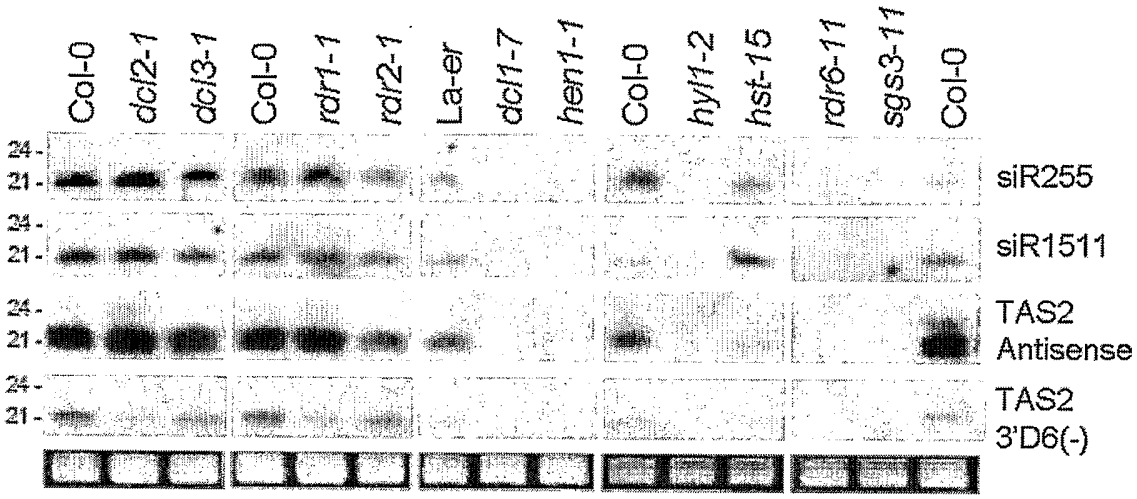


FIGURE 5E

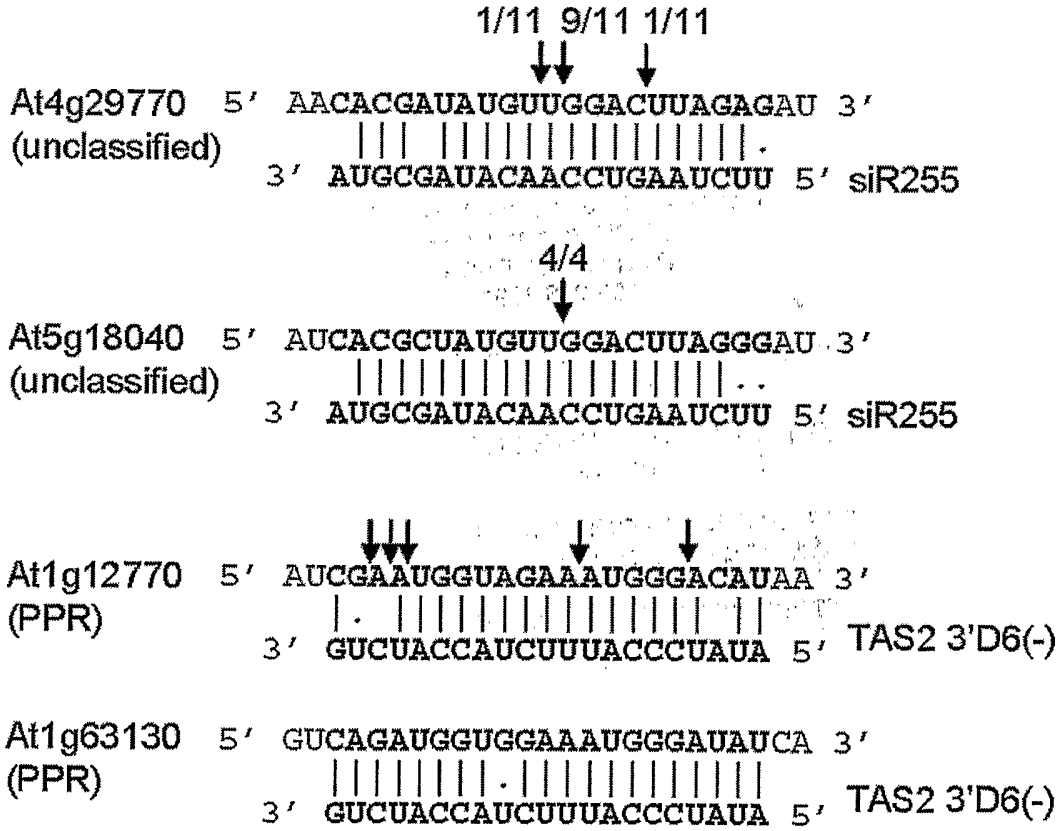


FIGURE 6A

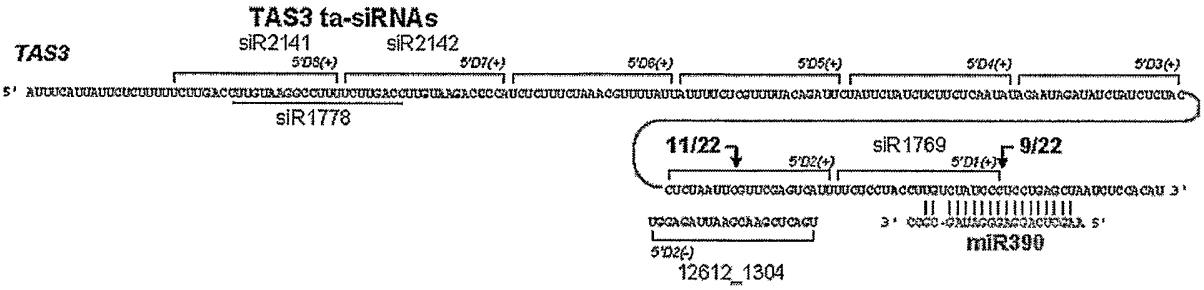


FIGURE 6B

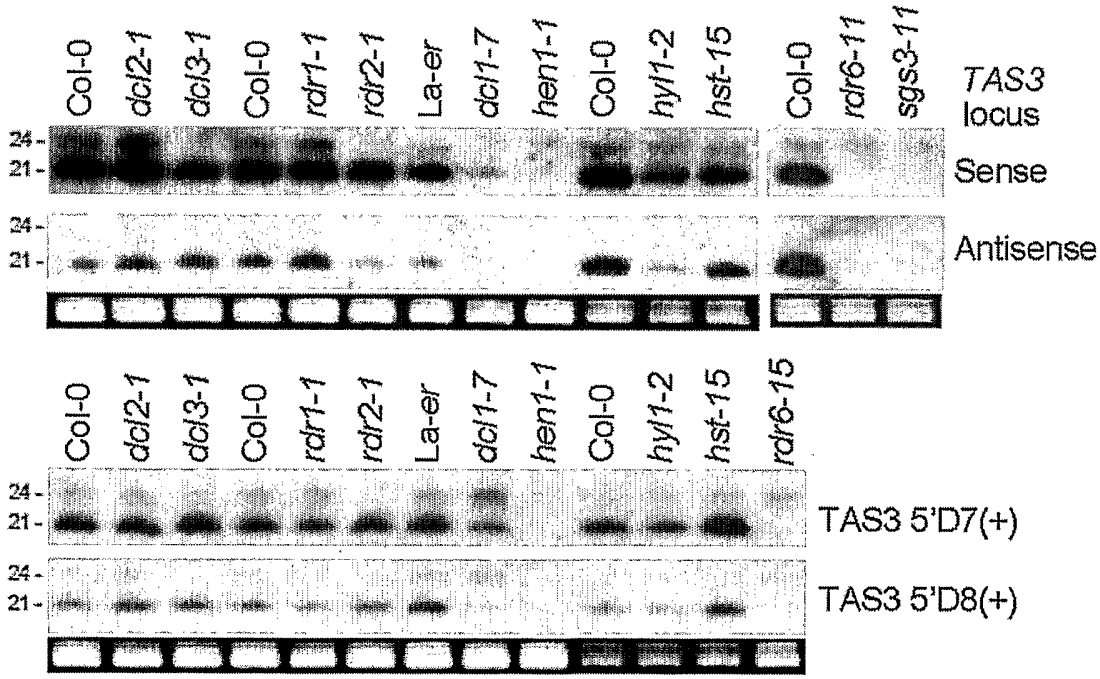


FIGURE 6C

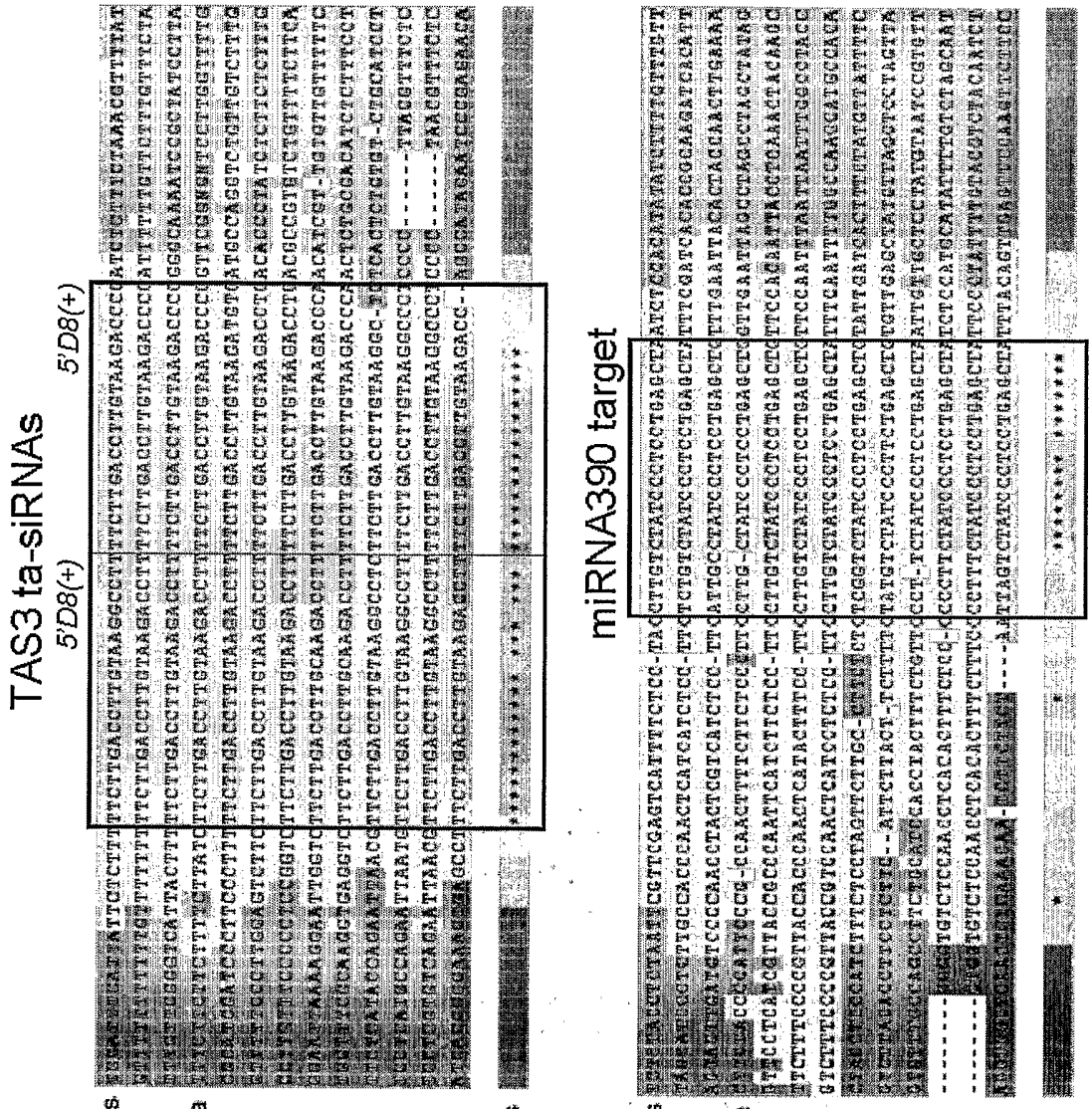

TAS3 ta-siRNAs
5'D8(+)
5'D8(+)
miRNA390 target
Arabidopsis
Populus
Vitis
Theobroma
Malus
Glycine
Lotus
Oryza
Sorghum
Zea
Triticum
Hordeum
Picea
Consensus

Figure 6D

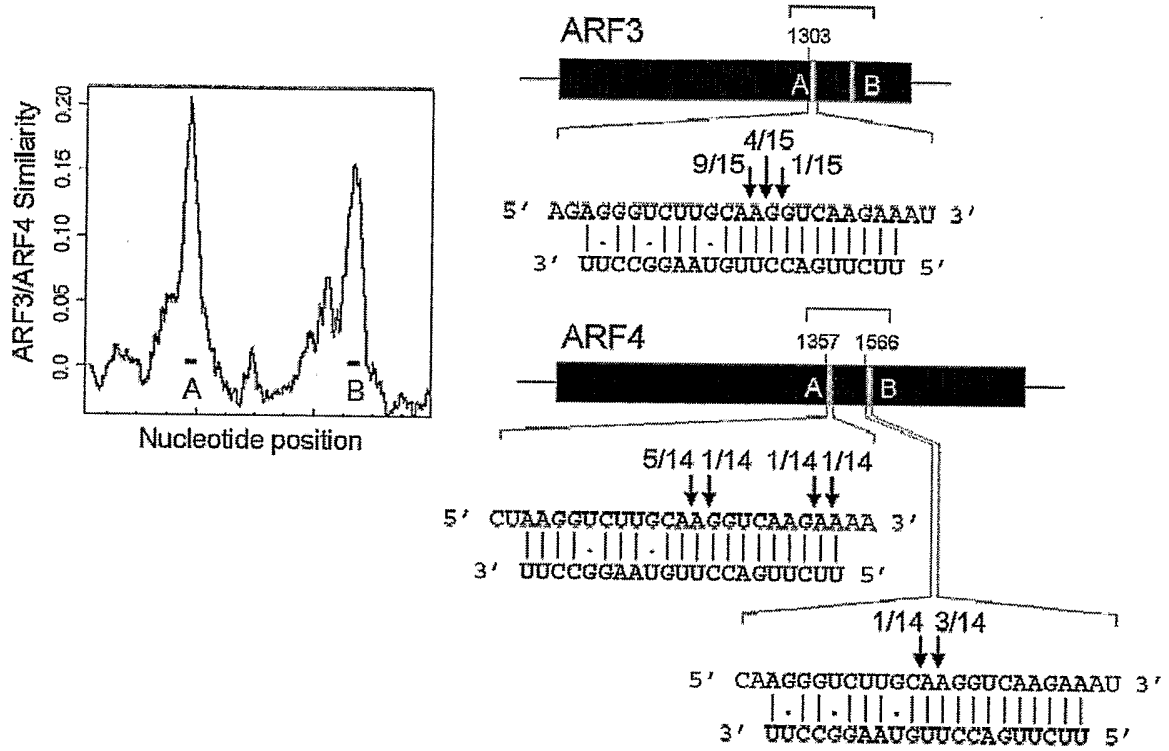


Figure 6E

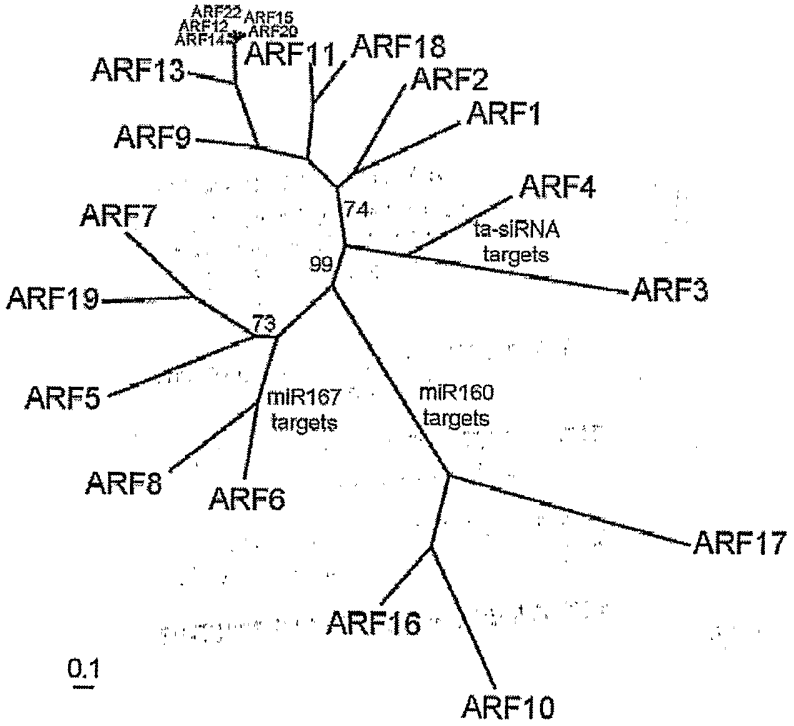


FIGURE 7

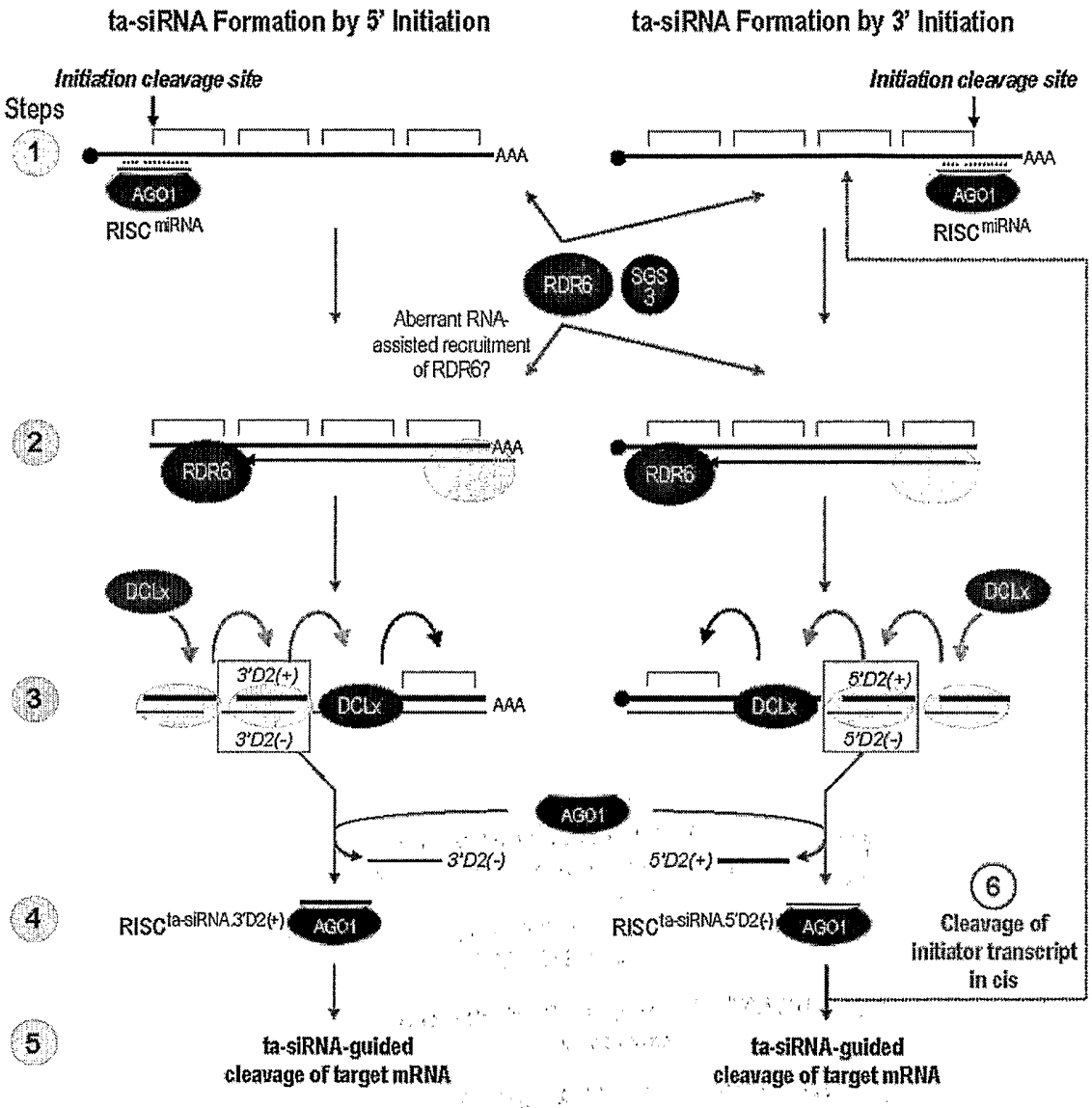

ta-siRNA Formation by 5' Initiation
ta-siRNA Formation by 3' Initiation
Initiation cleavage site
Initiation cleavage site
Steps
1
AAA
AAA
AGO1
AGO1
RISC miRNA
RISC miRNA
RDR6
SGS 3
Aberrant RNA-assisted recruitment of RDR6?
2
RDR6
RDR6
AAA
DCLx
DCLx
3
3'D2(+)
3'D2(-)
DCLx
AAA
DCLx
5'D2(+)
5'D2(-)
AGO1
3'D2(-)
5'D2(+)
4
RISC ta-siRNA 3'D2(+)
AGO1
RISC ta-siRNA 5'D2(-)
AGO1
6
Cleavage of initiator transcript in cis
5
ta-siRNA-guided cleavage of target mRNA
ta-siRNA-guided cleavage of target mRNA

FIGURE 8A

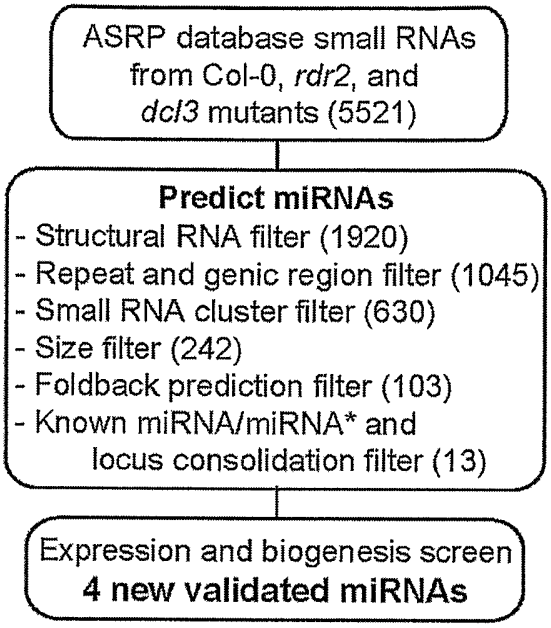


FIGURE 8B

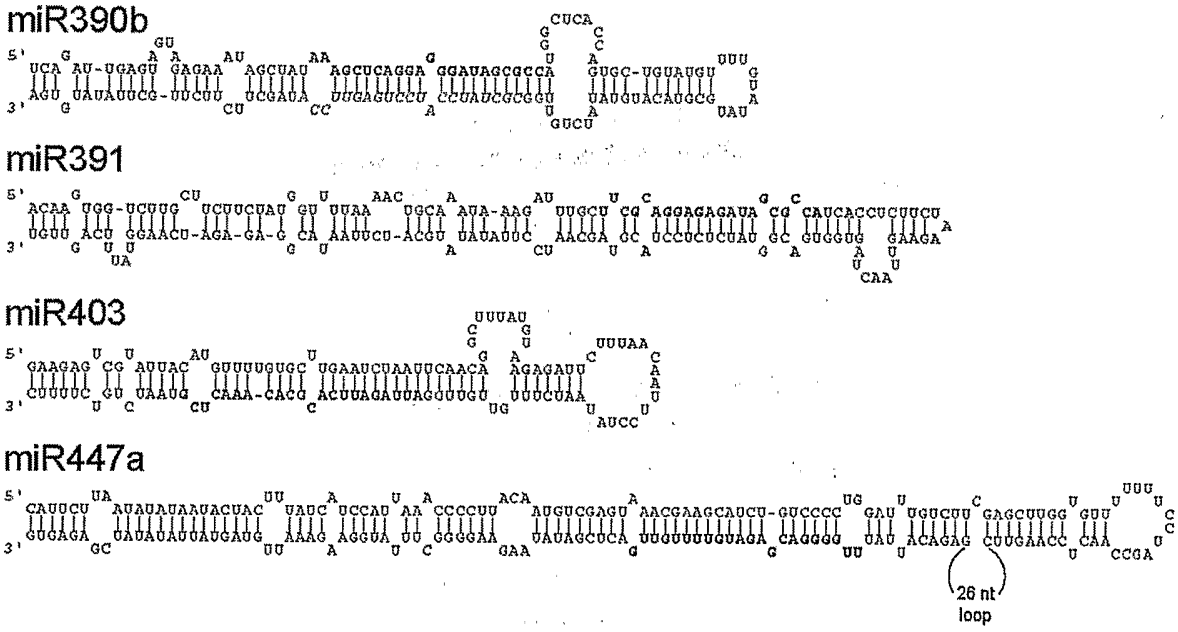


FIGURE 8C

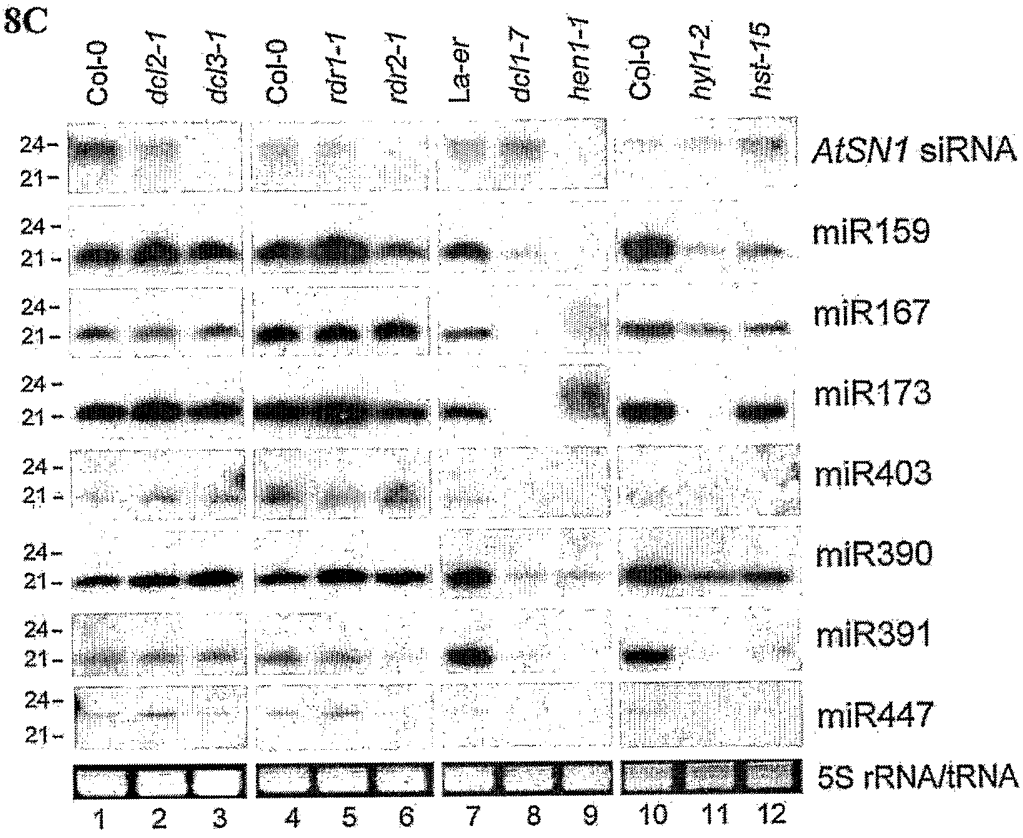


FIGURE 8D

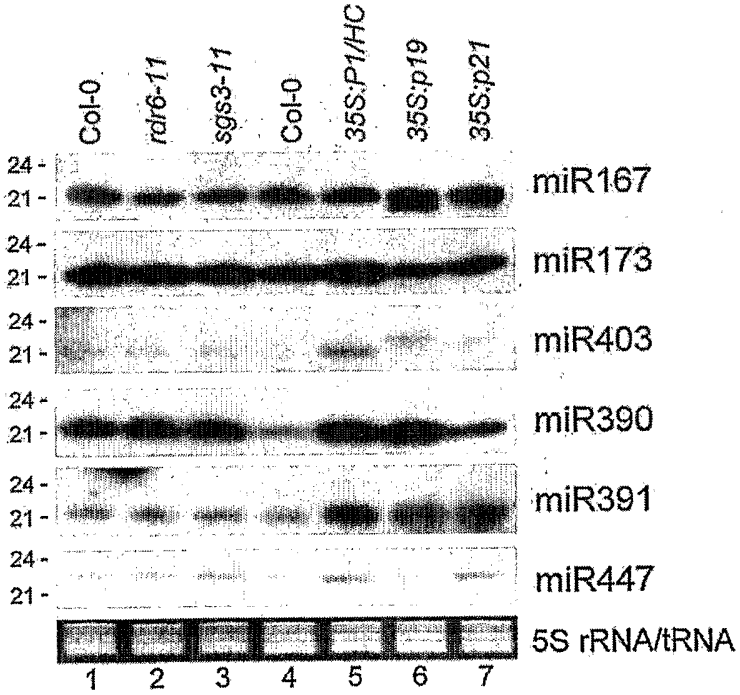


FIGURE 9A

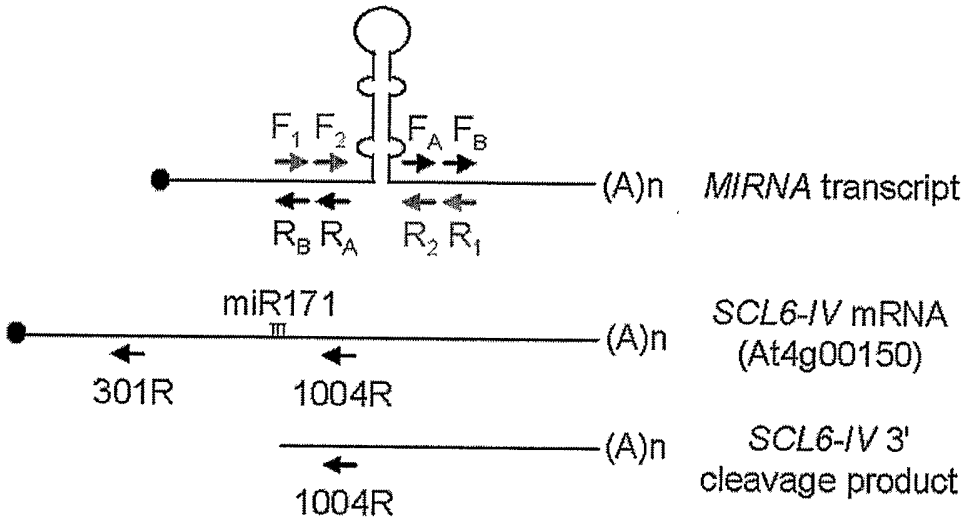


FIGURE 9B

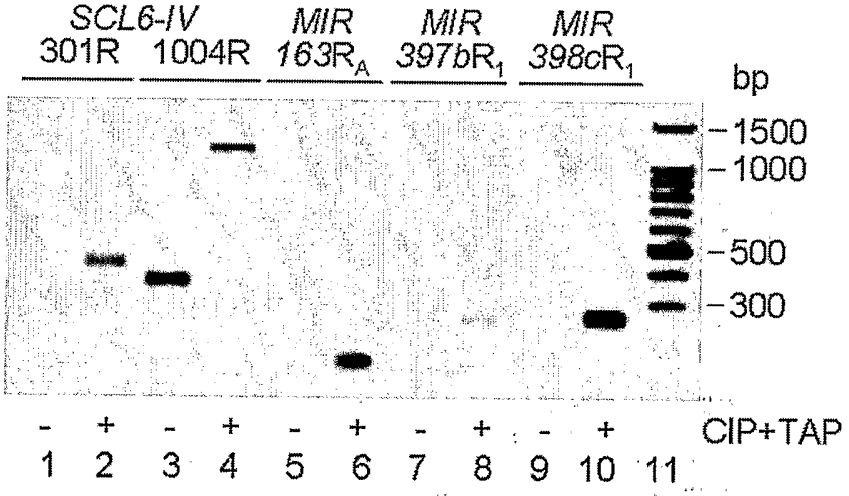


FIGURE 10A

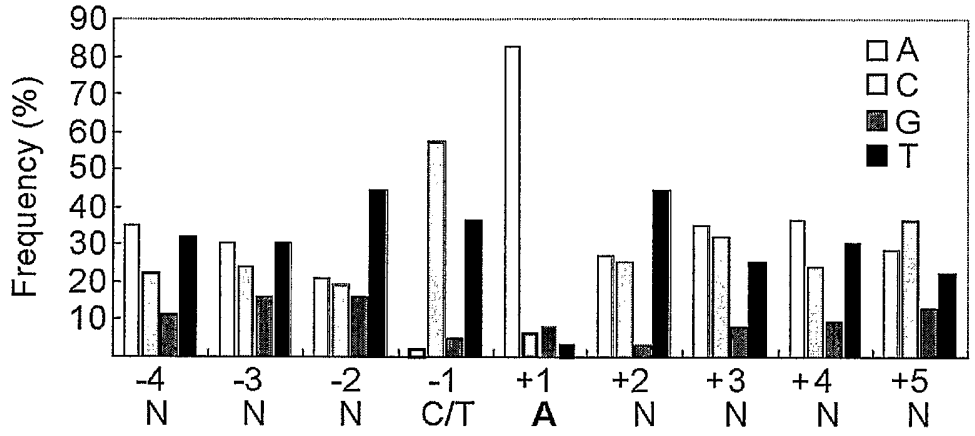


FIGURE 10C

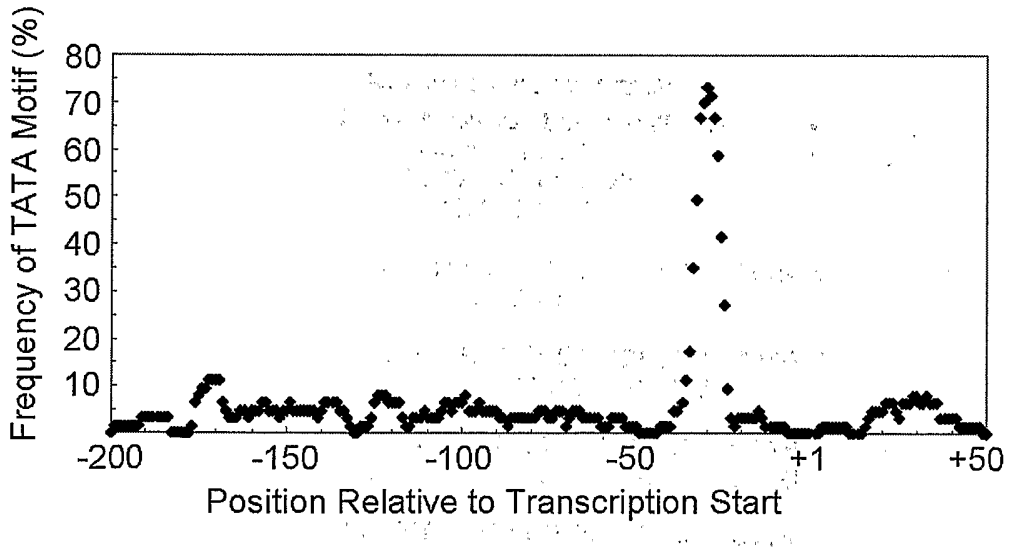


Figure 10B

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MIR156a ataaagaaaggtaagactctttgaaatagagagagataaggttttctcttAtcttcttct
MIR156c tcttctcctctcctcttattaatctaatcctcctccccgaatatttctctGcctttagtt
MIR156c ctcttctccttcggtTATAAATAttctctccggttttgcttgtttaacctAaaagcctca
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MIR157d tggtctcaaatctttccTATATATAcacaccactttccattacaccatttActcttcacc
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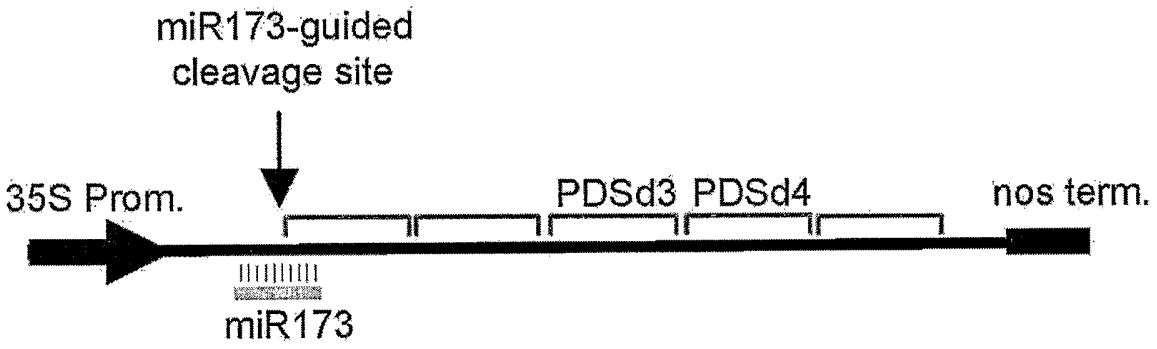
FIGURE 11



35S:TAS1cPDSd3d4

FIGURE 12A

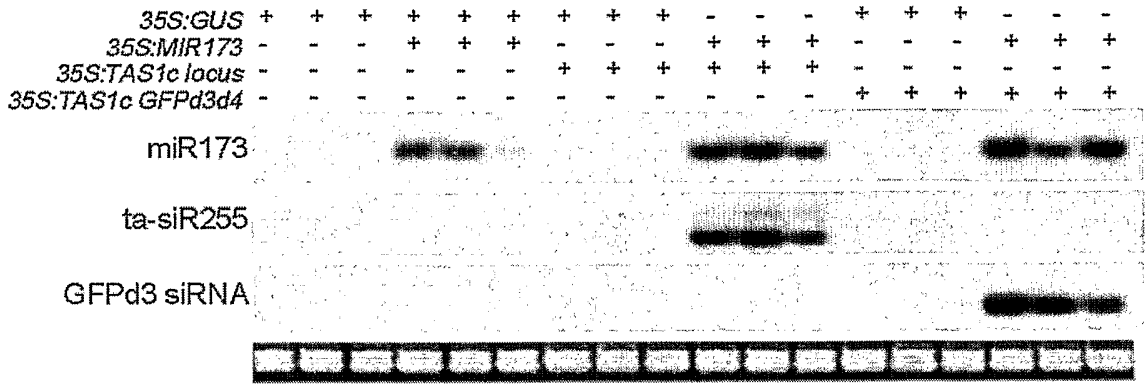


FIGURE 12B

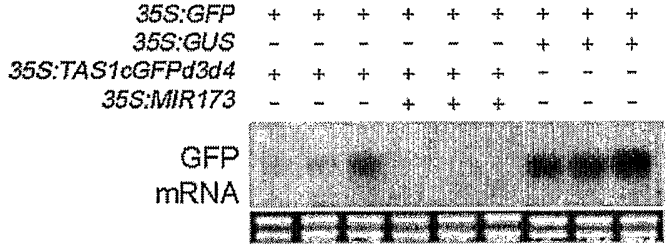


FIGURE 12C

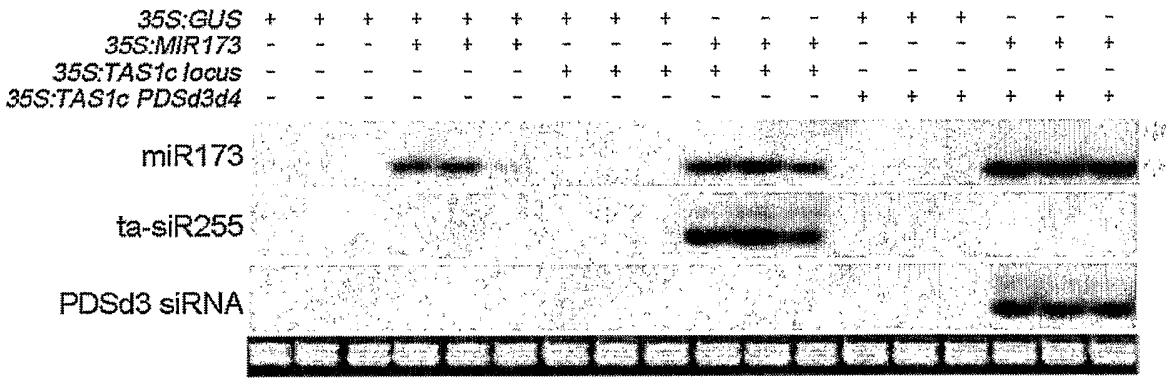


FIGURE 12D

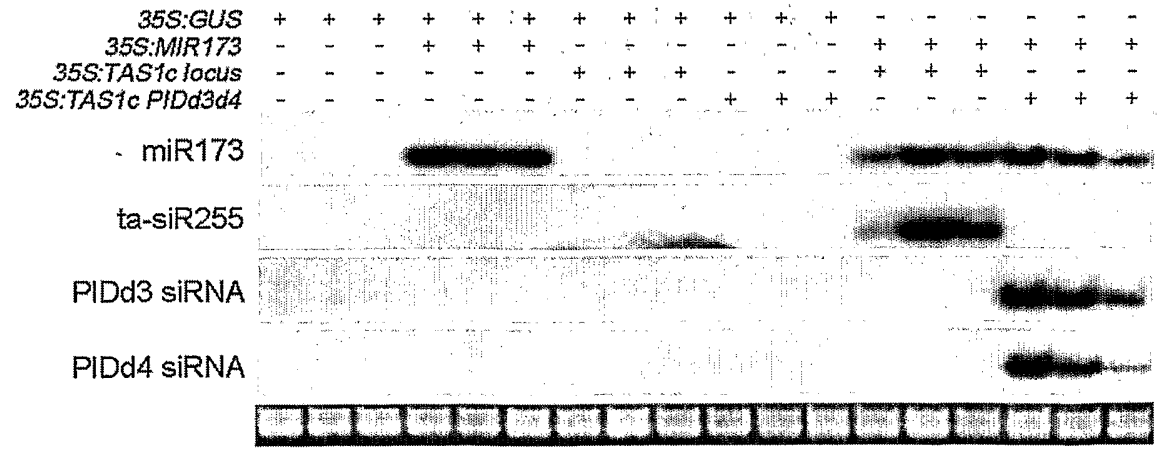
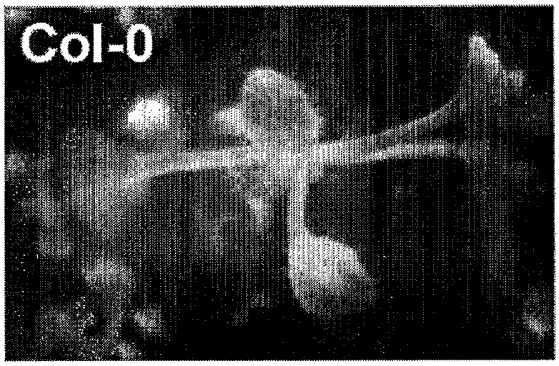


FIGURE 13



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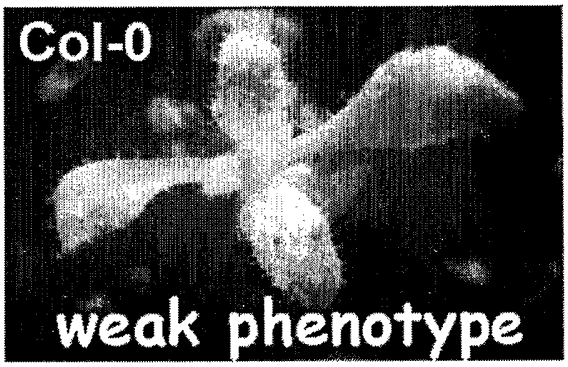

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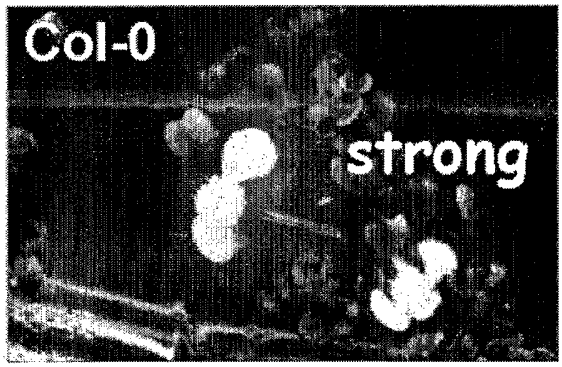

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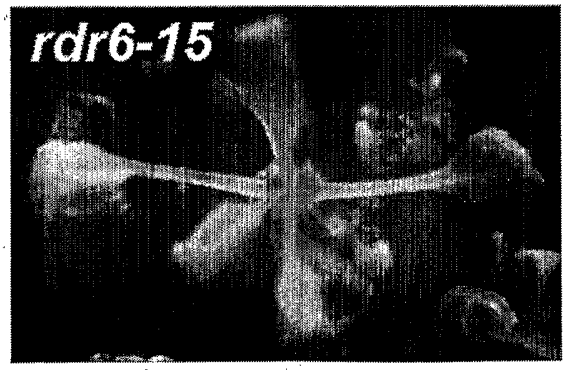

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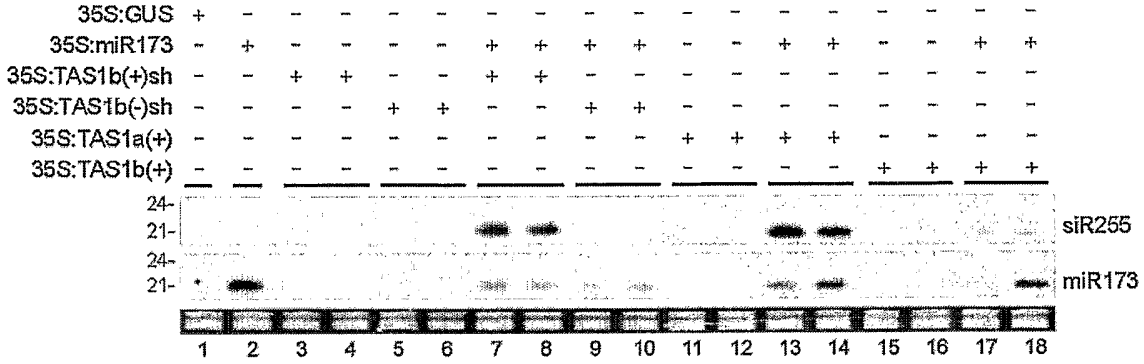

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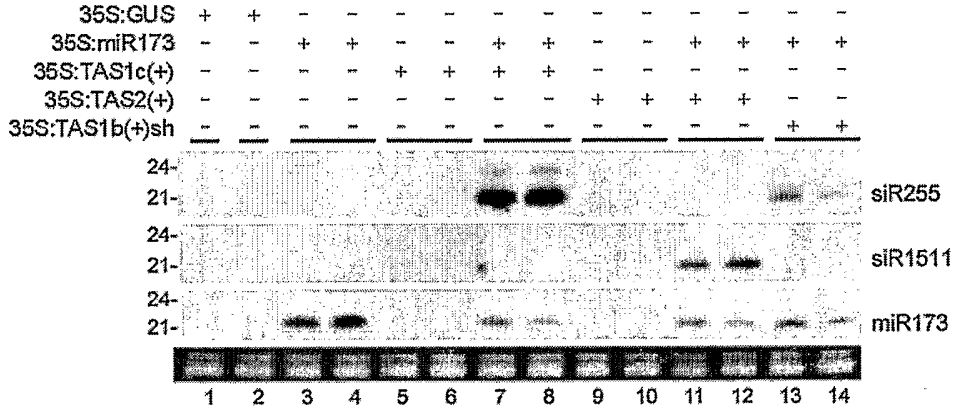

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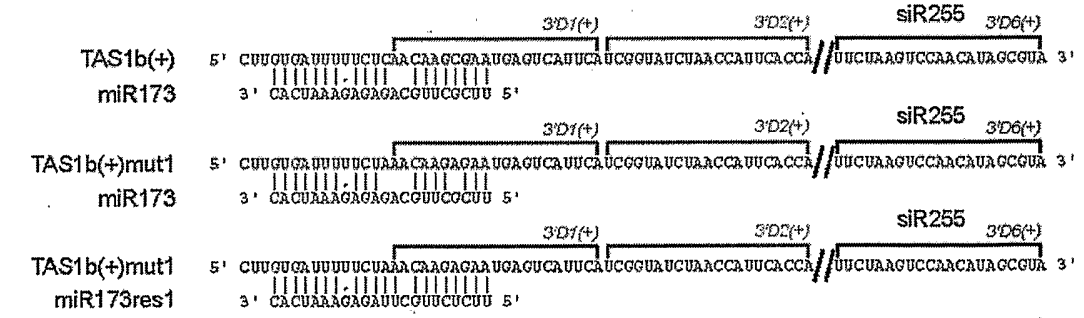

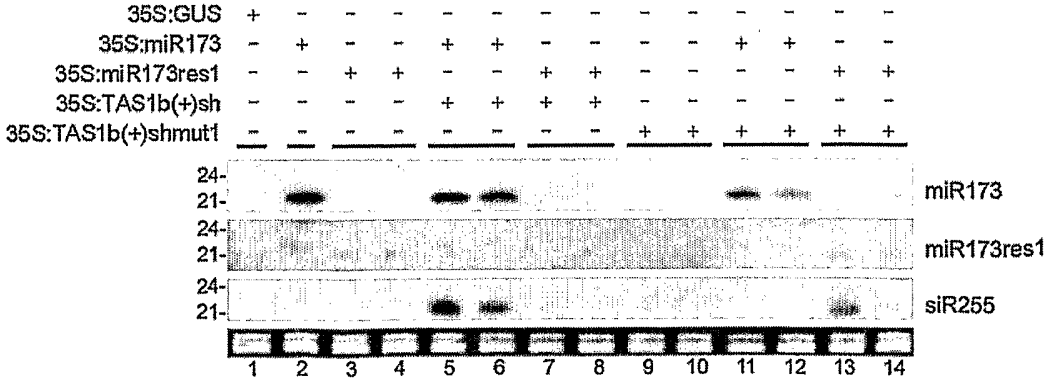

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Allen, Edwards

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uagccaagga ugacuucccu u 21

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uagccaagga ugacuugccu 20

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ugauugagcc gcgccaauau 20

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gcagcaccau uaagauucac 20

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gcagcaccau uaagauucac a 21

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ugauucucug uguaagcgaa a 21

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aagcucagga gggauagcgc 20

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gcucaggagg gauagcgcc 19

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agcucaggag ggauagcgcc a 21

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acgguaucuc uccuacguag c 21

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gguucaauaa agcuguggga ag 22

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<400> 274
ucauugagug cagcguugau gu 22

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<400> 275
ccugccaaag gagaguugcc c 21

<210> 276
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<220>
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<400> 276
uucgaugucu agcagugcca 20

<210> 277
<211> 1185
<212> DNA
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<220>
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ttgtacaaaa aagcaggctc cgcggccgcc cgcttcacca aacctaaacc taaacggcta 120

agcccgacgt caaataccaa aaagagaaaa acaagagcgc cgtcaagctc tgcaaatacg 180

atctgtaagt ccatcttaac acaaaagtga gatgggttct tagatcatgt tccgccgtta 240

gatcgagtca tggtcttgtc tcatagaaag gtactttcgt ttacttcttt tgagtatcga 300

gtagagcgtc gtctatagtt agtttgagat tgcgtttgtc agaagttagg ttcaatgtcc 360

cggtccaatt ttcaccagcc atgtgtcagt ttcgttcctt cccgtcctct tctttgattt 420

cgttgggtta cggatgtttt cgagatgaaa cagcattgtt ttgttgtgat ttttctctac 480

aagcgaatag accatttatc ggtggatctt agaaaattat gatgtatacg ttgtgggagt 540

ttatttgtat agttcatcca tgaactagaa aagacattgg acatattcca ggatatgcaa 600

aagaaaacaa tgaatattgt tttgaatgtg ttcaagtaaa tgagattttc aagtcgtcta 660

aagaacagtt gctaatacag ttacttattt caataaataa ttggttctaa taatacaaaa 720

catattcgag gatatgcaga aaaaaagatg tttgttattt tgaaaagctt gagtagtttc 780

tctccgaggt gtagcgaaga agcatcatct actttgtaat gtaattttct ttatgttttc 840

actttgtaat tttatttgtg ttaatgtacc atggccgata tcggttttat tgaaagaaaa 900

tttatgttac ttctgttttg gctttgcaat cagttatgct agttttctta tacccttttcg 960

taagcttcct aaggaatcgt tcattgattt ccactgcttc attgtatatt aaaactttac 1020

aactgtatcg accatcatat aattctgggt caagagatga aaatagaaca ccacatcgta 1080

aagtgaaata agggtgggcg cgccgaccca gctttcttgt acaaagtggt tcgataattc 1140

cttaattaac tagttctaga gcggccgccc accgcggtgg agctc 1185

<210> 278
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<212> DNA
<213> artificial sequence

<220>
<223> construct with artificial ta-siRNAs targeting Arabidopsis gene encoding PDS

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ttgtacaaaa aagcaggctc cgcggccgcc cgcttcacca aacctaaacc taaacggcta 120

agcccgacgt caaataccaa aaagagaaaa acaagagcgc cgtcaagctc tgcaaatacg 180

atctgtaagt ccatcttaac acaaaagtga gatgggttct tagatcatgt tccgccgtta 240

gatcgagtca tggtcttgtc tcatagaaag gtactttcgt ttacttcttt tgagtatcga 300

gtagagcgtc gtctatagtt agtttgagat tgcgtttgtc agaagttagg ttcaatgtcc 360

cggtccaatt ttcaccagcc atgtgtcagt ttcgttcctt cccgtcctct tctttgattt 420

cgttgggtta cggatgtttt cgagatgaaa cagcattgtt ttgttgtgat ttttctctac 480

aagcgaatag accatttatc ggtggatctt agaaaattat aagtagcact tccccaccta 540

ttagctttgc tttggtcagc tgaactagaa aagacattgg acatattcca ggatatgcaa 600

aagaaaacaa tgaatattgt tttgaatgtg ttcaagtaaa tgagattttc aagtcgtcta 660

aagaacagtt gctaatacag ttacttattt caataaataa ttggttctaa taatacaaaa 720

catattcgag gatatgcaga aaaaaagatg tttgttattt tgaaaagctt gagtagtttc 780

tctccgaggt gtagcgaaga agcatcatct actttgtaat gtaattttct ttatgttttc 840

actttgtaat tttatttgtg ttaatgtacc atggccgata tcggttttat tgaaagaaaa 900

tttatgttac ttctgttttg gctttgcaat cagttatgct agttttctta tacccttttcg 960

taagcttcct aaggaatcgt tcattgattt ccactgcttc attgtatatt aaaactttac 1020

aactgtatcg accatcatat aattctgggt caagagatga aaatagaaca ccacatcgta 1080

aagtgaaata agggtgggcg cgccgaccca gctttcttgt acaaagtggt tcgataattc 1140

cttaattaac tagttctaga gcggccgccc accgcggtgg agctc 1185

<210> 279
<211> 1185
<212> DNA
<213> artificial sequence

<220>
<223> construct with artificial ta-siRNAs targeting Arabidopsis gene encoding PID

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ttgtacaaaa aagcaggctc cgcggccgcc cgcttcacca aacctaaacc taaacggcta 120

agcccgacgt caaataccaa aaagagaaaa acaagagcgc cgtcaagctc tgcaaatacg 180

atctgtaagt ccatcttaac acaaaagtga gatgggttct tagatcatgt tccgccgtta 240

gatcgagtca tggtcttgtc tcatagaaag gtactttcgt ttacttcttt tgagtatcga 300

gtagagcgtc gtctatagtt agtttgagat tgcgtttgtc agaagttagg ttcaatgtcc 360

cggtccaatt ttcaccagcc atgtgtcagt ttcgttcctt cccgtcctct tctttgattt 420

cgttgggtta cggatgtttt cgagatgaaa cagcattgtt ttgttgtgat ttttctctac 480

aagcgaatag accatttatc ggtggatctt agaaaattat caaaagtaat cgaacgccgc 540

tcattagtcg gcgcaacgaa cgaactagaa aagacattgg acatattcca ggatatgcaa 600

aagaaaacaa tgaatattgt tttgaatgtg ttcaagtaaa tgagattttc aagtcgtcta 660

aagaacagtt gctaatacag ttacttattt caataaataa ttggttctaa taatacaaaa 720

catattcgag gatatgcaga aaaaaagatg tttgttattt tgaaaagctt gagtagtttc 780

tctccgaggt gtagcgaaga agcatcatct actttgtaat gtaattttct ttatgttttc 840

actttgtaat tttatttgtg ttaatgtacc atggccgata tcggttttat tgaaagaaaa 900

tttatgttac ttctgttttg gctttgcaat cagttatgct agttttctta tacccttttcg 960

taagcttcct aaggaatcgt tcattgattt ccactgcttc attgtatatt aaaactttac 1020

aactgtatcg accatcatat aattctgggt caagagatga aaatagaaca ccacatcgta 1080

aagtgaaata agggtgggcg cgccgaccca gctttcttgt acaaagtggt tcgataattc 1140

cttaattaac tagttctaga gcggccgccc accgcggtgg agctc 1185

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<220>
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<400> 280
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<210> 281
<211> 21
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<220>
<223> Synthetic oligonucleotide

<400> 281
ccaagggaag aggcagugca u 21

<210> 282
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<220>
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<400> 282
accagugaag aggcugugca g 21

<210> 283
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<220>
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<400> 283
gccagggaag aggcagugca u 21

<210> 284
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<220>
<223> Synthetic oligonucleotide

<400> 284
gccggugaag aggcugugca a 21

<210> 285
<211> 21
<212> RNA
<213> artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 285
gccggugaag aggcugugca g 21

<210> 286
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<212> DNA
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<400> 286
aaacgggttc ctaatcttta tatatacctt ccatcatcaa aagaccattc attgttcact 60

<210> 287
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 287
ataaagaaag gtaagactct ttgaaataga gagagataag gttttctctt atcttcttct 60

<210> 288
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 288
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<210> 289
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 289
ctcttctcct tcggttataa atattctctc cggttttgct tgtttaacct aaaagcctca 60

<210> 290
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<211> 60
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<210> 292
<211> 60
<212> DNA
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<210> 293
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<212> DNA
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<400> 293
tggtctcaaa tctttcctat atatacacac cactttccat tacaccattt actcttcacc 60

<210> 294
<211> 60
<212> DNA
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<400> 294
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<210> 295
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 295
acgaatatct aaaccactat aattacgact ctctatctat catttcttcc aaaacatgac 60

<210> 296
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<212> DNA
<213> Arabidopsis thaliana

<400> 296
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<210> 297
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 297

gttctaaatt tgaagcttat aaaaacccat cactactttt gcatacttgt atccgcagtg 60

<210> 298
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 298
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<210> 299
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<400> 299
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<210> 300
<211> 60
<212> DNA
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<210> 301
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 301
tttatcattt ttctcctcta tatatatgtg ccaccattcc tcttatactc ataactctcc 60

<210> 302
<211> 60
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<210> 303
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<212> DNA
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<400> 304
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<210> 305
<211> 60
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<213> Arabidopsis thaliana

<400> 305
tttagataca ttttagttat aaatatgaat caaatacatt ttagttctag aagaaaaaac 60

<210> 306
<211> 60
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<210> 307
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 307
catcaaagga aaattagtat aaataagcat agaggcgtcc atggattatc acagttctca 60

<210> 308
<211> 60
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ctagcaacct agcacttata tatgtagagt ttgtgaaatt tagggcagac aagcccccac 60

<210> 309
<211> 60
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<210> 310
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<210> 311
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<210> 313
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<210> 315
<211> 60
<212> DNA
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<210> 316
<211> 60
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<210> 317
<211> 60
<212> DNA
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tctcctcctt atcccttcta tataaacact cgtcttcttc ttcacttgat gaacagaaaa 60

<210> 318
<211> 60
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<400> 318
caaatcctct ctcatctctt tctataagta tctatagcgc ctcttaaacc acaaagcatc 60

<210> 319
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<210> 320
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<210> 326
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<210> 327
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acatctctta ctgtgcatat atatttagac aaacacatct ctctctctct atctctctca 60

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<210> 332
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<212> DNA
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<210> 333
<211> 60
<212> DNA
<213> Arabidopsis thaliana

<400> 333
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<210> 334
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<210> 335
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<210> 336
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<210> 337
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<212> DNA
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<210> 338
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<210> 340
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<210> 344
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<210> 345

<211> 60
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<210> 346
<211> 60
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<210> 347
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ccacttttat taaaactcat atatatacac tgagccatta gtccatgaat aaccaaccag 60

<210> 348
<211> 60
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<213> Arabidopsis thaliana

<400> 348
aaacactttc atcaactata tatacatact ttgctagtcc aacttccaat aactcaaaat 60

<210> 349
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic oligonucleotide

<400> 349
gctcctccca atcacccaac ccaat 25

<210> 350
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<220>
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<400> 350
ggagcggcgt tgtcgcggag aacaa 25

<210> 351
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gcgtttctct tgtcccaact ctttca 26

<210> 352
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