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(54) Titre : MOLECULES D'ARN POUR MODULER LA FLORAISON DANS DES PLANTES
 (54) Title: RNA MOLECULES FOR MODULATING FLOWERING IN PLANTS

(57) **Abrégé/Abstract:**

The present invention relates to new double stranded RNA (dsRNA) structures and their use in modulating flowering in plants. The present invention also relates to methods of modulating the time of plant flowering.

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(54) Title: RNA MOLECULES FOR MODULATING FLOWERING IN PLANTS

(57) Abstract: The present invention relates to new double stranded RNA (dsRNA) structures and their use in modulating flowering in plants. The present invention also relates to methods of modulating the time of plant flowering.

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RNA MOLECULES FOR MODULATING FLOWERING IN PLANTS

FIELD OF THE INVENTION

The present invention relates to new double stranded RNA (dsRNA) structures
5 and their use in modulating flowering in plants. The present invention also relates to
methods of modulating the time of plant flowering.

BACKGROUND OF THE INVENTION

RNA silencing is an evolutionarily conserved gene silencing mechanism in
10 eukaryotes that is induced by double-stranded RNA (dsRNA) which may be of a form
designated hairpin structured RNA (hpRNA). In the basic RNA silencing pathway,
dsRNA is processed by Dicer proteins into short, 20-25 nucleotide (nt) small RNA
duplexes, of which one strand is bound to Argonaute (AGO) proteins to form an RNA-
induced silencing complex (RISC). This silencing complex uses the small RNA as a
15 guide to find and bind to complementary single-stranded RNA, where the AGO protein
cleaves the RNA resulting in its degradation.

In plants, multiple RNA silencing pathways exist, including microRNA
(miRNA), trans-acting small interfering RNA (tasiRNA), repeat-associated siRNA
(rasiRNA) and exogenic (virus and transgene) siRNA (exosiRNA) pathways. miRNAs
20 are 20-24 nt small RNAs processed in the nucleus by Dicer-like 1 (DCL1) from short
stem-loop precursor RNAs that are transcribed by RNA polymerase II from MIR genes.
tasiRNAs are phased siRNAs of primarily 21 nt in size derived from DCL4 processing
of long dsRNA synthesized by RNA-dependent RNA polymerase 6 (RDR6) from
miRNA-cleaved TAS RNA fragment. The 24-nt rasiRNAs are processed by DCL3, and
25 the precursor dsRNA is generated by the combined function of plant-specific DNA-
dependent RNA polymerase IV (PolIV) and RDR2 from repetitive DNA in the
genome. The exosiRNA pathway overlaps with the tasiRNA and rasiRNA pathways
and both DCL4 and DCL3 are involved in exosiRNA processing. In addition to DCL1,
DCL3 and DCL4, the model plant *Arabidopsis thaliana* and other higher plants
30 encodes DCL2 or equivalent, which generates 22-nt siRNAs including 22-nt
exosiRNAs, and plays a key role in systemic and transitive gene silencing in plants. All
of these plant small RNAs are methylated at the 2'-hydroxyl group of the 3' terminal
nucleotide by HUA Enhancer 1 (HEN1), and this 3' terminal 2'-O-methylation is
thought to stabilize the small RNAs in plant cells. miRNAs, tasiRNAs and exosiRNAs
35 are functionally similar to small RNAs in animal cells which are involved in
posttranscriptional gene silencing or sequence-specific degradation of RNA in animals.

The rasiRNAs, however, are unique to plants and function to direct *de novo* cytosine methylation at the cognate DNA, a transcriptional gene silencing mechanism known as RNA-directed DNA methylation (RdDM).

RNA silencing induced by dsRNA has been extensively exploited to reduce gene activity in various eukaryotic systems, and a number of gene silencing technologies has been developed. Different organisms are often amenable to different gene silencing approaches. For instance, long dsRNA (at least 100 basepairs in length) is less suited to inducing RNA silencing in mammalian cells due to dsRNA-induced interferon responses, and so shorter dsRNAs (less than 30 basepairs) are generally used in mammalian cells, whereas in plants hairpin RNA (hpRNA) with a long dsRNA stem is highly effective. In plants, the different RNA silencing pathways have led to different gene silencing technologies, such as artificial miRNA, artificial tasiRNA and virus-induced gene silencing technologies. However, successful applications of RNA silencing in plants has so far been achieved primarily by using long hpRNA transgenes. A hpRNA transgene construct typically consists of an inverted repeat made up of fully complementary sense and antisense sequences of a target gene sequence (which when transcribed form the dsRNA stem of hpRNA) separated by a spacer sequence (forming the loop of hpRNA), which is inserted between a promoter and a transcription terminator for expression in plant cells. The spacer sequence functions to stabilize the inverted-repeat DNA in bacteria during construct preparation. The dsRNA stem of the resulting hpRNA transcript is processed by DCL proteins into siRNAs that direct target gene silencing. hpRNA transgenes have been widely used to knock down gene expression, modify metabolic pathways and enhance disease and pest resistance in plants for crop improvement, and many successful applications of the technology in crop improvement have now been reported (Guo et al., 2016; Kim et al., 2019).

Recent studies have suggested, however, that hpRNA transgenes are subject to self-induced transcriptional repression compromising the stability and efficacy of target gene silencing. While all transgenes are potentially subject to position or copy number-dependent transcriptional silencing, hpRNA transgenes are unique as they generate siRNAs that can direct DNA methylation to their own sequence via the RdDM pathway, and this has the potential to cause transcriptional self-silencing.

Whilst dsRNA induced gene silencing has proven to be a valuable tool in altering the phenotype of an organism, there is a need for alternate, preferably improved, dsRNA molecules which can be used for RNAi.

SUMMARY OF THE INVENTION

The inventors conceived of new designs of genetic constructs for producing RNA molecules which include one or more double-stranded RNA regions which
5 comprise multiple non-canonically basepaired nucleotides or non-basepaired nucleotides, or both, including forms which have two or more loop sequences, herein called loop-ended dsRNA (ledRNA). These RNA molecules have one or more of the following features; they are easily synthesized, they accumulate to higher levels in plant cells upon transcription of the genetic constructs encoding them, they more
10 readily form a dsRNA structure and induce efficient silencing of target RNA molecules in plant cells, and they may form circular RNA molecules upon processing in plant cells.

The present inventors have also identified that the activity of genes that regulate flowering time in plants may be modulated by using RNA molecules applied either
15 endogenously, or preferably exogenously to plant cells at an earlier time, for example to seeds that give rise to the plants. The RNA molecules may reduce or abolish the function of one or more genes involved in the timing of flowering, for example a repressor of flowering, and so promote flowering. Thus, the present disclosure also provides a method of influencing the timing of flowering of a plant. This may be used
20 to reduce or suppress activity of a gene with ability to influence a flowering characteristic through reduced expression of the gene by targeting its RNA transcripts. This modulation may be used to promote synchronous flowering of male and female parent lines in hybrid seed production, for example. Another use is to advance or retard flowering according to the variation of weather, or to extend or reduce the growing
25 season. The activity of the plant gene is preferably reduced as a result of under-expression within at least some cells of the plant.

One goal of classical breeding and cultivation of plants is to select varieties with a definite time of flowering. Early flowering varieties make it possible to cultivate important crops in regions in which the plant species would not normally reach
30 complete maturity. Later flowering varieties allow for increased or improved production of vegetative parts such as leaves, stems and tubers. Seed production in a previous generation of a late flowering variety is advantageously promoted by the use of RNA molecules of the invention. The selection of early flowering or late flowering varieties by classical breeding is however a very time-intensive process. The RNA
35 molecules and methods of the present disclosure are advantageous in this context.

In a first aspect, the present invention provides an RNA molecule comprising a first RNA component, a second RNA component which is covalently linked to the first RNA component and, optionally, one or more or all of (i) a linking ribonucleotide sequence which covalently links the first and second RNA components, (ii) a 5' leader sequence and (iii) a 3' trailer sequence,

5 wherein the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair with each other in the first RNA component, wherein the first RNA sequence comprises a first sense ribonucleotide sequence of at least 20

10 contiguous ribonucleotides, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence of at least 20 contiguous ribonucleotides, wherein the first antisense ribonucleotide sequence hybridises with the first sense ribonucleotide sequence in the RNA molecule, wherein the first antisense ribonucleotide sequence is capable of hybridising to a first region of a target RNA molecule which modulates the

15 timing of plant flowering,

wherein the second RNA component is covalently linked, via the linking ribonucleotide sequence if present or directly if the linking ribonucleotide sequence is not present, to the first 5' ribonucleotide or the first 3' ribonucleotide,

wherein the second RNA component consists of, in 5' to 3' order, a second 5'

20 ribonucleotide, a second RNA sequence and a second 3' ribonucleotide, wherein the second 5' and 3' ribonucleotides basepair to each other in the RNA molecule, wherein the second RNA sequence comprises a second sense ribonucleotide sequence, a second loop sequence of at least 4 ribonucleotides and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence hybridises with the second

25 antisense ribonucleotide sequence in the RNA molecule,

wherein the 5' leader sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide, and

30 wherein the 3' trailer sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the second 3' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the first 3' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide.

In a second aspect, the present invention provides an RNA molecule comprising

35 a first RNA component, a second RNA component which is covalently linked to the first RNA component and, optionally, one or more or all of (i) a linking ribonucleotide

sequence which covalently links the first and second RNA components, (ii) a 5' leader sequence and (iii) a 3' trailer sequence,

wherein the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair, wherein the first RNA sequence comprises a first sense ribonucleotide sequence, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence, wherein the first sense ribonucleotide sequence and first antisense ribonucleotide sequence each of at least 20 contiguous ribonucleotides whereby the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence fully basepair with the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence, wherein the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence are identical in sequence to a first region of a target RNA molecule which modulates the timing of plant flowering,

wherein the second RNA component is covalently linked, via the linking ribonucleotide sequence if present, to the first 5' ribonucleotide or the first 3' ribonucleotide,

wherein the second RNA component consists of, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide, wherein the second 5' and 3' ribonucleotides basepair, wherein the second RNA sequence comprises a second sense ribonucleotide sequence, a second loop sequence of at least 4 ribonucleotides and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence basepairs with the second antisense ribonucleotide sequence,

wherein the 5' leader sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide, and

wherein the 3' trailer sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the second 3' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the first 3' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide.

In these aspects, at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence are all capable of basepairing to nucleotides of the first region of the target RNA molecule. In an embodiment, the first sense ribonucleotide sequence is linked covalently to the first 5' ribonucleotide without any intervening nucleotides, or the first antisense ribonucleotide sequence is linked covalently to the first 3' ribonucleotide without any intervening nucleotides, or both. In another embodiment,

the RNA molecule comprises the linking ribonucleotide sequence, wherein the linking ribonucleotide sequence is less than 20 ribonucleotides. In an embodiment, the linking ribonucleotide sequence hybridizes to the target RNA molecule. In an embodiment, the linking ribonucleotide sequence is identical to a portion of the complement of the target RNA molecule. In another embodiment, the linking ribonucleotide sequence is between 1 and 10 ribonucleotides in length. In another embodiment, the RNA molecule comprises two or more sense ribonucleotide sequences, and antisense ribonucleotide sequences fully base paired thereto, which are identical in sequence to a region of a target RNA molecule. In an embodiment, the two or more sense ribonucleotide sequences are identical in sequence to different regions of the same target RNA molecule. In another embodiment, the two or more sense ribonucleotide sequences are identical in sequence to a region of different target RNA molecules. In another embodiment, the two or more sense ribonucleotide sequences have no intervening loop sequences. In an embodiment, the RNA molecule comprises two or more antisense ribonucleotide sequences, and sense ribonucleotide sequences fully base paired thereto, which are each complementary to a region of a target RNA molecule. In an embodiment, the two or more antisense ribonucleotide sequences are complementary to different regions of the same target RNA molecule. In another embodiment, the second of the two or more antisense ribonucleotide sequences are complementary to region of a different target RNA molecule than the first of the two or more antisense ribonucleotide sequences. In another embodiment, the two or more sense ribonucleotide sequences have no intervening loop sequences. In another embodiment, the RNA molecule is a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end. In another embodiment, the RNA molecule is a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end. In another embodiment, the RNA molecule is a single strand of ribonucleotides comprising a 5' end, the first RNA component comprising a first sense ribonucleotide sequence which is at least 21 nucleotides in length, at least one loop sequence, a first antisense ribonucleotide sequence which hybridises with the first sense ribonucleotide sequence over a length of at least 21 contiguous nucleotides, and the second RNA component comprising a

second sense ribonucleotide sequence which is at least 21 nucleotides in length, a loop sequence, a second antisense ribonucleotide sequence which hybridises with the second sense ribonucleotide sequence over a length of at least 21 contiguous nucleotides, and a 3' end, wherein the RNA molecule has only one 5' end and only one 3' end. In an embodiment, the ribonucleotide at the 5' end and the ribonucleotide at the 3' end are adjacent, each base paired and are not directly covalently bonded. In another embodiment, the RNA molecule comprises a first antisense ribonucleotide sequence which hybridizes to a first region of a target RNA, a second antisense ribonucleotide sequence which hybridizes to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one sense ribonucleotide sequence which hybridizes to the target RNA, wherein the two antisense sequences are not contiguous in the RNA molecule. In another embodiment, the RNA molecule comprises a first sense ribonucleotide sequence which is at least 60% identical to a first region of a target RNA, a second sense ribonucleotide sequence which is at least 60% identical to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one antisense ribonucleotide sequence which hybridizes to the target RNA, wherein the two sense sequences are not contiguous in the RNA molecule. In another embodiment, the RNA molecule has the 5' leader sequence. In another embodiment, the RNA molecule has the 3' trailer sequence. In an embodiment, each ribonucleotide is covalently linked to two other nucleotides. In another embodiment, at least one or all of the loop sequences are longer than 20 nucleotides. In an embodiment, the RNA molecules has none, or one, or two or more bulges, or a double-stranded region of the RNA molecule comprises one, or two, or more nucleotides which are not basepaired in the double-stranded region. In another embodiment, the RNA molecule has three, four or more loops. In another embodiment, the RNA molecule only has two loops. In an embodiment, all of the loops are between 4 and 1,000 ribonucleotides, or between 4 and 200 ribonucleotides, in length. In another embodiment, all of the loops are between 4 and 50 ribonucleotides in length. In another embodiment, each loop is between 20 and 30 ribonucleotides in length.

In a preferred embodiment, the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence are all capable of basepairing to nucleotides of the first region of the target RNA molecule. In this context, basepairing may be canonical or non-canonical, for example with at least some G:U basepairs. Independently for each G:U basepair, the G may be in the first region of the target RNA molecule or preferably in the first antisense ribonucleotide sequence. In an embodiment, the at least 20

contiguous ribonucleotides of the first antisense ribonucleotide sequence that are all capable of basepairing to nucleotides of the first region of the target RNA molecule do so by a canonical base pair. Alternatively, not all of the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence basepair to nucleotides of the first region of the target RNA molecule. For example, 1, 2, 3, 4 or 5 of the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence are not basepaired to the first region of the target RNA molecule.

In an embodiment, the first sense ribonucleotide sequence is linked covalently to the first 5' ribonucleotide without any intervening nucleotides, or the first antisense ribonucleotide sequence is linked covalently to the first 3' ribonucleotide without any intervening nucleotides, or both.

In an embodiment, the RNA molecule comprises one or more linking ribonucleotide sequence, wherein the linking ribonucleotide sequence is related in sequence to the target RNA molecule, either identical at least in part to a region of the target RNA molecule or to its complement. In a preferred embodiment, the linking ribonucleotide sequence together with sense sequences in the first and second RNA components form part of one contiguous sense sequence, or together with antisense sequences in the first and second RNA components form part of one contiguous antisense sequence. In an embodiment, the RNA molecule comprises the linking ribonucleotide sequence, wherein the linking ribonucleotide sequence is less than 20 ribonucleotides. In an embodiment, the linking ribonucleotide sequence hybridizes to the target RNA molecule. In an embodiment, the linking ribonucleotide sequence is identical to a portion of the complement of the target RNA molecule. In an embodiment, the linking ribonucleotide sequence is between 1 and 50, or between 1 and 10 ribonucleotides, in length.

In an embodiment, the RNA molecule comprises two or more sense ribonucleotide sequences, and antisense ribonucleotide sequences fully based paired thereto, which are identical in sequence to a region of a target RNA molecule. In an embodiment, the two or more sense ribonucleotide sequences are identical in sequence to different regions of the same target RNA molecule. In an alternate embodiment, the two or more sense ribonucleotide sequences are identical in sequence to a region of different target RNA molecules. In an embodiment, the two or more sense ribonucleotide sequences have no intervening loop sequences, i.e. they are contiguous relative to the target RNA molecule.

In an embodiment, the RNA comprises two or more antisense ribonucleotide sequences, and sense ribonucleotide sequences fully based paired thereto, which are

each complementary to a region of a target RNA molecule. In an embodiment, the two or more antisense ribonucleotide sequences are complementary to different regions of the same target RNA molecule.

In an embodiment, the second of the two or more antisense ribonucleotide
5 sequences are complementary to region of a different target RNA molecule than the first of the two or more antisense ribonucleotide sequences.

In an embodiment, the RNA molecule is a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired
10 with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end.

In an embodiment, the RNA molecule is a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired
15 with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end.

In an embodiment, the RNA molecule is a a single strand of ribonucleotides comprising a 5' end, the first RNA component comprising a first sense ribonucleotide sequence which is at least 21 nucleotides in length, at least one loop sequence, a first
20 antisense ribonucleotide sequence which hybridises with the first sense ribonucleotide sequence over a length of at least 21 contiguous nucleotides, and the second RNA component comprising a second sense ribonucleotide sequence which is at least 21 nucleotides in length, a loop sequence, a second antisense ribonucleotide sequence which hybridises with the second sense ribonucleotide sequence over a length of at
25 least 21 contiguous nucleotides, and a 3' end, wherein the RNA molecule has only one 5' end and only one 3' end.

In an embodiment, the ribonucleotide at the 5' end and the ribonucleotide at the 3' end are adjacent, each base paired and are not directly covalently bonded.

In an embodiment, the RNA molecule comprises a first antisense ribonucleotide
30 sequence which hybridizes to a first region of a target RNA, a second antisense ribonucleotide sequence which hybridizes to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one sense ribonucleotide sequence which hybridizes to the target RNA, wherein the two antisense sequences are not contiguous
35 in the RNA molecule.

In an embodiment, the RNA molecule comprises a first sense ribonucleotide sequence which is at least 60% identical to a first region of a target RNA, a second sense ribonucleotide sequence which is at least 60% identical to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one antisense ribonucleotide sequence which hybridizes to the target RNA, wherein the two sense sequences are not contiguous in the RNA molecule.

In an embodiment, the RNA molecule has the 5' leader sequence.

In an embodiment, the RNA molecule has the 3' trailer sequence.

10 In an embodiment, each ribonucleotide is covalently linked to two other nucleotides. Alternatively, the RNA molecule may be represented as a dumbbell shape (Figure 1) but have a gap or nick in one part of the double-stranded structure.

In an embodiment, at least one or all of the loop sequences are longer than 20 nucleotides.

15 In an embodiment, the RNA molecules has none, or one, or two or more bulges, or a double-stranded region of the RNA molecule comprises one, or two, or more nucleotides which are not basepaired in the double-stranded region.

In an embodiment, the RNA molecules has three, four or more loops.

In an embodiment, the RNA molecules has only has two loops.

20 In an embodiment, the target RNA is in a plant cell. Examples of such plants cells include, but are not limited to, those from *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. The plant cell may be from a legume such as alfalfa or clover, a leafy vegetable e.g. lettuce, or a grass e.g. turfgrass.

25 In an embodiment, the RNA molecule is present in a plant cell.

In an embodiment, the RNA molecule of the invention is produced/expressed in a cell, such as for example a bacterial cell or other microbial cell, which is different to the cell comprising the target RNA. In a preferred embodiment, the microbial cell is a cell in which the RNA molecule is produced by transcription from a genetic construct encoding the RNA molecule, wherein the RNA molecule is substantially, or preferably predominantly, not processed in the microbial cell by cleavage within one or more loop sequences, one or more dsRNA regions, or both. For example, the microbial cell is a yeast cell or another fungal cell which does not have a Dicer enzyme. A greatly preferred cell for production of the RNA molecule is a *Saccharomyces cerevisiae* cell.

30

35 The microbial cell may be living, or may have been killed by some treatment such as heat treatment, or may be in the form of a dried powder.

In an embodiment, at least one or all of the loop sequences of the RNA molecule are longer than 20 nucleotides. In a preferred embodiment, at least one of the loops of the RNA molecule is between 4 and 1,200 ribonucleotides in length, or between 4 and 1000 ribonucleotides in length. In a more preferred embodiment, all of the loops are
5 between 4 and 1,000 ribonucleotides in length. In a more preferred embodiment, at least one of the loops of the RNA molecule is between 4 and 200 ribonucleotides in length. In an even more preferred embodiment, all of the loops are between 4 and 200 ribonucleotides in length. In an even more preferred embodiment, at least one of the loops of the RNA molecule is between 4 and 50 ribonucleotides in length. In a most
10 preferred embodiment, all of the loops are between 4 and 50 ribonucleotides in length. In embodiments, the minimum length of the loop is 20 nucleotides, 30 nucleotides, 40 nucleotides, or 50 nucleotides. In an embodiment, each loop of the RNA molecule is independently between 20 and 50 ribonucleotides, or between 20 and 40 ribonucleotides or between 20 and 30 ribonucleotides in length.

15 In an embodiment, the target RNA encodes a protein.

In another embodiment, the RNA molecule may comprise a region of a nucleotide sequence set forth in SEQ ID NO:146, SEQ ID NO:147, or SEQ ID NOs:151-152 (wheat), SEQ ID NOs:154-155 (barley), SEQ ID NOs:156-164 (rice), SEQ ID NOs:165-178 (maize), SEQ ID NOs:179-185 (*Brassica napus*), SEQ ID
20 NOs:186-187 and SEQ ID NO:210 (*Medicago truncatula*), SEQ ID NOs:188-190 (alfalfa), SEQ ID NOs:191-204 (soybean), SEQ ID NOs:205-207 (sugarbeet), SEQ ID NOs:208-209 (*Brassica rapa*), SEQ ID NOs:211-220 (onion) and SEQ ID NOs:221-228 (lettuce), or a complement (antisense) of a region of the sequence, or both the region and the complement, or a nucleotide sequence 95% identical thereto. In an
25 embodiment, the RNA molecule of the invention comprises a sense and an antisense sequence from a region of an RNA transcript from a gene whose cDNA corresponds to one of the SEQ ID NOs listed above, or a nucleotide sequence 95% or preferably 99% identical thereto. Such sequence is preferably derived from the RNA transcript of a naturally occurring homolog of the gene in that plant species. In another embodiment,
30 RNA molecules of the invention may comprise a a region of a nucleotide sequence set forth in SEQ ID NO:146, SEQ ID NO:147 or SEQ ID NOs:151-228.

In an embodiment of the aspects, the second RNA component is characterised in that:

i) the second sense ribonucleotide sequence consists of at least 20 contiguous
35 ribonucleotides covalently linked, in 5' to 3' order, the second 5' ribonucleotide, a third RNA sequence and a third 3' ribonucleotide,

ii) the second antisense ribonucleotide sequence consists of at least 20 contiguous ribonucleotides covalently linked, in 5' to 3' order, a third 5' ribonucleotide, a fourth RNA sequence and the second 3' ribonucleotide,

iii) the second 5' ribonucleotide basepairs with the second 3' ribonucleotide,

5 iv) the third 3' ribonucleotide basepairs with the third 5' ribonucleotide,

wherein the chimeric RNA molecule is capable of being processed in a plant cell or *in vitro* whereby the second antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length. Most preferably, the asRNA molecules produced from the second antisense sequence are
10 capable of reducing expression of the target RNA, either without or in combination with asRNAs produced from the first antisense sequence of the first RNA component. It is more preferred that between 5% and 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, and/or the
15 second sense ribonucleotide sequence and the second antisense ribonucleotide sequence, and/or every sense ribonucleotide sequence and its corresponding antisense ribonucleotide sequence which hybridise, in total, are either basepaired in a non-canonical basepair or are not basepaired, and/or the dsRNA region formed between the complementary sense and antisense sequences does not comprise 20 contiguous canonical basepairs. More preferably, about 12%, about 15%, about 18%, about 21%,
20 about 24%, about 27%, about 30%, between 10% and 30%, or between 15% and 30%, or even more preferably between 16% and 25%, of the ribonucleotides of a sense ribonucleotide sequence and its corresponding antisense ribonucleotide sequence, preferably for every dsRNA region in the RNA molecule, in total, are either basepaired in a non-canonical basepair or are not basepaired. Even more preferably, about 12%,
25 about 15%, about 18%, about 21%, about 24%, about 27%, about 30%, between 10% and 30%, or between 15% and 30%, or even more preferably between 16% and 25%, of the ribonucleotides of the dsRNA region(s) in the RNA molecule, in total, are basepaired in non-canonical basepairs and all of the other ribonucleotides of the dsRNA region(s) in the RNA molecule are basepaired in canonical basepairs. In
30 preferred embodiments, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or 100% of the non-canonical basepairs in the first or second dsRNA region, or all dsRNA regions in total, are G:U basepairs. Most preferably, in these embodiments,

(a) the chimeric RNA molecule or at least some of the asRNA molecules, or
35 both, are capable of reducing the expression or activity of a target RNA molecule which modulates the timing of plant flowering, or

(b) the first and second antisense ribonucleotide sequences, preferably every antisense ribonucleotide sequence in the RNA molecule, comprises a sequence of at least 20 contiguous ribonucleotides which is at least 50% identical in sequence to a region of the complement of the target RNA molecule, preferably at least 60% identical, more preferably at least 70% identical, even more preferably at least 80% identical, most preferably at least 90% identical or 100% identical to the region of the complement of the target RNA molecule, or both (a) and (b).

In a third aspect, the present invention provides a chimeric ribonucleic acid (RNA) molecule, comprising a double-stranded RNA (dsRNA) region which comprises a first sense ribonucleotide sequence of at least 20 contiguous nucleotides in length and a first antisense ribonucleotide sequence of at least 20 contiguous nucleotides in length, whereby the first sense ribonucleotide sequence and the first antisense ribonucleotide sequences are capable of hybridising to each other to form the dsRNA region, wherein

i) the first sense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide,

ii) the first antisense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide,

iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide to form a terminal basepair of the dsRNA region,

iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide to form a terminal basepair of the dsRNA region,

v) between about 5% and about 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired,

vi) the dsRNA region does not comprise 20 contiguous canonical basepairs,

vii) the RNA molecule is capable of being processed in a plant cell or *in vitro* whereby the first antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length,

viii) the RNA molecule or at least some of the asRNA molecules, or both, are capable of reducing the expression or activity of a target RNA molecule which modulates the timing of plant flowering, and

ix) the RNA molecule is capable of being made enzymatically by transcription *in vitro* or in a cell, or both.

In an embodiment, the first sense ribonucleotide sequence is covalently linked to the first antisense ribonucleotide sequence by a first linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides, or between 4 and 1,000 ribonucleotides, or between 4 and 200 ribonucleotides, or between 4 and 50 ribonucleotides, or at least 10 nucleotides, or between 10 and 1,000 ribonucleotides, or between 10 and 200 ribonucleotides, or between 10 and 50 ribonucleotides, in length, whereby the first linking ribonucleotide sequence is covalently linked to either the second 3' ribonucleotide and the first 5' ribonucleotide or, preferably, to the first 3' ribonucleotide and the second 5' ribonucleotide, so that the sequences are comprised in a single, contiguous strand of RNA. In another embodiment, the first linking ribonucleotide sequence is covalently linked to either the second 3' ribonucleotide and the first 5' ribonucleotide or, preferably, to the first 3' ribonucleotide and the second 5' ribonucleotide, so that the sequences are comprised in a single, contiguous strand of RNA.

In an embodiment, the loop sequence in the chimeric RNA molecule comprises one or more binding sequences which are complementary to an RNA molecule which is endogenous to the plant cell, and/or the loop sequence in the RNA molecule comprises an open reading frame which encodes a polypeptide or a functional polynucleotide.

In its simplest form, such a chimeric RNA molecule is referred to as a hairpin RNA (hpRNA). In a more preferred embodiment, between about 5% and about 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence of the dsRNA, in total, are basepaired in non-canonical basepairs, preferably G:U basepairs. That is, all of the ribonucleotides of the first sense ribonucleotide sequence are basepaired to ribonucleotides of the first antisense ribonucleotide sequence, either in canonical basepairs or non-canonical basepairs, whereby the dsRNA region comprises 20 contiguous basepairs including some non-canonical basepairs. The dsRNA region thereby does not comprise 20 contiguous canonical basepairs. In a more preferred embodiment of the hpRNA of the invention, the first antisense ribonucleotide sequence is fully complementary to a region of the target RNA. In this embodiment, the first sense ribonucleotide sequence is different in sequence to the region of the target RNA by the substitution of C nucleotides in the region of the target RNA with U nucleotides in the hpRNA. Such molecules are exemplified in the hairpin RNAs comprising G:U basepairs in Examples 6-11. In preferred embodiments, the length of the first antisense ribonucleotide sequence is 20 to about 1000 nucleotides, or 20 to about 500 nucleotides, or other lengths as described herein. More preferably, the hpRNA is produced in, or introduced into, a plant cell. In

this embodiments, the target RNA may be a transcript of an endogenous gene in the plant cell.

In an embodiment, the first antisense ribonucleotide sequence is fully complementary to a region of the target RNA and the first sense ribonucleotide
5 sequence is different in sequence to the region of the target RNA by the substitution of C nucleotides in the region of the target RNA with U nucleotides.

In a more preferred embodiment, the chimeric RNA molecule comprises a second sense ribonucleotide sequence and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide
10 sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the first 3' ribonucleotide and the second 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and
15 the second sense ribonucleotide sequence, thereby forming an ledRNA structure. In an alternative preferred embodiment, the chimeric RNA molecule comprises a second antisense ribonucleotide sequence and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the
20 first linking ribonucleotide sequence is covalently linked to the second 3' ribonucleotide and the first 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and the second antisense ribonucleotide sequence.

In another preferred embodiment, the chimeric RNA molecule comprises a
25 second sense ribonucleotide sequence and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence and the second antisense ribonucleotide sequences are capable of hybridising to each other to form a second dsRNA region, and the first sense ribonucleotide sequence and the first antisense
30 ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the first 3' ribonucleotide and the second 5' ribonucleotide, and the RNA molecule further, or optionally, comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4
35 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and

the second sense ribonucleotide sequence or which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence.

In an embodiment, the chimeric RNA molecule comprises a second sense ribonucleotide sequence and a second antisense ribonucleotide sequence and the first
5 sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the second 3' ribonucleotide and the first 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises
10 a loop sequence of at least 4 nucleotides in length and which is covalently linked to the first 3' ribonucleotide and the second antisense ribonucleotide sequence, or which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence.

In an embodiment, the chimeric RNA molecule comprises a second sense
15 ribonucleotide sequence and a second antisense ribonucleotide sequence and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the second 3' ribonucleotide and the first 5' ribonucleotide, and the RNA
20 molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the first 3' ribonucleotide and the second antisense ribonucleotide sequence, or which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence.

25 In an embodiment, the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence each comprise at least 20 contiguous nucleotides in length.

In an embodiment, the first and second sense ribonucleotide sequences are covalently linked by an intervening ribonucleotide sequence which is unrelated in
30 sequence to the target RNA molecule, or which is related in sequence to the target RNA molecule, or the first and second sense ribonucleotide sequences are covalently linked without an intervening ribonucleotide sequence.

In an embodiment, the first and second antisense ribonucleotide sequences are covalently linked by an intervening ribonucleotide sequence which is unrelated in
35 sequence to the complement of a target RNA molecule, or which is related in sequence to the complement of a target RNA molecule, or the first and second antisense

ribonucleotide sequences are covalently linked without an intervening ribonucleotide sequence.

In an embodiment, the first and second sense ribonucleotide sequences may form one contiguous sense ribonucleotide region having at least 50% identity in sequence to a target RNA molecule. In another embodiment, the first and second antisense sense ribonucleotide sequences may form one contiguous antisense ribonucleotide region having at least 50% identity in sequence to the complement of a target RNA molecule. In another embodiment, the RNA molecule comprises a first sense ribonucleotide sequence which is at least 60% identical to a first region of a target RNA, a second sense ribonucleotide sequence which is at least 60% identical to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one antisense ribonucleotide sequence which hybridizes to the target RNA, wherein the two sense sequences are not contiguous in the RNA molecule. In an embodiment, the first and second regions of the target RNA are contiguous in the target RNA molecule. Alternatively, they are not contiguous. In preferred embodiments, the first and second sense ribonucleotide sequences are each, independently, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% identical to the respective region of target RNA i.e. the first sense sequence may be at least 70% identical to its target region and the second sequence at least 80% identical to its target sequence, etc.

In an embodiment, between 5% and 40% of the ribonucleotides of the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, preferably basepaired in G:U basepairs, wherein the second dsRNA region does not comprise 20 contiguous canonical basepairs, and wherein the RNA molecule is capable of being processed in a eukaryotic cell or *in vitro* whereby the second antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length.

In an embodiment, each linking ribonucleotide sequence is independently between 4 and about 2000 nucleotides in length, preferably between 4 and about 1200 nucleotides in length, more preferably between 4 and about 200 nucleotides in length and most preferably between 4 and about 50 nucleotides in length.

In an embodiment, the chimeric RNA molecule further comprises a 5' leader sequence or a 3' trailer sequence, or both.

- In a fourth aspect, the present invention provides a chimeric RNA molecule comprising a first RNA component and a second RNA component which is covalently linked to the first RNA component,
- wherein the first RNA component comprises a first double-stranded RNA (dsRNA) region, which comprises a first sense ribonucleotide sequence and a first antisense ribonucleotide sequence which are capable of hybridising to each other to form the first dsRNA region, and a first intervening ribonucleotide sequence of at least 4 nucleotides which covalently links the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence,
- wherein the second RNA component comprises a second sense ribonucleotide sequence, a second antisense ribonucleotide sequence and a second intervening ribonucleotide sequence of at least 4 ribonucleotides which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence hybridises with the second antisense ribonucleotide sequence in the RNA molecule,
- wherein in the first RNA component,
- i) the first sense ribonucleotide sequence consists of at least 20 contiguous ribonucleotides covalently linked, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide,
 - ii) the first antisense ribonucleotide sequence consists of at least 20 contiguous ribonucleotides covalently linked, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide,
 - iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide,
 - iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide,
 - v) between 5% and 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, and
 - vi) the first dsRNA region does not comprise 20 contiguous canonical basepairs, wherein the chimeric RNA molecule is capable of being processed in a plant cell or *in vitro* whereby the first antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length, and wherein
 - (a) the chimeric RNA molecule or at least some of the asRNA molecules, or both, are capable of reducing the expression or activity of a target RNA molecule which modulates plant flowering, or
 - (b) the first antisense ribonucleotide sequence comprises a sequence of at least 20 contiguous ribonucleotides which is at least 50% identical in sequence,

preferably at least 90% or 100% identical in sequence, to a region of the complement of the target RNA molecule, or

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

In an embodiment of the two above aspects, the at least 20 contiguous
5 ribonucleotides of the first antisense ribonucleotide sequence are all capable of basepairing to nucleotides of a first region of the target RNA molecule.

In an embodiment of the two above aspects, the chimeric RNA molecule comprises two or more antisense ribonucleotide sequences, and sense ribonucleotide sequences based paired thereto, which antisense sequences are each complementary,
10 preferably fully complementary, to a region of a target RNA molecule. The regions of the target RNA molecule to which they are complementary may or may not be contiguous in the target RNA molecule. In an embodiment, the two or more antisense ribonucleotide sequences are complementary to different regions of the same target RNA molecule. In an alternate embodiment, the two or more antisense ribonucleotide
15 sequences are complementary to regions of different target RNA molecules.

In an embodiment, the two or more antisense ribonucleotide sequences have no intervening loop sequences, i.e. they are contiguous relative to the complement of the target RNA molecule. In a preferred embodiment, one or both of the two or more antisense ribonucleotide sequences and sense ribonucleotide sequences basepair along
20 their full length through canonical basepairs, or through some canonical and some non-canonical basepairs, preferably G:U basepairs.

The RNA molecule may comprise a 5'-leader sequence and/or a 3'-trailer sequence.

In an embodiment of the two above aspects, the chimeric RNA molecule
25 comprises a hairpin RNA (hpRNA) structure having a 5' end, a sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired with the sense ribonucleotide sequence over at least 21 contiguous nucleotides, an intervening loop sequence and a 3' end.

The RNA molecule may comprise a 5'-leader sequence and/or a 3'-trailer
30 sequence.

In an embodiment of the two above aspects, the chimeric RNA molecule comprises a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired with each sense ribonucleotide
35 sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end.

The order 5' to 3' may be the sense ribonucleotide sequence and then the antisense ribonucleotide sequence, or *vice versa*. In an embodiment, the ribonucleotide at the 5' end and the ribonucleotide at the 3' end are adjacent, each base paired and are not directly covalently bonded, see for example Figure 1.

5 In an embodiment of the two above aspects, between about 15% and about 30%, or between about 16% and about 25%, of the ribonucleotides of the sense ribonucleotide sequence and the antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, preferably basepaired in non-canonical basepairs, more preferably basepaired in G:U basepairs.

10 In an embodiment of the two above aspects, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or 100% of the non-canonical basepairs are G:U basepairs.

In an embodiment of the two above aspects, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 1% or none, of the ribonucleotides in
15 the dsRNA region are not basepaired.

In an embodiment of the two above aspects, every one in four to every one in six ribonucleotides in the dsRNA region form a non-canonical basepair or are not basepaired, preferably form a G:U basepair.

In an embodiment of the two above aspects, the dsRNA region does not
20 comprise 8 contiguous canonical basepairs.

In an embodiment of the two above aspects, the dsRNA region comprises at least 8 contiguous canonical basepairs, preferably at least 8 but not more than 12 contiguous canonical basepairs.

In an embodiment of the two above aspects, all of the ribonucleotides in the
25 dsRNA region, or in each dsRNA region, are base-paired with a canonical basepair or a non-canonical basepair.

In an embodiment of the two above aspects, one or more ribonucleotides of the sense ribonucleotide sequence or one or more ribonucleotides of the antisense ribonucleotide sequence, or both, are not basepaired.

30 In an embodiment of the two above aspects, the antisense RNA sequence is less than 100% identical, or between about 80% and 99.9% identical, or between about 90% and 98% identical, or between about 95% and 98% identical, in sequence to the complement of a region of the target RNA molecule.

In an embodiment of the two above aspects, the antisense RNA sequence is
35 100% identical in sequence to a region of the target RNA molecule.

In an embodiment of the two above aspects, the sense and/or antisense ribonucleotide sequence, preferably both, is at least 50, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1,000, or about 100 to 5 about 1,000, or 20 to about 1000 nucleotides, or 20 to about 500 nucleotides, in length.

In an embodiment of the two above aspects, the number of ribonucleotides in the sense ribonucleotide sequence is between about 90% and about 110% of the number of ribonucleotides in the antisense ribonucleotide sequence.

In an embodiment of the two above aspects, the number of ribonucleotides in 10 the sense ribonucleotide sequence is the same as the number of ribonucleotides in the antisense ribonucleotide sequence.

In an embodiment of the two above aspects, the chimeric RNA molecule further comprises a 5' extension sequence which is covalently linked to the first 5' ribonucleotide or a 3' extension sequence which is covalently linked to the second 3' 15 ribonucleotide, or both.

In an embodiment of the two above aspects, the chimeric RNA molecule further comprises a 5' extension sequence which is covalently linked to the second 5' ribonucleotide or a 3' extension sequence which is covalently linked to the first 3' ribonucleotide, or both.

In an embodiment of the two above aspects, the chimeric RNA molecule 20 comprises two or more dsRNA regions which are the same or different.

In an embodiment of the two above aspects, when expressed in a plant cell more asRNA molecules are formed that are 22 and/or 20 ribonucleotides in length when compared to processing of an analogous RNA molecule which has a corresponding 25 dsRNA region which is fully basepaired with canonical basepairs.

In an embodiment, an RNA molecule of the first or second aspect is also a chimeric RNA molecule of the third or fourth aspects.

In an embodiment of each of the above aspects, the target RNA encodes VERNALIZATION1 (VRN1), VERNALIZATION2 (VRN2), 30 EARLYINSHORTDAYS4, FLOWERING LOCUS T1 (FT1), FLOWERING LOCUS T2 (FT2), Flowering Locus C (FLC), FRIGIDA (FRI) or CONSTANS in the plant species of interest.

In an embodiment of each of the above aspects, the target RNA comprises a region of a nucleotide sequence set forth in any one or more of SEQ ID NO's 146, 147, 35 or 151 to 228 (where the T's are replaced with U's), or a complement (antisense) of the region of the sequence, or both the region and the complement, or a nucleotide

sequence 95%, preferably, 99%, identical thereto (where the T's are replaced with U's). In an embodiment, the region is at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or at least 21 nucleotides in length.

In an embodiment of each of the above aspects, target RNA is a gene transcript
5 of the following from wheat, with Accession Nos of the genes or proteins in parentheses: VRN1/VRN-A1 (KR422423.1; SEQ ID NO:151); VRN2 (ZCCT1, TaVRN2-B; SEQ ID NO:145) (AAS58481.1); TaFT (Accession No. AY705794.1; SEQ ID NO:152) or homologous genes in other species, preferably cereal species.

In an embodiment of each of the above aspects, the target RNA is a gene
10 transcript of the one of the following from barley: HvVRN1 (AY896051; SEQ ID NO:153), HvVRN2 (AY687931, AY485978; SEQ ID NO:154) or HvFT (DQ898519; SEQ ID NO:155), or homologous genes in other species, preferably cereal species.

In an embodiment of each of the above aspects, the target RNA is a gene
15 transcript of one of the following from canola, BnFLC1 (AY036888, Bna.FLC.A10, BnaA10g22080D; SEQ ID NO:179); BnFLC2 (AY036889; SEQ ID NO:180); BnFLC3 (AY036890; SEQ ID NO:181); BnFLC4 (AY036891; SEQ ID NO:182); BnFLC5 (AY036892; SEQ ID NO:183); BnFRI (BnaA03g13320D; SEQ ID NO:184); BnFT (BnaA02g12130D; SEQ ID NO:185) or homologous genes in other species. .

In an embodiment of each of the above aspects, the target RNA is a gene
20 transcript of one of the following from *Arabidopsis*, FRI (AT4G00650); FLC (AT5G10140); VRN1 (AT3G18990); VRN2 (AT4G16845); VIN3 (AT5G57380); FT (AT1G65480); SOC1 (AT2G45660); CO (constans) (AT5G15840); LFY (AT5G61850); AP1 (AT1G69120) or homologous genes in other species.

In an embodiment of each of the above aspects, the target RNA is a gene
25 transcript of one of the following from rice, OsPhyB (OSNPB_030309200; SEQ ID NO:156); OsCol4 (HC084637; SEQ ID NO:157); RFT1 (OSNPB_070486100; SEQ ID NO:158); OsSNB (OSNPB_070235800; SEQ ID NO:159); OsIDS1 (Os03g0818800; SEQ ID NO:160); OsGI (OSNPB_010182600; SEQ ID NO:161), OsMADS50 (SEQ ID NO:162), OsMADS55 (SEQ ID NO:163) or OsLFY (SEQ ID NO:164), or
30 homologous genes in other species.

In an embodiment of each of the above aspects, the target RNA is a gene
transcript of the one of the following from maize (*Zea mays*): ZmMADS1/ZmM5
(LOC542042, HM993639; SEQ ID NO:), PHYA1 (AY234826; SEQ ID NO:166),
PHYA2 (AY260865; SEQ ID NO:167), PHYB1 (AY234827; SEQ ID NO:168),
35 PHYB2 (AY234828; SEQ ID NO:169), PHYC1 (AY234829; SEQ ID NO:170),
PHYC2 (AY234830; SEQ ID NO:171), ZmLD (AF166527; SEQ ID NO:172), ZmFL1

(AY179882; SEQ ID NO:173), ZmFL2 (AY179881; SEQ ID NO:174), DWARF8 (AF413203; SEQ ID NO:175), ZmAN1 (L37750; SEQ ID NO:176), ZmID1 (AF058757; SEQ ID NO:177), ZCN8 (LOC100127519; SEQ ID NO:178), or homologous genes in other species, preferably cereal species.

5 In an embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following from *Medicago truncatula*, MtFTa1 (HQ721813; SEQ ID NO:186); MtFTb1 (HQ721815; SEQ ID NO:187), MtYFL (BT053010, SEQ ID NO:210), MtSOC1a (Medtr07g075870), MtSOC1b (Medtr08g033250), MtSOC1c (Medtr08g033220), or homologous genes in other species.

10 In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following from alfalfa (*Medicago sativa*), MsFRI-L (SEQ ID NO:188), MsSOC1a (SEQ ID NO:189), or MsFT (SEQ ID NO:190), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following from soybean (*Glycine max*): encoded
 15 by the gene GLYMA_05G148700 with any one or more of the following transcript variants GmFLC-X1 (SEQ ID NO:191), GmFLC-X2 (SEQ ID NO:192) GmFLC-X3 (SEQ ID NO:193), GmFLC-X4 (SEQ ID NO:194), GmFLC-X5 (SEQ ID NO:195), GmFLC-X6 (SEQ ID NO:196), GmFLC-X7 (SEQ ID NO:197), GmFLC-X8 (SEQ ID NO:198), or GmFLC-X9 (SEQ ID NO:199), or SUPPRESSOR OF FRI (SEQ ID
 20 NO:200), GmFRI (SEQ ID NO:201), GmFT2A (SEQ ID NO:202), GmPHYA3 (SEQ ID NO:203), or GIGANTEA (SEQ ID NO:204), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of the following from sugarbeet (*Beta vulgaris*), BvBTC1 (HQ709091, SEQ ID NO:205), preferably BvFT1 (HM448909.1, SEQ ID NO:206) and/or BvFT2
 25 (HM448911, SEQ ID NO:207), where RNAi-induced down-regulation of the BvFT1-BvFT2 module led to a strong delay in bolting after vernalization by several weeks, or BvFL1 (DQ189214, DQ189215), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following genes from *Brassica rapa*, which may be turnip, cabbage, bok choy,
 30 turnip rape or related crucifers: BrFLC2 (AH012704, SEQ ID NO:208), BrFT (Bra004928) or BrFRI (HQ615935, SEQ ID NO:209), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following from cotton (*Gossypium hirsutum*): GhCO (Gorai.008G059900), GhFLC (Gorai.013G069000), GhFRI (Gorai.003G118000),
 35 GhFT (Gorai.004G264600), GhLFY (Gorai.001G053900), GhPHYA (Gorai.007G292800, Gorai.013G203900), GhPHYB (Gorai.011G200200), GhSOC1

(Gorai.008G115200), GhVRN1 (Gorai.002G006500, Gorai.005G240900, Gorai.012G150900, Gorai.013G040000), GhVRN2 (Gorai.003G176300), GhVRN5 (Gorai.009G023200), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following

5 from onion (*Allium cepa*): AcGI (GQ232756, SEQ ID NO:211), AcFKF (GQ232754, SEQ ID NO:212), AcZTL (GQ232755, SEQ ID NO:213), AcCOL (GQ232751, SEQ ID NO:214), AcFTL (CF438000, SEQ ID NO:215), AcFT1 (KC485348, SEQ ID NO:216), AcFT2 (KC485349, SEQ ID NO:217), AcFT6 (KC485353, SEQ ID NO:218), AcPHYA (GQ232753, SEQ ID NO:219), AcCOP1 (CF451443, SEQ ID

10 NO:220), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following from asparagus (*Asparagus officinalis*): FPA (LOC109824259, LOC109840062), TWIN SISTER of FT-like (LOC109835987), MOTHER of FT (LOC109844838), FCA-like (LOC109841154, LOC109821266), PHOTOPERIOD-INDEPENDENT EARLY

15 FLOWERING 1 (LOC109834006), FLOWERING LOCUS T-like (LOC109830558, LOC109825338, LOC109824462), Flowering locus K (LOC109847537), Flowering time control protein FY (LOC109844014), flowering time control protein FCA-like (LOC109842562), or homologous genes in other species. In another embodiment of each of the above aspects, the target RNA is a gene transcript of one of the following

20 from lettuce (*Lactuca sativa*): LsFT (LOC111907824, SEQ ID NO:221), TFL1-like (LOC111903066, SEQ ID NO:222), TFL1 homolog 1-like (LOC111903054, SEQ ID NO:223), LsFLC (LOC111876490, JI588382, SEQ ID NO:224), LsSOC1-like (LOC111912847, SEQ ID NO:225, LOC111880753, SEQ ID NO:226, LOC111878575, SEQ ID NO:227), TsLFY (LC164345.1, XM_023888266.1, SEQ ID

25 NO:228), or homologous genes in other species.

In an embodiment of each of the above aspects, the target RNA is a miRNA. Examples of such targets include, but are not limited to, miR-156 or miR-172.

In an embodiment of each of the above aspects, the RNA molecule or chimeric RNA molecule reduces the time to flowering compared to an isogenic plant lacking the

30 RNA molecule or chimeric RNA molecule. In an embodiment, the plant is *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an embodiment, the plant is *Arabidopsis*, corn, canola, cotton, soybean, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. The plant may be from alfalfa or clover, a leafy vegetable e.g. lettuce,

35 or a grass e.g. turfgrass.

In an embodiment, the first and second regions of the target RNA are contiguous in the target RNA. Alternatively, they are not contiguous.

In an embodiment of each of the above aspects, the RNA molecule or chimeric RNA molecule delays the time to flowering compared to an isogenic plant lacking the RNA molecule or chimeric RNA molecule. In an embodiment, the plant is a grass, where the target gene is a homolog of a cereal gene, as above.

In an embodiment of each of the above aspects, the plant is genetically unmodified.

In an embodiment of each of the above aspects, the RNA molecule comprises a 5' leader sequence or 5' extension sequence. In an embodiment, the RNA molecule comprises a 3' trailer sequence or 3' extension sequence. In a preferred embodiment, the RNA molecule comprises both the 5' leader/extension sequence and the 3' trailer/extension sequence.

In an embodiment of each of the above aspects, at least one loop sequence in the RNA molecule comprises one or more binding sequences which are complementary to an RNA molecule which is endogenous to the plant cell, such as, for example, an miRNA or other regulatory RNA in the plant cell. As would readily be understood, this feature may be in combination with any of the loop length features, non-canonical basepairing and any of the other features described above for the RNA molecule. In an embodiment, at least one loop sequence comprises multiple binding sequences for a miRNA, or binding sequences for multiple miRNAs, or both. In an embodiment, at least one loop sequence in the RNA molecule comprises an open reading frame which encodes a polypeptide or a functional polynucleotide. The open reading frame is preferably operably linked to a translation initiation sequence, whereby the open reading frame is capable of being translated in a plant cell of interest. For example, the translation initiation sequence comprises, or is comprised in, an internal ribosome entry site (IRES). The IRES is preferably a plant IRES. The translated polypeptide is preferably 50-400 amino acid residues in length, or 50-300 or 50-250, or 50-150 amino acid residues in length. Such RNA molecules, when produced in a plant cell, are capable of being processed to form circular RNA molecules comprising most or all of the loop sequence and which are capable of being translated to provide high levels of the polypeptide.

In an embodiment of each of the above aspects, the RNA molecule has none, or one, or two or more bulges in a double-stranded region. In this context, a bulge is a nucleotide, or two or more contiguous nucleotides, in the sense or antisense ribonucleotide sequence which is not basepaired in the dsRNA region and which does

not have a mismatched nucleotide at the corresponding position in the complementary sequence in the dsRNA region. The dsRNA region of the RNA molecule may comprise a sequence of more than 2 or 3 nucleotides within the sense or antisense sequence, or both, which loops out from the dsRNA region when the dsRNA structure forms. The
5 sequence which loops out may itself form some internal basepairing, for example it may itself form a stem-loop structure.

In an embodiment of each of the above aspects, the RNA molecule has none, or one, or two or more bulges in a double-stranded region. In this context, a bulge is a nucleotide, or two or more contiguous nucleotides, in the sense or antisense
10 ribonucleotide sequence which is not basepaired in the dsRNA region and which does not have a mismatched nucleotide at the corresponding position in the complementary sequence in the dsRNA region. The dsRNA region of the RNA molecule may comprise a sequence of more than 2 or 3 nucleotides within the sense or antisense sequence, or both, which loops out from the dsRNA region when the dsRNA structure forms. The
15 sequence which loops out may itself form some internal basepairing, for example it may itself form a stem-loop structure.

In an embodiment of each of the above aspects, the RNA molecule has three, four or more loops. In a preferred embodiment, the RNA molecule has only two loops. In an embodiment, the first double-stranded region, or the first and second dsRNA
20 region, or every dsRNA region, of the RNA molecule comprises one, or two, or more nucleotides which are not basepaired in the double-stranded region, or independently up to 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% or 10% of the nucleotides in the double-stranded region which are not basepaired.

In an embodiment of each of the above aspects, about 12%, about 15%, about
25 18%, about 21%, about 24%, or between about 15% and about 30%, or preferably between about 16% and about 25%, of the ribonucleotides of the sense ribonucleotide sequence and its corresponding antisense ribonucleotide sequence, in total, that form a dsRNA region are either basepaired in a non-canonical basepair or are not basepaired. In a preferred embodiment, at least 50%, at least 60%, at least 70%, at least 80%, at
30 least 90%, at least 95%, at least 97%, or 100% of the non-canonical basepairs in a dsRNA region, or in all dsRNA regions in the RNA molecule, are G:U basepairs. The G nucleotide in each G:U basepair may independently be in the sense ribonucleotide sequence or preferably in the antisense ribonucleotide sequence. Regarding the G nucleotides in the G:U basepairs of a dsRNA region, preferably at least 50% are in the
35 antisense ribonucleotide sequence, more preferably at least 60% or 70%, even more preferably at least 80% or 90%, and most preferably at least 95% of them are in the

antisense ribonucleotide sequence in the dsRNA region. This feature may apply independently to one or more or all of the dsRNA regions in the RNA molecule. In an embodiment, less than 25%, less than 20%, less than 15%, less than 10%, preferably less than 5%, more preferably less than 1% or most preferably none, of the
5 ribonucleotides in the dsRNA region, or in all of the dsRNA regions in the RNA molecule in total, are not basepaired. In a preferred embodiment, every one in four to every one in six ribonucleotides in the dsRNA region, or in the dsRNA regions in total, form a non-canonical basepair or are not basepaired within the RNA molecule. In a preferred embodiment, the dsRNA region, or in the dsRNA regions in total, do not
10 comprise 10 or 9 or preferably 8 contiguous canonical basepairs. In an alternative embodiment, the dsRNA region comprises at least 8 contiguous canonical basepairs, for example 8 to 12 or 8 to 14 or 8 to 10 contiguous canonical basepairs. In a preferred embodiment, all of the ribonucleotides in the dsRNA region, or in all dsRNA regions in the RNA molecule, are base-paired with a canonical basepair or a non-canonical
15 basepair. In an embodiment, one or more ribonucleotides of the sense ribonucleotide sequence or one or more ribonucleotides of the antisense ribonucleotide sequence, or both, are not basepaired. In an embodiment, one or more ribonucleotides of each sense ribonucleotide sequence and one or more ribonucleotides of each antisense ribonucleotide sequence are not basepaired in the RNA molecule of the invention.

20 In an embodiment, one or more or all of the antisense ribonucleotide sequences of the RNA molecule is less than 100% identical, or between about 80% and 99.9% identical, or between about 90% and 98% identical, or between about 95% and 98% identical, preferably between 98% and 99.9% identical, in sequence to the complement of a region of the target RNA molecule or to two such regions, which may or may not
25 be contiguous in the target RNA molecule. In a preferred embodiment, one or more of the antisense RNA sequences is 100% identical in sequence to a region of the complement of the target RNA molecule, for example to a region comprising 21, 23, 25, 27, 30, or 32 contiguous nucleotides. In an embodiment, the sense or antisense ribonucleotide sequence, preferably both, is at least 40, at least 50, at least about 100, at
30 least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1,000, or about 100 to about 1,000, contiguous nucleotides in length. The lengths of at least 100 nucleotides are preferred when using the RNA molecule in plant cells. In an embodiment, the number of ribonucleotides in the sense ribonucleotide sequence is
35 between about 90% and about 110%, preferably between 95% and 105%, more preferably between 98% and 102%, even more preferably between 99% and 101%, of

the number of ribonucleotides in the corresponding antisense ribonucleotide sequence to which it hybridises. In a most preferred embodiment, the number of ribonucleotides in the sense ribonucleotide sequence is the same as the number of ribonucleotides in the corresponding antisense ribonucleotide sequence. These features can be applied to each
5 dsRNA region in the RNA molecule.

The overall length of the RNA molecule of the invention, produced as a single strand of RNA, after splicing out of any introns but before any processing of the RNA molecule by Dicer enzymes or other RNAses, is typically between 50 and 2000 ribonucleotides, preferably between 60 or 70 and 2000 ribonucleotides, more
10 preferably between 80 or 90 and 2000 ribonucleotides, even more preferably between 100 or 110 and 2000 ribonucleotides. In preferred embodiments, the minimum length of the RNA molecule is 120, 130, 140, 150, 160, 180, or 200 nucleotides, and the maximum length is 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1500 or 2000 ribonucleotides. Each combination of these mentioned minimum and maximum lengths
15 is contemplated. Production of RNA molecules of such lengths by transcription *in vitro* or in cells such as bacterial or other microbial cells, preferably *S. cerevisiae* cells, or in the eukaryotic cell where the target RNA molecule is to be down-regulated, is readily achieved.

In a further aspect, the present invention provides a chimeric ribonucleic acid
20 (RNA) molecule, comprising a double-stranded RNA (dsRNA) region which comprises a sense ribonucleotide sequence and an antisense ribonucleotide sequence which are capable of hybridising to each other to form the dsRNA region, wherein

i) the sense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide,

25 ii) the antisense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide,

iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide to form a terminal basepair of the dsRNA region,

30 iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide to form a terminal basepair of the dsRNA region,

v) between about 5% and about 40% of the ribonucleotides of the sense ribonucleotide sequence and the antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired,

35 vi) the dsRNA region does not comprise 20 contiguous canonical basepairs,

vii) the RNA molecule is capable of being processed in a plant cell *or in vitro* whereby the antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length,

viii) the RNA molecule or at least some of the asRNA molecules, or both, are
5 capable of reducing the expression or activity of a target RNA molecule which modulates the timing of plant flowering, and

ix) the RNA molecule is capable of being made enzymatically by transcription *in vitro* or in a cell, or both.

In another aspect, the present invention provides a chimeric RNA molecule
10 comprising a first RNA component and a second RNA component which is covalently linked to the first RNA component, wherein the first RNA component comprises a first double-stranded RNA (dsRNA) region, which comprises a first sense ribonucleotide sequence and a first antisense ribonucleotide sequence which are capable of hybridising to each other to form the first dsRNA region, and a first intervening ribonucleotide
15 sequence of at least 4 nucleotides which covalently links the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, wherein the second RNA component comprises a second sense ribonucleotide sequence, a second antisense ribonucleotide sequence and a second intervening ribonucleotide sequence of at least 4 ribonucleotides which covalently links the second sense ribonucleotide sequence and
20 the second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence hybridises with the second antisense ribonucleotide sequence in the RNA molecule, wherein in the first RNA component,

i) the first sense ribonucleotide sequence consists of at least 20 contiguous ribonucleotides covalently linked, in 5' to 3' order, a first 5' ribonucleotide, a first
25 RNA sequence and a first 3' ribonucleotide,

ii) the first antisense ribonucleotide sequence consists of at least 20 contiguous ribonucleotides covalently linked, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide,

iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide,

30 iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide,

v) between 5% and 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, and

vi) the first dsRNA region does not comprise 20 contiguous canonical basepairs,

wherein the chimeric RNA molecule is capable of being processed in a plant cell or *in vitro* whereby the first antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length, and wherein

- (a) the chimeric RNA molecule or at least some of the asRNA molecules, or both,
5 are capable of reducing the expression or activity of a target RNA molecule which modulates the timing of plant flowering, or
- (b) the first antisense ribonucleotide sequence comprises a sequence of at least 20 contiguous ribonucleotides which is at least 50% identical in sequence, preferably at least 90% or 100% identical in sequence, to a region of the
10 complement of the target RNA molecule, or
- (c) both (a) and (b).

In an embodiment where the chimeric RNA molecule has a first RNA component, the first 5' ribonucleotide and first 3' ribonucleotide of the first RNA component basepair to each other. That basepair is defined herein as the terminal
15 basepair of the dsRNA region formed by self-hybridisation of the first RNA component. In the embodiment where the first sense ribonucleotide sequence is linked covalently to the first 5' ribonucleotide without any intervening nucleotides and the first antisense ribonucleotide sequence is linked covalently to the first 3' ribonucleotide without any intervening nucleotides, the first 5' ribonucleotide is directly linked to one
20 of the sense sequence and antisense sequence and the first 3' ribonucleotide is directly linked to the other of the sense sequence and antisense sequence.

In embodiments of the above aspects, the RNA molecule comprises one or more or all of (i) a linking ribonucleotide sequence which covalently links the first and second RNA components, (ii) a 5' extension sequence and (iii) a 3' extension sequence,
25 wherein the 5' extension sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the first RNA component or to the second RNA component, and wherein the 3' extension sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the second RNA component or to the first RNA component, respectively. In an embodiment, the first RNA component and the
30 second RNA component are covalently linked via a linking ribonucleotide sequence. In an alternative embodiment, the first RNA component and the second RNA component are directly linked, without any linking ribonucleotide sequence present.

In preferred embodiments of the above aspects, the RNA molecule is capable of being made enzymatically by transcription *in vitro* or in a cell, or both. In an
35 embodiment, an RNA molecule of the present invention is expressed in a plant cell i.e. produced in the cell by transcription from one or more nucleic acids encoding the RNA

molecule. The one or more nucleic acids encoding the RNA molecule is preferably a DNA molecule, which may be present on a vector in the cell or integrated into the genome of the cell, either the nuclear genome of the cell or in the plastid DNA of the cell. The one or more nucleic acids encoding the RNA molecule may also be an RNA molecule such as a viral vector.

In a further aspect, the present invention provides an isolated and/or exogenous polynucleotide encoding an RNA molecule of the invention, or a chimeric RNA molecule of the invention.

In an embodiment, the polynucleotide is a DNA construct.

In an embodiment, the polynucleotide is operably linked to a promoter capable of directly expression of the RNA molecule in a plant cell. Examples of such promoters include, but are not limited to an RNA polymerase promoter such as an RNA polymerase III promoter, an RNA polymerase II promoter, or a promoter which functions *in vitro*.

In an embodiment, the polynucleotide encodes an RNA precursor molecule comprising an intron in at least one loop sequence which is capable of being spliced out during transcription of the polynucleotide in a plant cell or *in vitro*.

In an embodiment, the polynucleotide is a chimeric DNA which comprises in order, a promoter capable of initiating transcription of the RNA molecule in a host cell, operably linked to a DNA sequence which encodes the RNA molecule, preferably a hpRNA, and a transcription termination and/or polyadenylation region. In a preferred embodiment, the RNA molecule comprises a hairpin RNA structure which comprises a sense ribonucleotide sequence, a loop sequence and an antisense ribonucleotide sequence, more preferably wherein the sense and antisense ribonucleotide sequences basepair to form a dsRNA region wherein between about 5% and about 40% of the ribonucleotides in the dsRNA region are basepaired in non-canonical basepairs, preferably G:U basepairs.

In an embodiment, polynucleotides of the invention comprise a nucleotide sequence set forth in SEQ ID NO:150 or a nucleotide sequence 95% identical thereto. In an embodiment, polynucleotides of the invention comprise a nucleotide sequence set forth in SEQ ID NO:150.

Also provided is a vector comprising a polynucleotide of the invention.

In an embodiment, the vector is a viral vector. In an embodiment, the vector is a plasmid vector such as a binary vector suitable for use with *Agrobacterium tumefaciens*.

In an embodiment where the polynucleotide or vector of the invention is in a plant host cell, the promoter region of the polynucleotide or vector, which is operably linked to the region which encodes an RNA molecule of the invention, has a lower level of methylation when compared to the promoter of a corresponding polynucleotide or vector encoding an RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs. In an embodiment, the lower level of methylation is less than 50%, less than 40%, less than 30% or less than 20%, when compared to the promoter of the corresponding polynucleotide or vector. In an embodiment, the host cell comprises at least two copies of the polynucleotide or vector encoding an RNA molecule of the invention. In this embodiment:

- i) the level of reduction in the expression and/or activity of the target RNA molecule in the plant cell is at least the same relative to a corresponding plant cell having a single copy of the polynucleotide or vector, and/or
- ii) the level of reduction in the expression and/or activity of the target RNA molecule in the plant cell is lower when compared to a corresponding cell comprising an RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs.

In another aspect, the present invention provides a host cell comprising one or more or all of an RNA molecule of the invention, a chimeric RNA molecule of the invention, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, a polynucleotide of the invention, or a vector of the invention.

The host cell may be a bacterial cell such as *E. coli*, a fungal cell such as a yeast cell, for example, *S. cerevisiae*, or a eukaryotic cell such as a plant cell. In an embodiment, the promoter is heterologous relative to the polynucleotide. The polynucleotide encoding the RNA molecule may be a chimeric or recombinant polynucleotide, or an isolated and/or exogenous polynucleotide. In an embodiment, the promoter can function *in vitro*, for example a bacteriophage promoter such as a T7 RNA polymerase promoter or SP6 RNA polymerase promoter. In an embodiment, the promoter is an RNA polymerase III promoter such as a U6 promoter or an H1 promoter. In an embodiment, the promoter is an RNA polymerase II promoter, which may be a constitutive promoter, a tissue-specific promoter, a developmentally regulated promoter or an inducible promoter. In an embodiment, the polynucleotide encodes an RNA precursor molecule comprising an intron in at least one loop sequence which is capable of being spliced out during or after transcription of the polynucleotide in a host cell.

In an embodiment, the host cell is a plant cell.

In an embodiment, the promoter region of the polynucleotide has a lower level of methylation, such as less than about 50%, less than about 40%, less than about 30% or less than about 20%, when compared to the promoter of a corresponding
5 polynucleotide encoding an RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs.

In an embodiment, the host cell is a plant cell comprising the chimeric RNA molecule or small RNA molecules produced by processing of the chimeric RNA molecule, or both, wherein the chimeric RNA molecule comprises, in 5' to 3' order, the
10 first sense ribonucleotide sequence, the first linking ribonucleotide sequence which comprises a loop sequence, and the first antisense ribonucleotide sequence. In an embodiment, the plant cell may be from *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an embodiment, the plant cell may be from *Arabidopsis*, corn, canola, cotton, soybean,
15 wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye.

In an embodiment, the host cell comprises at least two copies of the polynucleotide, and wherein

i) the level of reduction in the expression or activity of the target RNA molecule in a plant cell is at least the same when compared to if the cell had a single copy of the
20 polynucleotide, and/or

ii) the level of reduction in the expression or activity of the target RNA molecule in a plant cell is lower when compared to a corresponding cell comprising an RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs.

25 In an embodiment, the cell encodes and/or comprises the chimeric RNA molecule of the invention and the level of sense ribonucleotide sequence in the cell is less than 50 to 99% the level of the antisense ribonucleotide.

In an embodiment, the RNA molecule is expressed in a eukaryotic cell i.e. produced by transcription in the cell. In these embodiments, a greater proportion of
30 dsRNA molecules are formed by processing of the RNA molecule that are 22 and/or 20 ribonucleotides in length when compared to processing of an analogous RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs. That is, the RNA molecules of these embodiments are more readily processed to provide 22- and/or 20-ribonucleotide short antisense RNAs than the
35 analogous RNA molecule whose dsRNA region is fully basepaired with canonical basepairs, as a proportion of the total number of 20-24 nucleotide asRNAs produced

from the RNA molecule. Expressed differently, a lesser proportion of dsRNA molecules are formed by processing of the RNA molecule that are 23 and/or 21 ribonucleotides in length when compared to processing of an analogous RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs. That is, the RNA molecules of these embodiments are less readily processed to provide 23- and/or 21-ribonucleotide short antisense RNAs than the analogous RNA molecule whose dsRNA region is fully basepaired with canonical basepairs, as a proportion of the total number of 20-24 nucleotide asRNAs produced from the RNA molecule. Preferably, at least 50% of the RNA transcripts produced in the cell by transcription from the genetic construct are not processed by Dicer. In an embodiment, when the RNA molecule is expressed in a eukaryotic cell i.e. produced by transcription in the cell, a greater proportion of the short antisense RNA molecules that are formed by processing of the RNA molecule have more than one phosphate covalently attached at the 5' terminus when compared to processing of an analogous RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs. That is, a greater proportion of the short antisense RNA molecules have an altered charge which can be observed as a mobility shift of the molecules in gel electrophoresis experiments.

In a further aspect, the present invention provides a plant comprising one or more or all of an RNA molecule of the invention, a chimeric RNA molecule of the invention, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, a polynucleotide of the invention, a vector of the invention, or a host cell of the invention which is a plant cell.

In an embodiment, the plant is transgenic insofar as it comprises a polynucleotide of the invention. In an embodiment, the polynucleotide is stably integrated into the genome of the plant. The invention also includes plant parts, and products obtained therefrom, comprising the RNA molecule or small RNA molecules (20-24nt in length) produced by processing of the chimeric RNA molecule, or both, and/or the polynucleotide or vector of the invention, for example to seeds, crops, harvested products and post-harvest products produced therefrom.

In a further aspect, the present invention provides a method of producing an RNA molecule of the invention, or a chimeric RNA molecule of the invention, the method comprising expressing the polynucleotide of the invention in a host cell or cell-free expression system.

In an embodiment, the method further comprises at least partially purifying the RNA molecule.

In another aspect, the present invention provides a method of producing the plant of the invention, the method comprising introducing the polynucleotide of the invention into a plant cell so that it is stably integrated into the genome of the cell, and generating the plant from the cell.

5 In another aspect, the present invention provides a method of producing a cell or plant, the method comprising introducing a polynucleotide or vector or RNA molecule of the invention into a plant cell, preferably so that the polynucleotide or vector or part thereof encoding the RNA molecule is stably integrated into the genome of the plant cell. In an embodiment, the plant is generated from the cell or a progeny cell, for
10 example by regenerating a transgenic plant and optionally producing progeny plants therefrom. In an embodiment, the plant is generated by introducing the cell or one or more progeny cells into the plant. Alternatively to the stable integration of the polynucleotide or vector into the genome of the plant cell, the polynucleotide or vector may be introduced into the cell without integration of the polynucleotide or vector into
15 the genome, for example to produce the RNA molecule transiently in the plant cell or plant. In an embodiment, the plant, is resistant to a pest or pathogen, e.g. a plant pest or pathogen, preferably an insect pest or fungal pathogen. In an embodiment, the method comprises a step of testing one or more plants, comprising the polynucleotide or vector or RNA molecule of the disclosure for modulation of flowering. The plants that are
20 tested may be progeny from the plant, into which the polynucleotide or vector or RNA molecule of the disclosure was first introduced, and therefore the method may comprise a step of obtaining such progeny. The method may further comprise a step of identifying and/or selecting the plant with desired time to flowering such as early flowering. For example, multiple plants, which each comprise the polynucleotide or
25 vector or RNA molecule of the invention may be tested to identify those with desired time to flowering, and progeny obtained from the identified plant(s).

In a further aspect, the present invention provides an extract of a host cell of the invention, wherein the extract comprises the RNA molecule of the invention, a chimeric RNA molecule of the invention, small RNA molecules (20-24nt in length)
30 produced by processing of the RNA molecule or chimeric RNA molecule, or both, and/or the polynucleotide of the invention.

In a further aspect, the present invention provides a composition comprising one or more of an RNA molecule of the invention, a chimeric RNA molecule of the invention, small RNA molecules (20-24nt in length) produced by processing of the
35 RNA molecule or chimeric RNA molecule, a polynucleotide of the invention, a vector

of the invention, a host cell of the invention, or an extract of the invention, and one or more suitable carriers.

In one embodiment, the composition is suitable for application to a field, e.g. as topical spray. In an embodiment, the field comprises plants. In an embodiment, the
5 composition is suitable for application to a crop, for example by spraying on crop plants in a field.

In a further embodiment, the composition further comprises at least one compound which enhances the stability of the RNA molecule, chimeric RNA molecule or polynucleotide and/or which assists in the RNA molecule, chimeric RNA molecule
10 or polynucleotide being taken up by a cell of a plant. In an embodiment, the compound is a transfection promoting agent.

In an aspect, the present invention provides a method for down-regulating the level and/or activity of a target RNA molecule which modulates plant flowering in a plant, the method comprising delivering to the plant one or more of an RNA molecule
15 of the invention, a chimeric RNA molecule of the invention, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, a polynucleotide of the invention, a vector of the invention, a host cell of the invention, an extract of the invention, or a composition of the invention.

In this context, delivering may be via contacting, exposing, transforming or
20 otherwise introducing an RNA molecule or chimeric RNA molecule disclosed herein or a mixture thereof, or small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule or the polynucleotide or vector of the invention to the plant cell or plant. The introduction may be enhanced by use of an agent that increases the uptake of the RNA molecule(s), polynucleotides or vectors of
25 the invention, for example with the aid of transfection promoting agents, DNA- or RNA-binding polypeptides, or may be done without adding such agents, for example by planting seed which is transgenic for a polynucleotide or vector of the invention and allowing the seed to grow into a transgenic plant which expresses the RNA molecules of the invention. In an embodiment, the target RNA molecule encodes a protein. In an
30 embodiment, the method reduces the level and/or activity of more than one target RNA molecule, the target RNA molecules being different, for example two or more target RNAs are reduced in level and/or activity which are related in sequence such as from a gene family. Thus, in an embodiment, the chimeric RNA molecule or small RNA molecules produced by processing of the chimeric RNA molecule, or both, are
35 contacted with the cell or organism, preferably a plant cell or plant by topical application to the cell or organism, or provided in a feed for the organism.

In an embodiment, the target RNA molecule encodes a protein. Alternatively, one or more of the target RNAs do not encode a protein, such as a rRNA, tRNA, snoRNA or miRNA.

In an embodiment, the chimeric RNA molecule, or small RNA molecules
5 produced by processing of the chimeric RNA molecule, or both, are contacted with the cell or plant by topical application to the cell or plant.

In another embodiment, the present disclosure encompasses a method of promoting flowering time of a plant, the method comprising expressing a polynucleotide heterologous to said plant, wherein said polynucleotide heterologous to
10 said plant is a polynucleotide of the invention such as an RNA molecule of the invention, wherein expression of said polynucleotide in said plant directs early flowering.

The present inventors have surprisingly found that RNA can be directly applied to a plant or seed to influence future flowering time. Thus, in a further aspect the
15 present invention provides a method of modulating the flowering time of a plant, or a plant produced from a seed, the method comprising contacting the plant or seed with a composition comprising an RNA molecule which comprises at least one double stranded RNA region, and/or a polynucleotide(s) encoding the RNA molecule, wherein the at least one double stranded RNA region comprises an antisense ribonucleotide
20 sequence which is capable of hybridising to a region of a target RNA molecule which modulates the timing of plant flowering.

In an embodiment, the composition is an aqueous composition.

In an embodiment, the composition comprises at least one compound which enhances the stability of the RNA molecule and/or which assists in the RNA molecule
25 being taken up by a cell of a plant. In an embodiment, the compound is a transfection promoting agent.

In an embodiment, the method comprises soaking the seed in the composition. In an alternate embodiment, the plant is a seedling, and the method comprises soaking at least a part of the seedling in the composition. In an embodiment, at least a part, or
30 all, of the cotyledon(s) and/or the hypocotyl are soaked in the composition.

In an embodiment, the plant is in a field and the method comprising spraying the composition on at least a part of the plant.

The RNA molecule can have any suitable structure for gene silencing. Examples include, but are not limited to, hairpin RNA, a microRNA, a siRNA or an
35 ledRNA. The RNA molecule of the above aspect can be a chimeric RNA molecule such as described herein.

The nature in which flowering time is modulated will depend on the target RNA molecule. In one embodiment, the plant has an early flowering time when compared to a control plant that has not been applied with the composition. In an alternate embodiment, the plant has a late flowering time when compared to a control plant that
5 has not been applied with the composition. Examples of target RNA molecules to be targeted to induce early or late flowering are discussed herein.

In an embodiment, the RNA molecule is complexed with a non-RNA molecule such as DNA, a protein or a polymer. In an embodiment, the complex comprises the RNA molecule conjugated to the non-RNA molecule such as by a covalent bond.

10 In an embodiment, the composition is topically applied to the plant or seed.

In an embodiment, the polynucleotide is present in the composition in a cell and/or a vector.

In another aspect, the present invention provides a kit comprising one or more of an RNA molecule of the invention, a chimeric RNA molecule of the invention, a
15 polynucleotide of the invention, a vector of the invention, a host cell of the invention, an extract of the invention, or a composition of the invention. The kit may further comprise instructions for use of the kit.

Whilst more widely used in transgenic expression systems, as discussed herein there are also applications of dsRNA technology which rely on the need for the large
20 scale production of dsRNA molecules, such as spraying a crop to modulate flowering. The present inventors have identified *S. cerevisiae* as a suitable organism to use in large scale production processes because dsRNA molecules expressed therein are not cleaved. Thus, in a further aspect, the present invention provides a process for producing dsRNA molecules, the process comprising

25 a) culturing *S. cerevisiae* expressing one or more polynucleotides encoding one or more dsRNA molecules, and

b) harvesting the *S. cerevisiae* producing the dsRNA molecules, or the dsRNA molecules from the *S. cerevisiae*,

wherein the *S. cerevisiae* are cultured in a volume of at least 1 litre.

30 The dsRNA can have any structure, such as an hairpin RNA (for example shRNA), a miRNA or a dsRNA of the invention.

In an embodiment, the *S. cerevisiae* are cultured in a volume of at least 10 litres, at least 100 litres, at least 1,000 litres, at least 10,000 litres or at least 100,000 litres.

In an embodiment, the process produces at least 0.1, at least 0.5 or at least 1
35 g/litre of an RNA molecule of the invention.

The *S. cerevisiae* produced using the process, or dsRNA molecules isolated therefrom (either in a purified or partially purified (such as an extract) state) can be used in methods described herein such as, but not limited to, a method for reducing or down-regulating the level and/or activity of a target RNA molecule in a cell or plant.

5 Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the
10 scope of the invention, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of
15 compositions of matter.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

20 **Figure 1.** Schematic designs of two ledRNA molecules. (A) This ledRNA molecule comprises a sense sequence which can be considered to be two adjacent sense sequences, covalently linked without an intervening spacer sequence and having identity to the target RNA, an antisense sequence which is complementary to the sense sequence and which is divided into two regions, a 5' region and a 3' region, and two
25 loops that separate the sense from the antisense sequences. (B) This ledRNA molecule comprises an antisense sequence which can be considered to be two adjacent antisense sequences, covalently linked without an intervening spacer sequence and having identity to the complement of a target RNA, a sense sequence which is complementary to the antisense sequence and which is divided into two regions, and two loops that
30 separate the sense from the antisense sequences. The RNA molecule produced by transcription, for example by *in vitro* transcription from a promoter such as a T7 or Sp6 promoter, self-anneals by basepairing between the complementary sense and antisense sequences to form a double-stranded region with a loop at each end and having a "nick" in either the antisense or sense sequence. Additional sequences may be linked to the 5'
35 and/or 3' ends as 5'- or 3'-extensions.

Figure 2. ledRNA is more efficient in forming dsRNA than sense/antisense annealing or hairpin RNA. Schematic representations of three forms of double-stranded RNA molecules are shown: A, conventional dsRNA formed by annealing of two separate strands; B, a hairpin RNA having a 5'- and a 3'-extension; and C, ledRNA molecule.

5 The lower panel shows a photograph after gel electrophoresis of the RNA transcripts for the three types of RNA molecules targeting either a GUS gene or a GFP gene.

Figure 3. Northern blot hybridization of treated (A and B) and untreated distal (C and D) tissues shows that ledRNA is more stable than dsRNA and spread through tobacco leaf tissue. In the distal tissues (C and D, top panel) the dsRNA signal could not be
10 detected, in contrast to strong ledRNA signals.

Figure 4. ledRNA treatment induced downregulation of GUS in both the treated area (1) and the untreated area above (3).

Figure 5. ledRNA induces silencing of the FAD2.1 gene in *N. benthamiana* leaves.

Figure 6. Northern blot hybridization confirms strong downregulation of FAD2.1
15 mRNA by treatment with ledFAD2.1 at 6 and 24 hours.

Figure 7. Alignment of the nucleotide sequences of a region of the *GUS* target gene (SEQ ID NO:14) and the sense sequence of the hpGUS[G:U] construct (nucleotides 9 to 208 of SEQ ID NO:11). 52 cytidine (C) nucleotides were substituted with thymidine (T) nucleotides. Conserved nucleotides are asterisked, substituted C's are not
20 asterisked.

Figure 8. Alignment of the nucleotide sequences of a region of the *GUS* target gene (SEQ ID NO:14) and the sense sequence of the hpGUS[1:4] construct (nucleotides 9 to 208 of SEQ ID NO:12). Every 4th nucleotide in hpGUS[1:4] was substituted relative to the corresponding wild-type sense sequence, whereby for every 4th nucleotide, C was
25 changed to G, G was changed to C, A was changed to T, and T was changed to A. Conserved nucleotides are asterisked, substituted G's and C's are not asterisked, substituted A's and T's are shown with semi-colons.

Figure 9. Alignment of the nucleotide sequences of a region of the *GUS* target gene (SEQ ID NO:14) and the sense sequence of the hpGUS[2:10] construct (nucleotides 9 to 208 of SEQ ID NO:13). Every 9th and 10th nucleotide in each block of 10
30 nucleotides in hpGUS[2:10] was substituted relative to the corresponding wild-type sense sequence, whereby for every 9th and 10th nucleotide, C was changed to G, G was changed to C, A was changed to T and T was changed to A. Conserved nucleotides are asterisked, substituted G's and C's are not asterisked, substituted A's and T's are
35 shown with semi-colons.

Figure 10. Schematic diagram showing structures of the genetic constructs encoding modified hairpin RNAs targeting GUS mRNA.

Figure 11. Schematic diagram of vector pWBPPGH used to transform tobacco plants, providing a GUS target gene. The T-DNA extends from the right border (RB) to the
5 left border (LB) of the vector. The selectable marker gene on the T-DNA is the 35S-HPT-tm1' gene encoding hygromycin resistance.

Figure 12. GUS activity in plants transformed with constructs encoding modified hairpin RNAs for reducing expression of a GUS target gene. No hp: control PPGH11 and PPGH24 plants with no hpGUS constructs. The number of plants showing less than
10 10% GUS activity compared to the corresponding control PPGH11 or PPGH24 plants and the percentage of such plants relative to the number of plants tested are given in brackets.

Figure 13. (A) Average GUS activity of all transgenic plants: 59 plants for hpGUS[wt], 74 for hpGUS[G:U], 33 for hpGUS[1:4] and 41 for hpGUS[2:10]. (B)
15 Average GUS activity of all silenced plants (32 for hpGUS[wt], 71 for hpGUS[G:U], 33 for hpGUS[1:4] and 28 for hpGUS[2:10].

Figure 14. GUS activity of transgenic progeny plants containing hpGUS[wt], hpGUS[G:U] or hpGUS[1:4].

Figure 15. Autoradiograph of a Southern blot of DNA from 16 plants transformed
20 with the hpGUS[G:U] construct. DNAs were digested with HindIII prior to gel electrophoresis and probed with an OCS-T probe. Lane 1: size markers (HindIII-digested lambda DNA); Lanes 2 and 3, DNA from parental plants PPGH11 and PPGH24; Lanes 4-19: DNAs from 16 different transgenic plants.

Figure 16. Autoradiogram of a Northern blot hybridisation experiment to detect sense
25 (upper panel) and antisense (lower panel) sRNAs derived from hairpin RNAs expressed in transgenic tobacco plants. Lanes 1 and 2 contained RNA obtained from the parental plants PPGH11 and PPGH24 lacking the hpGUS constructs. Lanes 3-11 contained RNA from hpGUS[wt] plants and lanes 12-20 contained RNA from hpGUS[G:U] plants.

Figure 17. Autoradiograph of a Northern blot hybridisation to detect antisense sRNAs
30 from transgenic plants. Lanes 1-10 were from hpGUS[wt] plants, lanes 11-19 were from hpGUS[G:U] plants. The antisense sRNAs have mobility corresponding to 20-24nt in length. The blot was reprobated with antisense to U6 RNA as a lane-loading control.

Figure 18. Autoradiograph of a repeat Northern blot hybridisation to detect antisense
35 sRNAs from transgenic plants

Figure 19. DNA methylation analysis of the junction region of the 35S promoter and sense GUS region in hpGUS constructs in transgenic plants. The junction fragments were PCR-amplified either with (+) or without (-) prior treatment of plant DNA with McrBC enzyme.

- 5 **Figure 20.** DNA methylation analysis of the 35S promoter region in hpGUS constructs in transgenic plants. The 35S fragments were PCR-amplified either with (+) or without (-) prior treatment of plant DNA with McrBC enzyme.

Figure 21. Size distribution and abundance of processed RNA. (A) EIN2 constructs. (B) GUS constructs.

- 10 **Figure 22.** Alignment of the sense sequence (upper sequence, nucleotides 17 to 216 of SEQ ID NO:22) of the hpEIN2[G:U] construct and the nucleotide sequence (lower sequence, SEQ ID NO:27) of a region of the cDNA corresponding to the *A. thaliana* EIN2 target gene. The sense sequence was made by replacing 43 cytidine (C) nucleotides in the wild-type sequence with thymidine (T) nucleotides. Conserved
15 nucleotides are asterisked, substituted C's are not asterisked.

- Figure 23.** Alignment of the sense sequence (upper sequence, nucleotides 13 to 212 of SEQ ID NO:24) of the hpCHS[G:U] construct with the nucleotide sequence of a region of the cDNA corresponding to the *A. thaliana* CHS target gene (SEQ ID NO:28, lower sequence). The sense sequence was made by replacing 65 cytidine (C) nucleotides in
20 the wild-type sequence with thymidine (T) nucleotides. Conserved nucleotides are asterisked, substituted C's are not asterisked.

- Figure 24.** Alignment of the antisense sequence (upper sequence, nucleotides 8 to 207 of SEQ ID NO:25) of the hpEIN2[G:U/U:G] construct and the nucleotide sequence (lower sequence, SEQ ID NO:29) of a region of the complement of the *A. thaliana*
25 EIN2 target gene and the. The antisense sequence was made by replacing 49 cytidine (C) nucleotides in the wild-type sequence with thymidine (T) nucleotides. Conserved nucleotides are asterisked, substituted C's are not asterisked.

- Figure 25.** Alignment of the antisense sequence (upper sequence, nucleotides 13 to 212 of SEQ ID NO:26) of the hpCHS[G:U/U:G] construct and the nucleotide sequence
30 (lower sequence, SEQ ID NO:30) of a region of the complement of the *A. thaliana* CHS target gene. The antisense sequence was made by replacing 49 cytidine (C) nucleotides in the wild-type sequence with thymidine (T) nucleotides. Conserved nucleotides are asterisked, substituted C's are not asterisked.

- Figure 26.** Schematic diagrams of the ethylene insensitive 2 (EIN2) and chalcone
35 synthase (CHS) hpRNA constructs. 35S: CaMV 35S promoter; EIN2 and CHS regions are show either as wild-type sequence (wt) or the G:U modified sequence (G:U). The

arrows indicate the orientation of the DNA fragments – right to left arrows indicate the antisense sequences. Restriction enzyme sites are also shown.

Figure 27. Hypocotyl lengths of transgenic *A. thaliana* seedlings in the EIN2 assay, containing either the hpEIN2[wt] or hpEIN2[G:U]

- 5 **Figure 28.** qRT-PCR for CHS mRNA in transgenic *A. thaliana* transgenic for the hpCHS[wt] or hpCHS[G:U] constructs, normalised to the levels of Actin2 RNA. Col-0 is the wild-type (nontransgenic) *A. thaliana*.

Figure 29. Autoradiograph of Northern blot hybridisation of RNA from plants transformed with hpEIN2[wt] or hpEIN2[G:U]. Upper panel shows the hypocotyl
10 length for the lines. The autoradiograph shows Northern blot probed with an EIN2 sense probe to detect antisense sRNAs. The same blot was re-probed with a U6 RNA probe as a loading control (U6 RNA).

Figure 30. DNA methylation analysis of 35S promoter and 35S-sense EIN2 sequences in genomic DNA of transgenic *A. thaliana* plants.

- 15 **Figure 31.** Levels of DNA methylation in the promoter and 5' region of hairpin RNA constructs.

Figure 32. 35S promoter in the least methylated lines of the hpEIN2[wt] population still shows significant methylation.

- Figure 33.** 35S promoter in the G:U hpEIN2 lines shows only weak methylation
20 (<10%).

Figure 34. ledRNA and hprRNA with G:U gene silencing in CHO and Vero cells at 72 hrs.

Figure 35. Dumbbell plasmids tested in Hela cells at 48 hrs.

Figure 36. Examples of possible modifications of dsRNA molecules.

- 25 **Figure 37.** Reduced aphid performance following feeding from artificial diet supplemented with ledRNA for down-regulating expression of the *MpC002* or *MpRack-1* genes in green peach aphid. Upper panel (A): the average number of nymphs per adult aphid after a ten day period with 100 µl of 50ng/µl ledRNA. Lower panel (B): percentage of aphids surviving over a five day time course after feeding on
30 100 µl containing 200ng/µl ledRNA of *MpC002*, *MpRack-1* or the control ledGFP.

Figure 38. Northern blot hybridization to detect ledGUS and hpGUS RNA using full-length sense GUS transcript as probe. “+” at the bottom indicates high GUS expression; “-” indicates low/no GUS expression i.e. strong GUS silencing.

- Figure 39.** Northern blot hybridization to detect long hpEIN2 and ledEIN2 RNA
35 (upper panel) and siRNAs derived from the two constructs (lower panel).

Figure 40. Schematic representation of stem-loop structures of transcripts expressed from GUS hpRNA constructs. The transcripts have complementary sense and antisense sequences which basepair to form GUS sequence-specific dsRNA stems, with the indicated lengths in basepairs (bp) for the stems, and the number of nucleotides (nt) in the loops. The GFP hpRNA constructs encoded transcripts that formed a GFP-specific dsRNA stem with completely canonical basepairing (GFPhp[WT]) or a dsRNA stem having about 25% of basepairs as G:U base-pairs (GFPhp[G:U], with a loop derived from a region of GUS coding sequence. The loop sequences for the GFPhp transcripts each comprised two sequences that were complementary to miR165/miR166 and therefore provide binding sites for these miRNAs.

Figure 41. Northern blot hybridisation analysis showing that transgenes encoding hpRNAs generate distinct fragments of the loop sequence when expressed in plant cells. (A) Expression of the GUS target gene (GUS) and the long hpRNA transgene GUShp1100 with a 1100 nt spacer/loop sequence. A construct encoding the cucumber mosaic virus 2b RNA silencing suppressor (CMV2b) was included to enhance transgene expression. (B) Northern blot analysis showing RNA from expression of the two short hpRNA transgenes GUShp93-1 and GUShp93-2 in stably transformed *A. thaliana* plants. RNA samples were either treated (+) or not treated (-) with RNase I. Both RNA blots were hybridized with loop-specific antisense RNA probes.

Figure 42. The loop of GUShp1100 accumulated to high levels in *N. benthamiana* cells and was resistant to RNase R digestion.

Figure 43. Transgenic *S. cerevisiae* expressing a GUShp1100 construct showed a single RNA molecular species corresponding to the full length hairpin RNA transcript. The lower panel shows the Northern blot hybridisation of RNA samples from the transgenic *S. cerevisiae*.

Figure 44. GUShp1100 transcript expressed in *S. cerevisiae* remains full-length and does not form circular loop RNA. The first four lanes used *in vitro* transcripts of full-length or the dsRNA stem of GUShp1100, supplemented with total RNA isolated from wild-type *N. benthamiana* leaves.

Figure 45. hpRNA loops may be used as an effective sequence-specific repressor of miRNAs. (A) The GFPhp[G:U] construct induced strong miR165/166 suppression phenotypes in transgenic *Arabidopsis* plants. (B) Northern blot hybridization to determine the abundance of GFPhp transcript in RNA from transgenic *Arabidopsis* plants. (C) RT-qPCR analysis of circular RNA of the GFPhp loop.

Figure 46. Treatment of wheat seedlings with ledTaVRN2 reduced the requirement of winter wheat for vernalisation prior to initiation of flowering. A) LedTaVRN2 treated

seeds of winter wheat variety CSIRO W7 flowered earlier than untreated or mock treated W7. B) The earlier flowering of W7 wheat treated with ledTaVRN2 caused fewer nodes to be dedicated to leaves. Meaning more nodes were dedicated to flowers/grain. Chinese Spring is a spring type wheat that does not require vernalisation, used as a control.

Figure 47. Treatment of the winter wheat variety CSIRO W7 with ledTaVRN2 induced earlier flowering compared to ledGFP, mock and no treatment controls. Early flowering parental genotypes Sunstate A (SSA) and Sunstate B (SSB) lacked a vernalisation response and were included as controls.

10

KEY TO THE SEQUENCE LISTING

- SEQ ID NO:1 – Ribonucleotide sequence of GFP ledRNA.
 SEQ ID NO:2 – Ribonucleotide sequence of GUS ledRNA.
 SEQ ID NO:3 – Ribonucleotide sequence of *N. benthamiana* FAD2.1 ledRNA.
 15 SEQ ID NO:4 – Nucleotide sequence encoding GFP ledRNA.
 SEQ ID NO:5 – Nucleotide sequence encoding GUS ledRNA.
 SEQ ID NO:6 – Nucleotide sequence encoding *N. benthamiana* FAD2.1 ledRNA.
 SEQ ID NO:7 – Nucleotide sequence encoding GFP.
 SEQ ID NO:8 – Nucleotide sequence encoding GUS.
 20 SEQ ID NO:9 – Nucleotide sequence encoding *N. benthamiana* FAD2.1.
 SEQ ID NO:10 – Nucleotide sequence used to provide the GUS sense region for constructs encoding hairpin RNA molecules targeting the GUS mRNA.
 SEQ ID NO:11 – Nucleotide sequence used to provide the GUS sense region for the construct encoding the hairpin RNA molecule hpGUS[G:U].
 25 SEQ ID NO:12 – Nucleotide sequence used to provide the GUS sense region for constructs encoding the hairpin RNA molecule hpGUS[1:4].
 SEQ ID NO:13 – Nucleotide sequence used to provide the GUS sense region for constructs encoding the hairpin RNA molecule hpGUS[2:10].
 SEQ ID NO:14 – Nucleotide sequence of nucleotides 781-1020 of the protein coding
 30 region of the GUS gene.
 SEQ ID NO:15 – Ribonucleotide sequence of the hairpin structure (including its loop) of the hpGUS[wt] RNA.
 SEQ ID NO:16 – Ribonucleotide of the hairpin structure (including its loop) of the hpGUS[G:U] RNA.
 35 SEQ ID NO:17 – Ribonucleotide of the hairpin structure (including its loop) of the hpGUS[1:4] RNA.

- SEQ ID NO:18 – Ribonucleotide of the hairpin structure (including its loop) of the hpGUS[2:10] RNA.
- SEQ ID NO:19 – Nucleotide sequence of the cDNA corresponding to the *A. thaliana* EIN2 gene, Accession No. NM_120406.
- 5 SEQ ID NO:20 – Nucleotide sequence of the cDNA corresponding to *A. thaliana* CHS gene, Accession No. NM_121396, 1703nt.
- SEQ ID NO:21 – Nucleotide sequence of a DNA fragment comprising a 200nt sense sequence from the cDNA corresponding to the *A. thaliana* EIN2 gene flanked by restriction enzyme sites.
- 10 SEQ ID NO:22 – Nucleotide sequence of a DNA fragment comprising the 200nt sense sequence of EIN2 as for SEQ ID NO:21 except that 43 C's were replaced with T's, used in constructing hpEIN2[G:U].
- SEQ ID NO:23 – Nucleotide sequence of a DNA fragment comprising a 200nt sense sequence from the cDNA corresponding to *A. thaliana* CHS gene flanked by restriction
- 15 enzyme sites.
- SEQ ID NO:24 – Nucleotide sequence of a DNA fragment comprising the 200nt sense sequence of CHS as for SEQ ID NO:23 except that 65 C's were replaced with T's, used in constructing hpCHS[G:U].
- SEQ ID NO:25 – Nucleotide sequence of a DNA fragment comprising the 200nt
- 20 antisense sequence of EIN2 with 50 C's replaced with T's, used in constructing hpEIN2[G:U/U:G].
- SEQ ID NO:26 – Nucleotide sequence of a DNA fragment comprising the 200nt antisense sequence of CHS with 49 C's replaced with T's, used in constructing hpCHS[G:U/U:G].
- 25 SEQ ID NO:27 – Nucleotide sequence of nucleotides 601-900 of the cDNA corresponding to the EIN2 gene from *A. thaliana* (Accession No. NM_120406).
- SEQ ID NO:28 – Nucleotide sequence of nucleotides 813-1112 of the cDNA corresponding to the CHS gene from *A. thaliana* (Accession No. NM_121396).
- SEQ ID NO:29 – Nucleotide sequence of the complement of nucleotides 652-891 of
- 30 the cDNA corresponding to the EIN2 gene from *A. thaliana* (Accession No. NM_120406).
- SEQ ID NO:30 – Nucleotide sequence of the complement of nucleotides 804-1103 of the cDNA corresponding to the CHS gene from *A. thaliana*.
- SEQ ID NO:31 – FANCM I protein coding region of the cDNA of *Arabidopsis*
- 35 *thaliana*, Accession No. NM_001333162. Target region nucleotides 675-1174 (500 nucleotides)

- SEQ ID NO:32 – FANCM I protein coding region of a cDNA of *Brassica napus*.
Target region nucleotides 896-1395 (500 bp)
- SEQ ID NO:33 – Nucleotide sequence encoding hpFANCM-At[wt] targeting the
FANCM I protein coding region of *A. thaliana*. FANCM sense sequence, nucleotides
5 38-537; loop sequence, nucleotides 538-1306; FANCM antisense sequence, nucleotides
1307-1806.
- SEQ ID NO:34 – Nucleotide sequence encoding hpFANCM-At[G:U] targeting the
FANCM I protein coding region of *A. thaliana*. FANCM sense sequence, nucleotides
38-537; loop sequence, nucleotides 538-1306; FANCM antisense sequence, nucleotides
10 1307-1806.
- SEQ ID NO:35 – Nucleotide sequence encoding hpFANCM-Bn[wt] targeting the
FANCM I protein coding region of *B. napus*. FANCM sense sequence, nucleotides 34-
533; loop sequence, nucleotides 534-1300; FANCM antisense sequence, nucleotides
1301-1800.
- 15 SEQ ID NO:36 – Nucleotide sequence encoding hpFANCM-Bn[G:U] targeting the
FANCM I protein coding region of *B. napus*. FANCM sense sequence, nucleotides 34-
533; loop sequence, nucleotides 534-1300; FANCM antisense sequence, nucleotides
1301-1800.
- SEQ ID NO:37 – Nucleotide sequence of the protein coding region of the cDNA
20 corresponding to the *B. napus* DDM1 gene; Accession No. XR_001278527.
- SEQ ID NO:38 – Nucleotide sequence of DNA encoding hpDDM1-Bn[wt] targeting
the DDM1 protein coding region of *B. napus*.
- SEQ ID NO:39 – Nucleotide sequence encoding hpDDM1-Bn[G:U] targeting the
DDM1 protein coding region of *B. napus*. DDM1 sense sequence, nucleotides 35-536;
25 loop sequence, nucleotides 537-1304; DDM1 antisense sequence, nucleotides 1305-
1805.
- SEQ ID NO:40 – EGFP cDNA.
- SEQ ID NO:41 – Nucleotide sequence of the coding region of hpEGFP[wt], with the
order antisense/ loop/ sense with respect to the promoter.
- 30 SEQ ID NO:42 – Nucleotide sequence of the coding region of hpEGFP[G:U] which
has 157 C to T substitutions in the EGFP sense sequence.
- SEQ ID NO:43 – Nucleotide sequence of the coding region of ledEGFP[wt] which has
no C to T substitutions in the EGFP sense sequence.
- SEQ ID NO:44 – Nucleotide sequence of the coding region of ledEGFP[G:U] which
35 has 162 C to T substitutions in the EGFP sense sequence.

- SEQ ID NO:45 – Nucleotide sequence used to provide the GUS sense region for the construct encoding the hairpin RNA molecule hpGUS[G:U] without flanking restriction enzyme sites.
- SEQ ID NO:46 – Nucleotide sequence used to provide the GUS sense region for
5 constructs encoding the hairpin RNA molecule hpGUS[1:4] without flanking restriction enzyme sites.
- SEQ ID NO:47 – Nucleotide sequence used to provide the GUS sense region for constructs encoding the hairpin RNA molecule hpGUS[2:10] without flanking restriction enzyme sites.
- 10 SEQ ID NO:48 – Nucleotide sequence of a DNA fragment comprising the 200nt sense sequence of EIN2 as for SEQ ID NO:21 except that 43 C's were replaced with T's, used in constructing hpEIN2[G:U] without flanking sequences.
- SEQ ID NO:49 – Nucleotide sequence of a DNA fragment comprising the 200nt sense
15 sequence of CHS as for SEQ ID NO:23 except that 65 C's were replaced with T's, used in constructing hpCHS[G:U] without flanking sequences.
- SEQ ID NO:50 – Nucleotide sequence of a DNA fragment comprising the 200nt antisense sequence of EIN2 with 50 C's replaced with T's, used in constructing hpEIN2[G:U/U:G] without flanking sequences
- SEQ ID NO:51 – Nucleotide sequence of a DNA fragment comprising the 200nt
20 antisense sequence of CHS with 49 C's replaced with T's, used in constructing hpCHS[G:U/U:G] without flanking sequences.
- SEQ ID NO:52 – Oligonucleotide primer used for amplifying the 200 bp GUS sense sequence (GUS-WT-F)
- SEQ ID NO:53 – Oligonucleotide primer used for amplifying the 200 bp GUS sense
25 sequence (GUS-WT-R)
- SEQ ID NO:54 – Oligonucleotide primer (forward) used for producing the hpGUS[G:U] fragment with every C replaced with T (GUS-GU-F)
- SEQ ID NO:55 – Oligonucleotide primer (reverse) used for producing the hpGUS[G:U] fragment with every C replaced with T (GUS-GU-R)
- 30 SEQ ID NO:56 – Oligonucleotide primer (forward) used for producing the hpGUS[1:4] fragment with every 4th nucleotide substituted (GUS-4M-F)
- SEQ ID NO:57 – Oligonucleotide primer (reverse) used for producing the hpGUS[1:4] fragment with every 4th nucleotide substituted (GUS-4M-R)
- SEQ ID NO:58 – Oligonucleotide primer (forward) used for producing the
35 hpGUS[2:10] fragment with every 9th and 10th nucleotide substituted (GUS-10M-F)

- SEQ ID NO:59 – Oligonucleotide primer (reverse) used for producing the hpGUS[2:10] fragment with every 9th and 10th nucleotide substituted (GUS-10M-R)
- SEQ ID NO:60 – Nucleotide sequence encoding forward primer (35S-F3)
- SEQ ID NO:61 – Nucleotide sequence encoding reverse primer (GUSwt-R2)
- 5 SEQ ID NO:62 – Nucleotide sequence encoding forward primer (GUSgu-R2)
- SEQ ID NO:63 – Nucleotide sequence encoding reverse primer (GUS4m-R2)
- SEQ ID NO:64 – Nucleotide sequence encoding forward primer (35S-F2)
- SEQ ID NO:65 – Nucleotide sequence encoding reverse primer (35S-R1)
- SEQ ID NO:66 – Oligonucleotide primer used for amplifying the wild-type 200 bp
- 10 EIN2 sense sequence (EIN2wt-F)
- SEQ ID NO:67 – Oligonucleotide primer used for amplifying the wild-type 200 bp EIN2 sense sequence (EIN2wt-R)
- SEQ ID NO:68 – Oligonucleotide primer used for amplifying the wild-type 200 bp CHS sense sequence (CHSwt-F)
- 15 SEQ ID NO:69 – Oligonucleotide primer used for amplifying the wild-type 200 bp CHS sense sequence (CHSwt-R)
- SEQ ID NO:70 – Oligonucleotide primer (forward) used for producing the hpEIN2[G:U] fragment, with every C replaced with T (EIN2gu-F)
- SEQ ID NO:71 – Oligonucleotide primer (reverse) used for producing the
- 20 hpEIN2[G:U] fragment, with every C replaced with T (EIN2gu-R)
- SEQ ID NO:72 – Oligonucleotide primer (forward) used for producing the hpCHS[G:U] fragment, with every C replaced with T (CHSgu-F)
- SEQ ID NO:73 – Oligonucleotide primer (reverse) used for producing the hpCHS[G:U] fragment, with every C replaced with T (CHSgu-R)
- 25 SEQ ID NO:74 – Oligonucleotide primer (forward) used for producing the hpEIN2[G:U/U:G] fragment, with every C replaced with T (asEIN2gu-F)
- SEQ ID NO:75 – Oligonucleotide primer (reverse) used for producing the hpEIN2[G:U/U:G] fragment with every C replaced with T (asEIN2gu-R)
- SEQ ID NO:76 – Oligonucleotide primer (forward) used for producing the
- 30 hpCHS[G:U/U:G] fragment, with every C replaced with T (asCHSgu-F)
- SEQ ID NO:77 – Oligonucleotide primer (reverse) used for producing the hpCHS[G:U/U:G] fragment, with every C replaced with T (asCHSgu-R)
- SEQ ID NO:78 – Nucleotide sequence encoding forward primer (CHS-200-F2)
- SEQ ID NO:79 – Nucleotide sequence encoding reverse primer (CHS-200-R2)
- 35 SEQ ID NO:80 – Nucleotide sequence encoding forward primer (Actin2-For)
- SEQ ID NO:81 – Nucleotide sequence encoding reverse primer (Actin2-Rev)

- SEQ ID NO:82 – Nucleotide sequence encoding forward primer (Top-35S-F2)
 SEQ ID NO:83 – Nucleotide sequence encoding reverse primer (Top-35S-R2)
 SEQ ID NO:84 – Nucleotide sequence encoding forward primer (Link-35S-F2)
 SEQ ID NO:85 – Nucleotide sequence encoding reverse primer (Link-EIN2-R2)
- 5 SEQ ID NO:86 – Ribonucleotide sequence of sense si22
 SEQ ID NO:87 – Ribonucleotide sequence of antisense si22
 SEQ ID NO:88 – Ribonucleotide sequence of forward primer
 SEQ ID NO:89 – Ribonucleotide sequence of reverse primer
 SEQ ID NO:90 – Ribonucleotide sequence of forward primer
- 10 SEQ ID NO:91 – Ribonucleotide sequence of reverse primer
 SEQ ID NO:92 – Possible modifications of dsRNA molecules
 SEQ ID NO:93 – Nucleotide sequence of a cDNA corresponding to the *Brassica napus* *DDMI* gene (Accession No. XR_001278527).
 SEQ ID NO:94 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi
- 15 (hpRNA) construct targeting a *DDMI* gene of *B. napus*.
 SEQ ID NO:95 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi (hpRNA) construct with G:U basepairs, targeting a *DDMI* gene of *B. napus*.
 SEQ ID NO:96 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct, targeting a *DDMI* gene of *B. napus*.
- 20 SEQ ID NO:97 – Nucleotide sequence of cDNA corresponding to *A. thaliana* *FANCM* gene (Accession No. NM_001333162).
 SEQ ID NO:98 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi (hpRNA) construct targeting a *FANCM* gene of *A. thaliana*.
 SEQ ID NO:99 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi
- 25 (hpRNA) construct with G:U basepairs, targeting a *FANCM* gene of *A. thaliana*.
 SEQ ID NO:100 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct, targeting a *FANCM* gene of *A. thaliana*.
 SEQ ID NO:101 – Nucleotide sequence of cDNA corresponding to *B. napus* *FANCM* gene (Accession No. XM_022719486.1).
- 30 SEQ ID NO:102 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi (hpRNA) construct targeting a *FANCM* gene of *B. napus*.
 SEQ ID NO:103 – Nucleotide sequence of a chimeric DNA encoding a hairpin RNAi (hpRNA) construct with G:U basepairs, targeting a *FANCM* gene of *B. napus*.
 SEQ ID NO:104 – Nucleotide sequence of a chimeric DNA encoding a ledRNA
- 35 construct, targeting a *FANCM* gene of *B. napus*.

- SEQ ID NO:105 – Nucleotide sequence of the protein coding region of the cDNA corresponding to the *Nicotiana benthamiana* TOR gene.
- SEQ ID NO:106 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a TOR gene of *N. benthamiana*.
- 5 SEQ ID NO:107 – Nucleotide sequence of the protein coding region of the cDNA corresponding to the acetolactate synthase (ALS) gene of barley, *Hordeum vulgare* (Accession No. LT601589).
- SEQ ID NO:108 – Nucleotide sequence of a chimeric DNA encoding a ledRNA targeting the ALS gene of barley (*H. vulgare*).
- 10 SEQ ID NO:109 – Nucleotide sequence of the protein coding region of the cDNA corresponding to the *HvNCED1* gene of barley *Hordeum vulgare* (Accession No. AK361999).
- SEQ ID NO:110 – Nucleotide sequence the protein coding region of the cDNA corresponding to the *HvNCED2* gene of barley *Hordeum vulgare* (Accession No.
- 15 DQ145931).
- SEQ ID NO:111 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting the *NCED1* genes of barley *Hordeum vulgare* and wheat *Triticum aestivum*.
- SEQ ID NO:112 – Nucleotide sequence of a chimeric DNA encoding a ledRNA
- 20 construct targeting the *NCED2* genes of barley *Hordeum vulgare* and wheat *Triticum aestivum*.
- SEQ ID NO:113 – Nucleotide sequence of the protein coding region of a cDNA corresponding to the barley gene encoding ABA-OH-2 (Accession No. DQ145933).
- SEQ ID NO:114 – Nucleotide sequence of a chimeric DNA encoding a ledRNA
- 25 construct targeting the ABA-OH-2 genes of barley *Hordeum vulgare* and wheat *Triticum aestivum*.
- SEQ ID NO:115 – Nucleotide sequence of the protein coding region of a cDNA corresponding to the *A. thaliana* gene encoding EIN2 (At5g03280).
- SEQ ID NO:116 – Nucleotide sequence of a chimeric DNA encoding a ledRNA
- 30 construct targeting the EIN2 gene of *A. thaliana*.
- SEQ ID NO:117 – Nucleotide sequence of the protein coding region of a cDNA corresponding to the *A. thaliana* gene encoding CHS (Accession No. NM_121396).
- SEQ ID NO:118 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting the *CHS* gene of *A. thaliana*.
- 35 SEQ ID NO:119 – Nucleotide sequence of the protein coding region of a cDNA corresponding to the *L. angustifolius* N-like gene (Accession No. XM_019604347).

- SEQ ID NO:120 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting the *L. angustifolius* N-like gene.
- SEQ ID NO:121 – Nucleotide sequence of the protein coding region of a cDNA corresponding to a *Vitis pseudoreticulata* MLO gene (Accession No. KR362912).
- 5 SEQ ID NO:122 – Nucleotide sequence of a chimeric DNA encoding a first ledRNA construct targeting a *Vitis* MLO gene.
- SEQ ID NO:123 – Nucleotide sequence of the protein coding region of the cDNA corresponding to the MpC002 gene of *Myzus persicae*.
- SEQ ID NO:124 – Nucleotide sequence of the protein coding region of the cDNA
10 corresponding to the MpRack-1 gene of *Myzus persicae*.
- SEQ ID NO:125 – Nucleotide sequence of the chimeric construct encoding the ledRNA targeting *M.persicae* C002 gene.
- SEQ ID NO:126 – Nucleotide sequence of the chimeric construct encoding the ledRNA targeting *M.persicae* Rack-1 gene.
- 15 SEQ ID NO:127 – Nucleotide sequence of the cDNA corresponding to the *Helicoverpa armigera* ABCwhite gene.
- SEQ ID NO:128 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a ABC transporter white gene of *Helicoverpa armigera*.
- SEQ ID NO:129 – Nucleotide sequence of the cDNA corresponding to the *Linepithema*
20 *humile* PBAN-type neuropeptides-like (XM_012368710).
- SEQ ID NO:130 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a PBAN gene in Argentine ants (Accession No. XM_012368710).
- SEQ ID NO:131 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding V-type proton ATPase catalytic subunit A
25 (Accession No. XM_023443547) of *L. cuprina*.
- SEQ ID NO:132 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding RNase 1/2 of *L. cuprina*.
- SEQ ID NO:133 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding chitin synthase of *L. cuprina*.
- 30 SEQ ID NO:134 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding ecdysone receptor (EcR) of *L. cuprina*.
- SEQ ID NO:135 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding gamma-tubulin 1/1-like of *L. cuprina*.
- SEQ ID NO:136 – *TaMlo* target gene (AF384144).
- 35 SEQ ID NO:137 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding *TaMlo*.

- SEQ ID NO:138 – Nucleotide sequence of the protein coding region of a cDNA corresponding to a *Vitis pseudoreticulata MLO* gene (Accession No. KR362912).
- SEQ ID NO:139 – Nucleotide sequence of a chimeric DNA encoding a first ledRNA construct targeting a *Vitis MLO* gene.
- 5 SEQ ID NO:140 – *Cyp51* homolog 1 (Accession No. KK764651.1, locus RSAG8_00934).
- SEQ ID NO:141 – *Cyp51* homolog 2 (Accession No. KK764892.1, locus number RSAG8_12664).
- SEQ ID NO:142 – Nucleotide sequence of a chimeric DNA encoding a ledRNA
- 10 construct targeting a gene encoding *Cyp51*.
- SEQ ID NO:143 – *CesA3* target gene (Accession No. JN561774.1).
- SEQ ID NO:144 – Nucleotide sequence of a chimeric DNA encoding a ledRNA construct targeting a gene encoding *CesA3*.
- SEQ ID NO:145 – *VRN2B* gene sequence (*Triticum monococcum*).
- 15 SEQ ID NO:146 – LED- VRN 2 construct.
- SEQ ID NO:147 – VRN2 stem sequence.
- SEQ ID NO:148 - LED- VRN 2 construct Loop sequence 1.
- SEQ ID NO:149 - LED- VRN 2 construct Loop sequence 2.
- SEQ ID NO:150 - Sequence encoding the LedVRN2 molecule.
- 20 SEQ ID NO:151 - Nucleotide sequence of the cDNA for *Triticum aestivum* cultivar Chinese Spring VRN-A1 cDNA protein coding sequence (TaVRN1-A1, Accession No. KR422423.1).
- SEQ ID NO:152 - Nucleotide sequence of the cDNA for *Triticum aestivum* flowering locus T cDNA sequence (TaFT, Accession No. AY705794.1). The protein coding
- 25 sequence is nucleotides 19-549.
- SEQ ID NO:153 - Nucleotide sequence of the cDNA sequence for *Hordeum vulgare* subsp. *spontaneum* MADS box transcription factor (HvVRN1, Accession No. AY896051) gene. The protein coding sequence is nucleotides 8-403.
- SEQ ID NO:154 - Nucleotide sequence of the cDNA for *Hordeum vulgare* cultivar
- 30 Dairokkaku ZCCT-Hb (HvVRN2, Accession No. AY485978) gene, partial cDNA.
- SEQ ID NO:155 - Nucleotide sequence of the cDNA for *Hordeum vulgare* cultivar Stander FT protein (HvFT, Accession No. DQ898519) gene.
- SEQ ID NO:156 - Nucleotide sequence of the cDNA for *Oryza sativa* Japonica Group phytochrome B-like gene, transcript variant X1 (OsPhyB, LOC4332623,
- 35 OSNPB_030309200).

- SEQ ID NO:157 - Nucleotide sequence of the cDNA for *Oryza sativa* Constans-like 4 gene, OsCol4 protein (Accession No. HC084637).
- SEQ ID NO:158 - Nucleotide sequence of the cDNA sequence of the *Oryza sativa* Japonica Group protein RFT1 homolog (OsRFT1, LOC4343254, OSNPB_070486100) gene. The protein coding sequence is nucleotides 167-1753.
- 5 SEQ ID NO:159 - Nucleotide sequence of the cDNA sequence of the *Oryza sativa* AP2-like ethylene-responsive transcription factor TOE3 OsSNB (OSNPB_070235800). The protein coding sequence is nucleotides 213-1520.
- SEQ ID NO:160 - Nucleotide sequence of the cDNA sequence for *Oryza sativa* Japonica Group AP2-like ethylene-responsive transcription factor TOE3 gene, transcript variant X1, (OsIDS1, LOC4334582, Os03g0818800). The protein coding sequence is nucleotides 575-1876.
- 10 SEQ ID NO:161 - Nucleotide sequence of the cDNA sequence for *Oryza sativa* Japonica Group GIGANTEA-like gene, transcript variant X1, (OsGI, LOC4325329, OSNPB_010182600). The protein coding sequence is nucleotides 440-3919.
- 15 SEQ ID NO:162 - Nucleotide sequence of the cDNA sequence for *Oryza sativa* OsMADS50 (homolog of AtSOC1) (HC084627). The protein coding sequence is nucleotides 23-712.
- SEQ ID NO:163 - Nucleotide sequence of the cDNA sequence for *Oryza sativa* Japonica Group OsMADS55 (homolog of AtSOC1) (Accession No. AY345223).
- 20 SEQ ID NO:164 - Nucleotide sequence of the cDNA sequence for *Oryza sativa* Japonica Group transcription factor FL (OsLFY, LOC4336857, Os04g0598300). The protein coding sequence is nucleotides 233-1399.
- SEQ ID NO:165 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* cultivar Assiniboine *ZmMADS1/ZmM5* (LOC542042, Accession No. HM993639), partial sequence.
- 25 SEQ ID NO:166 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* cultivar B73 phytochrome A1 apoprotein PHYA1 (Accession No. AY234826). Protein coding region is nucleotides 118-3510.
- 30 SEQ ID NO:167 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* phytochrome A2 apoprotein PHYA2 (LOC115101004, Accession No. AY260865). Protein coding region is nucleotides 141-3533.
- SEQ ID NO:168 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* phytochrome B1 apoprotein PHYB1 (LOC100383702, Accession No. AY234827). Protein coding region is nucleotides 1-3483.
- 35

- SEQ ID NO:169 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* phytochrome B2 apoprotein PHYB2 (Accession No. AY234828). Protein coding region is nucleotides 1-3498.
- SEQ ID NO:170 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* phytochrome C1 apoprotein PHYC1 (Accession No. AY234829). Protein coding region is nucleotides 48-3455.
- SEQ ID NO:171 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* phytochrome C2 apoprotein PHYC2 (Accession No. AY234830). Protein coding region is nucleotides 141-3533.
- 10 SEQ ID NO:172 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* flowering-time protein isoforms alpha and beta (ZmLD), alternatively spliced products (Accession No. AF166527). Protein coding region is nucleotides 122-3669.
- SEQ ID NO:173 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* cultivar A632 floricaula/leafy-like 1 (ZmFL1) (Accession No. AY179882).
- 15 Protein coding region is nucleotides 27-1199.
- SEQ ID NO:174 - Nucleotide sequence of the cDNA of gene encoding *Zea mays* cultivar A632 floricaula/leafy-like 2 (ZmFL2) (Accession No. AY789023).
- SEQ ID NO:175 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* cultivar A554 DWARF8 gene (Accession No. AF413203), partial cDNA.
- 20 SEQ ID NO:176 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* kaurene synthase A (ZmAN1 protein, Accession No. L37750). Protein coding region is nucleotides 105-2573.
- SEQ ID NO:177 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* zinc finger protein ID1 (ZmID1 protein, Accession No. AF058757). Protein coding region is nucleotides 112-1419.
- 25 SEQ ID NO:178 - Nucleotide sequence of the cDNA sequence of gene encoding *Zea mays* ZCN8 (ZmCN8 protein, LOC100127519). Protein coding region is nucleotides 60-672.
- SEQ ID NO:179 - Nucleotide sequence of the cDNA for *Brassica napus* MADS-box (FLC1) protein gene (BnFLC1-A10, Accession No. AY036888, BnaA10g22080D). The protein coding sequence is nucleotides 68-658.
- 30 SEQ ID NO:180 - Nucleotide sequence of the cDNA for *Brassica napus* MADS-box protein (FLC2) gene (BnFLC2, Accession No. AY036889). The protein coding sequence is nucleotides 34-621.

- SEQ ID NO:181 - Nucleotide sequence of the cDNA for *Brassica napus* MADS-box protein (FLC3) (BnFLC3, Accession No. AY036890). The protein coding sequence is nucleotides 46-636.
- SEQ ID NO:182 - Nucleotide sequence of the cDNA for *Brassica napus* MADS-box protein (FLC4) (BnFLC4, Accession No. AY036891). The protein coding sequence is nucleotides 147-734.
- SEQ ID NO:183 - Nucleotide sequence of the cDNA for *Brassica napus* MADS-box protein (FLC5) (BnFLC5, Accession No. AY036892). The protein coding sequence is nucleotides 63-736.
- 10 SEQ ID NO:184 - Nucleotide sequence of the cDNA for *Brassica napus* Frigida gene (BnFRI, BnaA03g13320D).
- SEQ ID NO:185 - Nucleotide sequence of the cDNA for *Brassica napus* linkage group A2 flowering locus T (FT) gene (BnFT, BnaA02g12130D).
- SEQ ID NO:186 - Nucleotide sequence of the cDNA sequence for *Medicago truncatula* cultivar Jester FTa1 protein (MtFTa1, Accession No. HQ721813) gene. The protein coding sequence is nucleotides 233-1399.
- 15 *truncatula* cultivar Jester FTb1 protein (MtFTb1, Accession No. HQ721815) gene. The protein coding sequence is nucleotides 233-1399.
- SEQ ID NO:187 - Nucleotide sequence of the cDNA sequence for *Medicago truncatula* cultivar Jester FTb1 protein (MtFTb1, Accession No. HQ721815) gene. The protein coding sequence is nucleotides 233-1399.
- 20 SEQ ID NO:188 - Nucleotide sequence of the cDNA sequence for *Medicago sativa* Frigida-like protein mRNA, (MsFRI-L, Accession No. JX173068, Chao et al., 2013). The protein coding sequence is nucleotides 7-1563.
- SEQ ID NO:189 - Nucleotide sequence of the cDNA sequence for *Medicago sativa* subsp. *caerulea* shatterproof mRNA, (MsSOC1a/ McaeSHP; Accession No. JX297565). The protein coding sequence is from nucleotide 31.
- 25 SEQ ID NO:190 - Nucleotide sequence of the cDNA sequence for *Medicago sativa* FT (FT) gene, (MsFT, Accession No. JF681135).
- SEQ ID NO:191 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C (GmFLC) encoded by the gene GLYMA_05G148700 (Accession No. XM_014775674, LOC100804540), transcript variant X1, mRNA. The protein coding sequence is nucleotides 90-686.
- 30 SEQ ID NO:192 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene GLYMA_05G148700 (Accession No. XM_003524857.4), transcript variant X2. The protein coding sequence is nucleotides 72-665.
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- SEQ ID NO:193 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene GLYMA_05G148700 (Accession No. XR_001388453), transcript variant X3. The protein coding sequence is nucleotides 90-653.
- 5 SEQ ID NO:194 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene GLYMA_05G148700 (Accession No. XM_006580064), transcript variant X4. The protein coding sequence is nucleotides 90-641.
- SEQ ID NO:195 - Nucleotide sequence of the cDNA sequence for *Glycine max*
- 10 MADS-box protein FLOWERING LOCUS C encoded by the gene GLYMA_05G148700 (Accession No. XM_006580065), transcript variant X5. The protein coding sequence is nucleotides 90-605.
- SEQ ID NO:196 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene
- 15 GLYMA_05G148700 (Accession No. XR_414429.3), transcript variant X6. The protein coding sequence is nucleotides 90-587.
- SEQ ID NO:197 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene
- 20 GLYMA_05G148700 (Accession No. XM_014775675), transcript variant X7. The protein coding sequence is nucleotides 90-587.
- SEQ ID NO:198 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene
- GLYMA_05G148700 (Accession No. XM_014775676), transcript variant X8. The protein coding sequence is nucleotides 90-587.
- 25 SEQ ID NO:199 - Nucleotide sequence of the cDNA sequence for *Glycine max* MADS-box protein FLOWERING LOCUS C encoded by the gene GLYMA_05G148700 (Accession No. XM_006580067), transcript variant X9. The protein coding sequence is nucleotides 90-575.
- SEQ ID NO:200 - Nucleotide sequence of the cDNA sequence of gene encoding
- 30 *Glycine max* protein SUPPRESSOR OF FRI 4 (LOC100819009), transcript variant X3. (Accession No. XM_003530888). The protein coding sequence is nucleotides 145-1257.
- SEQ ID NO:201 - Nucleotide sequence of the cDNA sequence of gene encoding
- Glycine max* protein FRIGIDA-like protein 4a (GmFRI4a, LOC100805780, Accession
- 35 No. NM_001360372). The protein coding sequence is nucleotides 77-1828.

- SEQ ID NO:202 - Nucleotide sequence of the cDNA sequence of gene encoding *Glycine max* protein protein FLOWERING LOCUS T (FT2A, GLYMA_16G150700, Accession No. NM_001253256). The protein coding sequence is nucleotides 78-605.
- 5 SEQ ID NO:203 - Nucleotide sequence of the cDNA sequence of gene encoding *Glycine max* protein phytochrome A, transcript variant X3 (GmPhyA3, Accession No. XM_014771785.2). The protein coding sequence is nucleotides 615-3899.
- SEQ ID NO:204 - Nucleotide sequence of the cDNA sequence of gene encoding *Glycine max* protein protein GIGANTEA, transcript variant 1 (GmGIGANTEA Accession No. NM_001354790). The protein coding sequence is nucleotides 419-3946.
- 10 SEQ ID NO:205 - Nucleotide sequence of the cDNA sequence of gene encoding *Beta vulgaris* subsp. *vulgaris* genotype KWS2320 bolting time control 1 (BTC1, Accession No. HQ709091). Protein coding region is nucleotides 307-2670.
- SEQ ID NO:206 - Nucleotide sequence of the cDNA sequence of gene encoding *Beta vulgaris* flowering locus T-like protein (FT1) gene (BvFT1, Accession No. 15 HM448909).
- SEQ ID NO:207 - Nucleotide sequence of the cDNA sequence of gene encoding *Beta vulgaris* flowering locus T-like protein (FT2) gene (BvFT2, Accession No. HM448911).
- SEQ ID NO:208 - Nucleotide sequence of the cDNA sequence of gene encoding 20 *Brassica rapa* cultivar IMB 218dh FLC2 (FLC2, Accession No. AH012704), partial sequence.
- SEQ ID NO:209 - Nucleotide sequence of the cDNA sequence of gene encoding *Brassica rapa* FRIGIDA (FRI, Accession No. HQ615935).
- SEQ ID NO:210 - Nucleotide sequence of the cDNA sequence of *Medicago truncatula* 25 clone MTYFL_FM_FN_FO1G-C-11 (MtYFL, Accession No. BT053010). Protein coding region is nucleotides 78-1136.
- SEQ ID NO:211 - Nucleotide sequence of the cDNA sequence of *Allium cepa* GIGANTEA (GIa) (AcGIa, Accession No. GQ232756). Protein coding region is nucleotides 27-3353.
- 30 SEQ ID NO:212 - Nucleotide sequence of the cDNA sequence of *Allium cepa* FKF1 (FKF1, Accession No. GQ232754). Protein coding region is nucleotides 53-1905.
- SEQ ID NO:213 - Nucleotide sequence of the cDNA sequence of *Allium cepa* ZEITLUPE (AcZTL, Accession No. GQ232755). Protein coding region is nucleotides 128-1963.

- SEQ ID NO:214 - Nucleotide sequence of the cDNA sequence of *Allium cepa* ACABR20 CONSTANS-like protein (AcCOL, Accession No. GQ232751). Protein coding region is nucleotides 22-972.
- SEQ ID NO:215 - Nucleotide sequence of the cDNA sequence of *Allium cepa*
5 ACAEE96 protein (AcFTL, Accession No. CF438000). Protein coding region is nucleotides 396-818.
- SEQ ID NO:216 - Nucleotide sequence of the cDNA sequence of *Allium cepa* cultivar CUDH2150 FT1 (AcFT1, Accession No. KC485348). Protein coding region is nucleotides 1-534.
- 10 SEQ ID NO:217 - Nucleotide sequence of the cDNA sequence of *Allium cepa* cultivar CUDH2150 FT2 (AcFT2, Accession No. KC485349). Protein coding region is nucleotides 42-566.
- SEQ ID NO:218 - Nucleotide sequence of the cDNA sequence of *Allium cepa* cultivar CUDH2150 FT6 (AcFT6, Accession No. KC485353). Protein coding region is
15 nucleotides 6-560.
- SEQ ID NO:219 - Nucleotide sequence of the cDNA sequence of *Allium cepa* clone ACAGK28 phytochrome A (PHYA) (AcPHYA, Accession No. GQ232753), partial sequence. Protein coding region is nucleotides 1-1119.
- SEQ ID NO:220 - Nucleotide sequence of the cDNA sequence of *Allium cepa* clone
20 ACADQ29 COP1 (AcCOP1, Accession No. CF451443). Protein coding region is nucleotides 249-647.
- SEQ ID NO:221 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* protein HEADING DATE 3A-like protein (LsFT, LOC111907824). Protein coding region is nucleotides 71-595.
- 25 SEQ ID NO:222 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* protein MOTHER of FT and TFL1-like (LsFL1-like, LOC111903066, Accession No. XM_023898861).
- SEQ ID NO:223 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* protein MOTHER of FT and TFL1 homolog 1-like (LsTFL1, LOC111903054,
30 Accession No. XM_023898849).
- SEQ ID NO:224 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* FLC (LsFLC, LOC111876490, Accession No. JI588382).
- SEQ ID NO:225 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* MADS-box protein SOC1-like (LsSOC1, LOC111912847, Accession No.
35 XM_023908569). Protein coding region is nucleotides 159-809.

SEQ ID NO:226 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* MADS-box protein SOC1-like (LsSOC1-like, LOC111880753, Accession No. XM_023877169), transcript variant X1. Protein coding region is nucleotides 129-782.

5 SEQ ID NO:227 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* MADS-box protein SOC1-like (LsSOC1-like, LOC111878575). Protein coding region is nucleotides 166-819.

SEQ ID NO:228 - Nucleotide sequence of the cDNA sequence of *Lactuca sativa* floricaula/leafy homolog (LsLFY, LOC111892192, Accession No. XM_023888266). Protein coding region is nucleotides 1-1278.

10 SEQ ID NO:229 and SEQ ID NO:230 – Oligonucleotide primers.

DETAILED DESCRIPTION OF THE INVENTION

General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used
15 herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, gene silencing, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures,
20 well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, *A Practical Guide to Molecular Cloning*, John Wiley and Sons (1984), J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), *Essential Molecular Biology: A Practical Approach*, Volumes 1 and 2, IRL
25 Press (1991), D.M. Glover and B.D. Hames (editors), *DNA Cloning: A Practical Approach*, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) *Antibodies: A Laboratory Manual*, Cold Spring Harbour Laboratory, (1988),
30 and J.E. Coligan *et al.* (editors) *Current Protocols in Immunology*, John Wiley & Sons (including all updates until present).

The term “antisense regulatory element” or “antisense ribonucleic acid sequence” or “antisense RNA sequence” as used herein means an RNA sequence that is
35 hybridizes. In certain embodiments, an antisense RNA sequence modulates (increases or decreases) the expression or amount of a target RNA molecule or its activity, for

example through reducing translation of the target RNA molecule. In certain embodiments, an antisense RNA sequence alters splicing of a target pre-mRNA resulting in a different splice variant. Exemplary components of antisense sequences include, but are not limited to, oligonucleotides, oligonucleosides, oligonucleotide
5 analogues, oligonucleotide mimetics, and chimeric combinations of these.

The term “antisense activity” is used in the context of the present disclosure to refer to any detectable and/or measurable activity attributable to the hybridization of an antisense RNA sequence to its target RNA molecule. Such detection and/or measuring may be direct or indirect. In an embodiment, antisense activity is assessed by detecting
10 and or measuring the amount of target RNA molecule transcript. Antisense activity may also be detected as a change in a phenotype associated with the target RNA molecule.

As used herein, the term “target RNA molecule” refers to a gene transcript that is modulated by an antisense RNA sequence according to the present disclosure.
15 Accordingly, “target RNA molecule” can be any RNA molecule the expression or activity of which is capable of being modulated by an antisense RNA sequence. Exemplary target RNA molecules include, but are not limited to, RNA (including, but not limited to pre-mRNA and mRNA or portions thereof) transcribed from DNA encoding a target protein, rRNA, tRNA, small nuclear RNA, and miRNA, including
20 their precursor forms. The target RNA may be the genomic RNA of a plant, or an RNA molecule derived therefrom. For example, the target RNA molecule can be an RNA from an endogenous gene (or mRNA transcribed from the gene) or a gene which is introduced or may be introduced into the plant cell whose expression is associated with a particular phenotype, trait, disorder or disease state, or a nucleic acid molecule
25 from an infectious agent. In an embodiment, the target RNA molecule is in a plant cell. In another example, the target RNA molecule encodes a protein. In this context, antisense activity can be assessed by detecting and or measuring the amount of target protein, for example through its activity such as enzyme activity, or a function other than as an enzyme, or through a phenotype associated with its function. As used
30 herein, the term “target protein” refers to a protein that is modulated by an antisense RNA sequence according to the present disclosure.

In certain embodiments, antisense activity is assessed by detecting and/or measuring the amount of target RNA molecules and/or cleaved target RNA molecules and/or alternatively spliced target RNA molecules.

Antisense activity can be detected or measured using various methods. For example, antisense activity can be detected or assessed by comparing activity in a particular sample and comparing the activity to that of a control sample.

The term “targeting” is used in the context of the present disclosure to refer to
5 the association of an antisense RNA sequence to a particular target RNA molecule or a particular region of nucleotides within a target RNA molecule. In an example, an antisense RNA sequence according to the present disclosure shares complementarity with at least a region of a target RNA molecule. In this context, the term “complementarity” refers to a sequence of ribonucleotides that is capable of base
10 pairing with a sequence of ribonucleotides on a target RNA molecule, through hydrogen bonding between bases on the ribonucleotides. For example, in RNA, adenine (A) is complementary to uracil (U) and guanine (G) to cytosine (C).

In certain embodiments, “complementary base” refers to a ribonucleotide of an antisense RNA sequence that is capable of base pairing with a ribonucleotide of a sense
15 RNA sequence in an RNA molecule of the invention or of its target RNA molecule. For example, if a ribonucleotide at a certain position of an antisense RNA sequence is capable of hydrogen bonding with a ribonucleotide at a certain position of a target RNA molecule, then the position of hydrogen bonding between the antisense RNA sequence and the target RNA molecule is considered to be complementary at that
20 ribonucleotide. In contrast, the term “non-complementary” refers to a pair of ribonucleotides that do not form hydrogen bonds with one another or otherwise support hybridization. The term “complementary” can also be used to refer to the capacity of an antisense RNA sequence to hybridize to another nucleic acid through complementarity. In certain embodiments, an RNA sequence and its target are
25 complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by ribonucleotides that can bond with each other to allow stable association between the antisense RNA sequence and a sense RNA sequence in the RNA molecule of the invention and/or the target RNA molecule. One skilled in the art recognizes that the inclusion of mismatches is possible without eliminating the
30 ability of the antisense RNA sequence and target to remain in association. Therefore, described herein are antisense RNA sequence that may comprise up to about 20% nucleotides that are mismatched (i.e., are not complementary to the corresponding nucleotides of the target). Preferably the antisense compounds contain no more than about 15%, more preferably not more than about 10%, most preferably not more than
35 5% or no mismatches. The remaining ribonucleotides are complementary or otherwise do not disrupt hybridization (e.g., G:U or A:G pairs) between the antisense RNA

sequence and the sense RNA sequence or the target RNA molecule. One of ordinary skill in the art would recognise the antisense RNA sequences described herein are at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% (fully) complementary to at least a region of a target RNA molecule.

The term "RNA molecules of the invention" is used herein to refer to RNA molecules and chimeric RNA molecules. In addition, an RNA molecule of the invention can be a chimeric RNA molecule.

As used herein, "chimeric RNA molecule" refers to any RNA molecule that is not naturally found in nature. In an example, chimeric RNA molecules disclosed herein have been modified to create mismatches in region(s) of dsRNA. For example, chimeric RNA molecules may be modified to convert cytosines to uracils. In an example, chimeric RNA molecules have been modified via treatment with bisulfite for a time and under conditions sufficient to convert non-methylated cytosines to uracils.

One of skill in the art would appreciate that various ribonucleotide combinations can base pair. Both canonical and non-canonical base pairings are contemplated by the present disclosure. In an example, a base pairing can comprise A:T or G:C in a DNA molecule or U:A or G:C in an RNA molecule. In another example, a base pairing may comprise A:G or G:T or U:G.

The term "canonical base pairing" as used in the present disclosure means base pairing between two nucleotides which are A:T or G:C for deoxyribonucleotides or A:U or G:C for ribonucleotides.

The term "non-canonical base pairing" as used in the present disclosure means an interaction between the bases of two nucleotides other than canonical base pairings, in the context of two DNA or two RNA sequences. For example, non-canonical base pairing includes pairing between G and U (G:U) or between A and G (A:G). Examples of non-canonical base pairing include purine – purine or pyrimidine – pyrimidine. Most commonly in the context of this disclosure, the non-canonical base pairing is G:U. Other examples of non-canonical base pairs, less preferred, are A:C, G:T, G:G and A:A.

The present disclosure refers to RNA components that "hybridize" across a series of ribonucleotides. Those of skill in the art will appreciate that terms such as "hybridize" and "hybridizing" are used to describe molecules that anneal based on complementary nucleic acid sequences. Such molecules need not be 100% complementary in order to hybridize (i.e. they need not "fully base pair"). For example, there may be one or more mismatches in sequence complementarity. In an

example, RNA components defined herein hybridise under stringent hybridization conditions. The term "stringent hybridization conditions" refers to parameters with which the art is familiar, including the variation of the hybridization temperature with length of an RNA molecule. Ribonucleotide hybridization parameters may be found in 5 references which compile such methods, Sambrook, et al. (*supra*), and Ausubel, et al. (*supra*). For example, stringent hybridization conditions, as used herein, can refer to hybridization at 65°C in hybridization buffer (3.5xSSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin (BSA), 2.5 mM NaH₂PO₄ (pH7), 0.5% SDS, 2 mM EDTA), followed by one or more washes in 0.2.xSSC, 0.01% BSA at 10 50°C. Shorter RNA components such as RNA sequences of 20-24 nucleotides in length hybridise under lower stringency conditions. The term "low stringency hybridization conditions" refers to parameters with which the art is familiar, including the variation of the hybridization temperature with length of an RNA molecule. For example, low stringency hybridization conditions, as used herein, can refer to hybridization at 42°C 15 in hybridization buffer (3.5xSSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin (BSA), 2.5 mM NaH₂PO₄ (pH7), 0.5% SDS, 2 mM EDTA), followed by one or more washes in 0.2.xSSC, 0.01% BSA at 30°C.

The present invention also encompasses RNA components that "fully base pair" across contiguous ribonucleotides. The term "fully base pair" is used in the context of 20 the present disclosure to refer to a series of contiguous ribonucleotide base pairings. A fully base paired series of contiguous ribonucleotides does not comprise gaps or non-basepaired nucleotides within the series. The term "contiguous" is used to refer to a series of ribonucleotides. Ribonucleotides comprising a contiguous series will be joined by a continuous series of phosphodiester bonds, each ribonucleotide being 25 directly bonded to the next.

RNA molecules of the present invention comprise a sense sequence and a corresponding antisense sequence. The relationship between these sequences is defined herein. The sequence relationship and activity of the antisense sequence in relation to a target RNA molecule is also defined herein.

30 The term "covalently linked" is used in the context of the present disclosure to refer to the link between the first and second RNA components or any RNA sequences or ribonucleotides. As one of skill in the art would appreciate, a covalent link or bond is a chemical bond that involves the sharing of electron pairs between atoms. In an example, the first and second RNA components or the sense RNA sequence and the 35 antisense RNA sequence are covalently linked as part of a single RNA strand which

may fold back on itself through self-complementarity. In this example, the components are covalently linked across one or more ribonucleotides by phosphodiester bonds.

In the context of the present disclosure, the term “hybridization” means the pairing of complementary polynucleotides through basepairing of complementary
5 bases. While not limited to a particular mechanism, the most common mechanism of pairing involves hydrogen bonding, which may be Watson-Crick hydrogen bonding, between complementary ribonucleotides.

As used herein, the phrase “the RNA molecule reduces the target gene activity in the plant cell” or similar phrases means that the target gene transcript is present in
10 the plant cell and exposure or contact of the cell expressing the target gene transcript to the target RNA molecule results in reduced levels and/or activity of the target gene transcript when compared to the same cell lacking the RNA molecule. In an embodiment, the target RNA molecule encodes a protein important for flowering. As an example, the RNA molecule can have a modulating effect on flowering by the plant.
15 For example, the modulating effect can be early flowering. In another example, the modulating effect can be late flowering.

In an example, RNA molecules according to the present disclosure and compositions comprising the same can be administered to a plant.

As used herein, the term “unrelated in sequence to a target” refers to molecules
20 having less than 50% identity along the full-length of the intervening RNA sequence. On the other hand, the term “related in sequence to a target” refers to molecules having 50% or more identity along the full-length of the intervening RNA sequence.

As used herein, the term “genetically unmodified” or “non-transgenic” refers to plants that have not been modified by genetic engineering methods.

As used herein, a “control” or “control plant” or “control plant cell” provides a
25 reference point for measuring changes in phenotype of a subject plant or plant cell to which a RNA molecule disclosed herein has been delivered. In an example, the control plant or plant cell is a genetically similar plant or plant cell lacking an RNA molecule disclosed herein, preferably an isogenic plant or plant cell. For the avoidance of doubt,
30 a control may be a single plant or a group of plants or a crop. Identification of a suitable control to provide a reference point for measuring changes in phenotype is considered well within the purview of those of skill in the art.

RNA molecules herein that “modulate the timing of plant flowering” are RNA molecules that are able to increase or decrease the time to flowering of a plant. In an
35 example, RNA molecules disclosed herein direct early flowering in plants compared to

a control(s). In another example, RNA molecules disclosed herein direct late flowering in plants compared to a control(s).

Flowering time of plants can be assessed by counting the number of days ("time to flower") between sowing or transplanting and the emergence of a first inflorescence.

5 For example, the "flowering time" of a plant can be determined using the method as described in WO 2007/093444. In another example, flowering time can be measured indirectly based on the number of rosette leaves before bolting. The term "time of flower" and related terms has the common meaning in the art for each plant type being
10 feature that indicates the onset of flowering may be different for different plant species. It generally means that the first flower of the plant opens or is fertilisable if the flower does not open. For grasses such as wheat, barley and rice, for example, the term "flowering" means that heads or (panicles) emerge.

Terms such as "early flowering" or "early flowering time" are used herein to
15 refer to plants which start to flower earlier than control plants. Hence these terms refer to plants that show an earlier start of flowering. In contrast, terms such as "late flowering" or "late flowering time" are used herein to refer to plants which start to flower later than control plants. Hence these terms refer to plants that show a later start to flowering. In an example, "early flowering" and "late flowering" can be determined
20 by at least a statistically significantly change (decrease or increase) in flowering time compared to a control plant(s) as determined by a two-tailed Student's t-test or other appropriate statistical analysis, P-value < 0.05.

As would be understood by those of skill in the art, the time to flower varies between plant species and between different plants lines or varieties within a species.
25 Accordingly, in an example and depending on species, "early flowering" can refer to a reduction in time to flower by at least about 2 days, 3 days, 5 days, 10 days, 15 days, 20 days, 30 days, 40 days or more. In an example, early flowering refers to a reduction in time to flower by at least 5 to 40 days. In another example, early flowering refers to a reduction in time to flower by at least 5 to 40 days. In another example, early
30 flowering refers to a reduction in time to flower by at least 10 to 30 days. For example, a reduction in time to flower of at least about 2 days, 3 days, 5 days, 10 days, 15 days, 20 days, 30 days or more can indicate early flowering in wheat. In an example, a reduction in time to flower of between 5 and 40 days indicates early flowering in wheat. In another example, a reduction in time to flower of between 10 and 30 days
35 indicates early flowering in wheat. In another example, an early flowering plant has fewer rosette leaves before bolting than control plants. In contrast, in an example and

depending on species, "late flowering" can refer to an increase in time to flower by at least about 2 days, 3 days, 5 days, 10 days, 15 days, 20 days, 30 days, 40 days or more. For example, an increase in time to flower of at least about 2 days, 3 days, 5 days, 10 days, 15 days, 20 days, 30 days, 40 days or more can indicate late flowering in wheat.

- 5 In another example, a late flowering plant has fewer rosette leaves before bolting than control plants.

As used herein, "vernalization" refers to a process by which flowering is accelerated in plants via exposure of the plant or seed from which the plant is grown to a temperature stimulus or an artificial equivalent. In one example, the artificial
10 equivalent is delivering RNA molecule(s) described herein to a plant or a plant part, for example to seed.

As used herein, a "target RNA or gene that modulates the timing of plant flowering" or an "RNA molecule that modulates the timing of plant flowering" is a target RNA, gene or RNA molecule which is involved in the genetic control of
15 flowering in a plant and/or which influences, regulates or modulates the timing of flowering, including affecting the age or developmental stage of a plant at which it flowers and including genes which are involved in sensing environmental cues that lead to promotion or suppression of flowering.

As used herein, the phrase "long-day conditions" refers to photoperiodic
20 conditions where a dark period in a day is shorter than a threshold dark period required for photoperiodic responses (critical dark period). A 14-hour light/10-hour dark photoperiod is typically used as a long-day condition.

"Plants" included in the invention are any flowering plants, including both monocotyledonous and dicotyledonous plants. Examples of monocotyledonous plants
25 include, but are not limited to, cereals such as wheat, barley, maize, rice, sorghum, pearl millet, rye and oats, grasses such as forage grasses and turfgrasses, vegetables such as asparagus, onions and garlic. Examples of dicotyledonous plants include, but are not limited to, vegetables such as such as tomato, legumes such as alfalfa, beans, peas, chickpeas, lupins and soybeans, peppers, lettuce, forage or feed plants such as
30 alfalfa, clover, *Brassica* species e.g. cabbage, broccoli, cauliflower, brussel sprouts, rapeseed, mustard and radish, carrot, beets, eggplant, spinach, cucumber, squash, melons, cantaloupe, sunflowers, fiber crops such as cotton, ornamentals such as flowers and shrubs, and trees used in forestry such as poplar, eucalyptus and pine. Various other examples of plants and crops are discussed further below.

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

As used herein, the term about, unless stated to the contrary, refers to +/- 20%,
5 more preferably +/- 10%, of the designated value.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

10

ledRNA Molecule

In certain embodiments, RNA molecules of the present invention comprise a first RNA component which is covalently linked to a second RNA component. In preferred embodiments, the RNA molecule self-hybridizes or folds to form a
15 “dumbbell” or ledRNA structure, for example see Figure 1. In an embodiment, the molecule further comprises one or more of the following:

- a linking ribonucleotide sequence which covalently links the first and second RNA components;
- a 5' leader sequence; and,
- 20 - a 3' trailer sequence.

In an embodiment, the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair to each other in the RNA molecule, wherein the first RNA sequence comprises a first sense ribonucleotide sequence of at least 20
25 contiguous ribonucleotides, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence of at least 20 contiguous ribonucleotides, wherein the first antisense ribonucleotide sequence hybridises with the first sense ribonucleotide sequence in the RNA molecule, wherein the first antisense ribonucleotide sequence is capable of hybridising to a first region of a target RNA molecule which modulates the
30 timing of plant flowering.

In another embodiment, the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair to each other in the RNA molecule, wherein the first RNA sequence comprises a first sense ribonucleotide sequence of at least 20
35 contiguous ribonucleotides, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence of at least 20 contiguous ribonucleotides, wherein

the first antisense ribonucleotide sequence fully basepairs with the first sense ribonucleotide sequence in the RNA molecule, wherein the first antisense ribonucleotide sequence is identical in sequence to the complement of a first region of a target RNA molecule. An example of this first RNA component of these two
5 embodiments is shown schematically in the left-hand half of Figure 1A or the right-hand half of Figure 1B.

In another embodiment, the first RNA component consists of a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair with each other in the first RNA component, wherein
10 the first RNA sequence comprises a first sense ribonucleotide sequence, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence, wherein the first sense ribonucleotide sequence and first antisense ribonucleotide sequence each of at least 20 contiguous ribonucleotides whereby the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence fully basepair
15 with the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence, wherein the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence are substantially identical in sequence to a first region of a target RNA molecule.

In these embodiments, the basepair formed between the first 5' ribonucleotide
20 and the first 3' ribonucleotide is considered to be the terminal basepair of the dsRNA region formed by self-hybridization of the first RNA component, i.e it defines the end of the dsRNA region.

In an embodiment, the first sense sequence has substantial sequence identity to a region of the target RNA, which identity may be to a sequence of less than 20
25 nucleotides in length. In an embodiment at least 15, at least 16, at least 17, at least 18, or at least 19 contiguous ribonucleotides, preferably at least 20 contiguous ribonucleotides, of the first sense ribonucleotide sequence and a first region of a target RNA molecule are at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or
30 99% identical in sequence. In another embodiment, the at least 15, at least 16, at least 17, at least 18, at least 19 contiguous ribonucleotides of the first sense ribonucleotide sequence and a first region of a target RNA molecule are 100% identical. In an embodiment, the first 3, first 4, first 5, first 6, or first 7 ribonucleotides from the 5' end of the first sense ribonucleotide sequence are 100% identical to the region of the target
35 RNA molecule, with the remaining ribonucleotides being at least 60%, at least 65%, at

least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the target RNA molecule.

In an embodiment the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence and a first region of a target RNA molecule are at least 60%, at
5 least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical. Again, in this embodiment, the first 3, first 4, first 5, first 6, or first 7 ribonucleotides can be 100% identical to the region of the target RNA molecule, with the remaining ribonucleotides being at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%,
10 at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to target RNA molecule. In another embodiment, the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence and a first region of a target RNA molecule are 100% identical.

In an embodiment, the first antisense sequence has substantial sequence identity
15 to the complement of a region of the target RNA, which identity may be to a sequence of less than 20 nucleotides in length of the complement. In an embodiment at least 15, at least 16, at least 17, at least 18, or at least 19 contiguous ribonucleotides, preferably at least 20 contiguous ribonucleotides, of the first antisense ribonucleotide sequence and the complement of a first region of a target RNA molecule are at least 60%, at least
20 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or 99% identical in sequence. In another embodiment, the at least 15, at least 16, at least 17, at least 18, at least 19 contiguous ribonucleotides of the first antisense ribonucleotide sequence and the complement of the first region of the target RNA molecule are 100% identical. In an embodiment, the
25 first 3, first 4, first 5, first 6, or first 7 ribonucleotides from the 5' end of the first antisense ribonucleotide sequence are 100% identical to the complement of the region of the target RNA molecule, with the remaining ribonucleotides being at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the complement
30 of the target RNA molecule.

In an embodiment the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence and the complement of a first region of the target RNA molecule are at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at
35 least 99% identical. Again, in this embodiment, the first 3, first 4, first 5, first 6, or first 7 ribonucleotides are 100% identical to the complement of the region of the target RNA

molecule, with the remaining ribonucleotides being at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the complement of the target RNA molecule. In another embodiment, the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence and a first region of a target RNA molecule are 100% identical.

In another embodiment, the second RNA component consists of, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide, wherein the second 5' and 3' ribonucleotides basepair, wherein the second RNA sequence comprises a second sense ribonucleotide sequence, a second loop sequence of at least 4 ribonucleotides and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence basepairs with the second antisense ribonucleotide sequence. In this embodiment, the basepair formed between the second 5' ribonucleotide and the second 3' ribonucleotide is considered to be the terminal basepair of the dsRNA region formed by self-hybridization of the second RNA component.

In an embodiment, the RNA molecule comprises a 5' leader sequence, or 5' extension sequence, which may arise as a result of transcription from a promoter in the genetic construct, from the start site of transcription to the beginning of the polynucleotide encoding the remainder of the RNA molecule. It is preferred that this 5' leader sequence or 5' extension sequence is relatively short compared to the remainder of the molecule, and it may be removed from the RNA molecule post-transcriptionally, for embodiment by RNase treatment. The 5' leader sequence or 5' extension sequence may be mostly non-basepaired, or it may contain one or more stem-loop structures. In this embodiment, the 5' leader sequence can consist of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide. In an embodiment, the 5' leader sequence is at least 10, at least 20, at least 30, at least 100, at least 200 ribonucleotides long, preferably to a maximum length of 250 ribonucleotides. In another embodiment, the 5' leader sequence is at least 50 ribonucleotides long. In an embodiment, the 5' leader sequence can act as an extension sequence for amplification of the RNA molecule via a suitable amplification reaction. For embodiment, the extension sequence may facilitate amplification via polymerase.

In another embodiment, the RNA molecule comprises a 3' trailer sequence or 3' extension sequence which may arise as a result of transcription continuing until a

transcription termination or polyadenylation signal in the construct encoding the RNA molecule. The 3' trailer sequence or 3' extension sequence may comprise a polyA tail. It is preferred that this 3' trailer sequence or 3' extension sequence is relatively short compared to the remainder of the molecule, and it may be removed from the RNA molecule post-transcriptionally, for embodiment by RNase treatment. The 3' trailer sequence or 3' extension sequence may be mostly non-basepaired, or it may contain one or more stem-loop structures. In this embodiment, the 3' trailer sequence can consist of a sequence of ribonucleotides which is covalently linked to the second 3' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the first 3' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide. In an embodiment, the 3' leader sequence is at least 10, at least 20, at least 30, at least 100, at least 200 ribonucleotides long, preferably to a maximum length of 250 ribonucleotides. In another embodiment, the 3' leader sequence is at least 50 ribonucleotides long. In an embodiment, the 3' trailer sequence can act as an extension sequence for amplification of the RNA molecule via a suitable amplification reaction. For embodiment, the extension sequence may facilitate amplification via polymerase.

In an embodiment, all except for two of the ribonucleotides are covalently linked to two other nucleotides i.e. the RNA molecule consists of only one RNA strand which has self-complementary regions, and so has only one 5' terminal nucleotide and one 3' terminal nucleotide. In another embodiment, all except for four of the ribonucleotides are covalently linked to two other nucleotides i.e. the RNA molecule consists of two RNA strands which have complementary regions which hybridise, and so has only two 5' terminal nucleotides and two 3' terminal nucleotides. In another embodiment, each ribonucleotide is covalently linked to two other nucleotides i.e. the RNA molecule is circular as well as having self-complementary regions, and so has no 5' terminal nucleotide and no 3' terminal nucleotide.

In an embodiment, the double-stranded region of the RNA molecule can comprise one or more bulges resulting from unpaired nucleotides in the sense RNA sequence or the antisense RNA sequence, or both. In an embodiment, the RNA molecule comprises a series of bulges. For embodiment, the double-stranded region of the RNA molecule may have 2, 3, 4, 5, 6, 7, 8, 9, 10 or more bulges. Each bulge may be, independently, one, two or more unpaired nucleotides, to as many as 10 nucleotides. Longer sequences may loop out of the sense or antisense sequences in the dsRNA region, which may basepair internally or remain unpaired. In another embodiment, the double-stranded region of the RNA molecule does not comprise a bulge i.e. is fully basepaired along the full length of the dsRNA region.

In another embodiment, the first sense ribonucleotide sequence is covalently linked to the first 5' ribonucleotide without any intervening nucleotides, or the first antisense ribonucleotide sequence is covalently linked to the first 3' ribonucleotide without any intervening nucleotides, or both. In another embodiment, there are at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10 intervening nucleotides. It is understood that such intervening nucleotides are unrelated in sequence to the target RNA molecule but may assist in stabilising the basepairing of adjacent sense and antisense sequences.

In another embodiment, the 20 consecutive nucleotides of the first sense ribonucleotide sequence are covalently linked to the first 5' ribonucleotide without any intervening nucleotides, and the 20 consecutive nucleotides of the first antisense ribonucleotide sequence are covalently linked to the first 3' ribonucleotide without any intervening nucleotides. In another embodiment, there are at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10 intervening nucleotides. The intervening nucleotides may be basepaired as part of the double-stranded region of the RNA molecule but are unrelated in sequence to the target RNA. They may assist in providing increased stability to the double-stranded region or to hold together two ends of the RNA molecule and not leave an unbasepaired 5' or 3' end, or both.

In an embodiment, the above referenced first and second RNA components comprise a linking ribonucleotide sequence. In an embodiment, the linking ribonucleotide sequence acts as a spacer between the first sense ribonucleotide sequence that is substantially identical in sequence to a first region of a target RNA molecule and the other components of the molecule. For example, the linking ribonucleotide sequence may act as a spacer between this region and a loop. In another embodiment, the RNA molecule comprises multiple sense ribonucleotide sequences that are substantially identical in sequence to a first region of a target RNA molecule and a linking ribonucleotide sequence which acts as a spacer between these sequences. In an embodiment, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10 ribonucleotide sequences that are substantially identical in sequence to a first region of a target RNA molecule are provided in the RNA molecule, each being separated from the other(s) by a linking ribonucleotide sequence.

In an embodiment, the above referenced RNA molecules comprise a 5' leader sequence. In an embodiment, the 5' leader sequence consists of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second

RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide. In an embodiment, the RNA molecule has a modified 5' or 3' end, for embodiment by attachment of a lipid group such as cholesterol, or a vitamin such as biotin, or a polypeptide. Such modifications may assist in the uptake of the RNA molecule into the plant cell where the RNA is to function.

In an embodiment, the linking ribonucleotide sequence is less than 100 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is less than 50 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is less than 20 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is less than 10 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is less than 5 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is between 1 and 100 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is between 1 and 50 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is between 1 and 20 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is between 1 and 10 ribonucleotides in length. In an embodiment, the linking ribonucleotide sequence is between 1 and 5 ribonucleotides in length. In an embodiment, the ribonucleotides of the linking ribonucleotide sequence are not basepaired. In a preferred embodiment, the ribonucleotides of the linking ribonucleotide sequence are all basepaired, or all except for 1, 2 or 3 of the ribonucleotides are basepaired.

In an embodiment, the first or second RNA component comprises a hairpin structure. In a preferred embodiment, the first and second RNA components each comprise a hairpin structure. In these embodiments, the hairpin structure can be a stem-loop. Accordingly, in an embodiment, the RNA molecule can comprise first and second RNA components which each comprise a hairpin structure, wherein the hairpins are covalently bound by a linker sequence. See, for example, Figure 1. In an embodiment, the linker sequence is one or more unpaired ribonucleic acid(s). In an embodiment, the linker sequence is between 1 and 10 unpaired ribonucleotides.

In an embodiment, the RNA molecule has a double hairpin structure i.e. an "ledRNA structure" or "dumbbell structure". In this embodiment, the first hairpin is the first RNA component and the second hairpin is the second RNA component. In these embodiments, either the first 3' ribonucleotide and the second 5' ribonucleotide, or the second 3' ribonucleotide and the first 5' ribonucleotide, but not both, are covalently joined. In this embodiment, the other 5'/3' ribonucleotides can be separated

by a nick (i.e. a discontinuity in the dsRNA molecule where there is no phosphodiester bond between the 5'/3' ribonucleotides. An embodiment, of this type of arrangement is shown in Figure 1B. In another embodiment, the respective 5'/3' ribonucleotides can be separated by a loop. The lengths of the 5' leader and 3' trailer sequences may be the same or different. For embodiment, the 5' leader may be around 5, 10, 15, 20, 25, 50, 100, 200, 500 ribonucleotides longer than the 3' trailer sequence or *vice versa*.

In embodiments where the RNA molecule has a double hairpin structure, the second hairpin (in addition to the first hairpin structure) comprises a sense RNA sequence and an antisense RNA sequence that are substantially identical in sequence to a region of a target RNA molecule or its complement, respectively. In an embodiment, each hairpin has a series of ribonucleotides that are substantially identical in sequence to a region of the same target RNA molecule. In an embodiment, each hairpin has a series of ribonucleotides that are substantially identical in sequence to different regions of the same target RNA molecule. In an embodiment, each hairpin has a series of ribonucleotides that are substantially identical in sequence to a region of different target RNA molecules i.e. the RNA molecule can be used to reduce the expression and/or activity of two target RNA molecules which may be unrelated in sequence.

In each hairpin of the double hairpin structure of the RNA molecule, the order of the sense and antisense RNA sequences in each hairpin, in 5' to 3' order, may independently be either sense then antisense, or antisense then sense. In preferred embodiments, the order of the sense and antisense sequences in the double hairpin structure of the RNA molecule is either antisense-sense-sense-antisense where the two sense sequences are contiguous (Figure 1A), or sense-antisense-antisense-sense where the two antisense sequences are contiguous (Figure 1B).

In an embodiment, the RNA molecule can comprise, in 5' to 3' order, a 5' leader sequence, a first loop, a sense RNA sequence, a second loop and a 3' trailer sequence, wherein the 5' and 3' leader sequences covalently bond to the sense strand to form a dsRNA sequence. In an embodiment, the 5' leader and 3' trailer sequences are not covalently bound to each other. In an embodiment, the 5' leader and 3' trailer sequences are separated by a nick. In an embodiment, the 5' leader and 3' trailer sequences are ligated together to provide a RNA molecule with a closed structure. In another embodiment, the 5' leader and 3' trailer sequences are separated by a loop.

The term "loop" is used in the context of the present disclosure to refer to a loop structure in an RNA molecule disclosed herein that is formed by a series of non-complementary ribonucleotides. Loops generally follow a series of base-pairs between the first and second RNA components or join a sense RNA sequence and an antisense

RNA sequence in one or both of the first and second RNA components. In an embodiment, all of the loop ribonucleotides are non-complementary, generally for shorter loops of 4-10 ribonucleotides. In other embodiments, some ribonucleotides in one or more of the loops are complementary and capable of basepairing within the loop
5 sequence, so long as these basepairings enable a loop structure to form. For example, at least 5%, at least 10%, or at least 15% of the loop ribonucleotides are complementary. Embodiments of loops include stem loops or hairpins, pseudoknots and tetraloops.

In an embodiment, the RNA molecule comprises only two loops. In another
10 embodiment, the RNA molecule comprises at least two, at least three, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 loops, preferably to a maximum of 10 loops. For example, the RNA molecule can comprise 4 loops.

Loops of various sizes are contemplated by the present disclosure. For example, loops can comprise 4, 5, 6, 7, 8, 9, 10, 11 or 12 ribonucleotides. In other embodiments,
15 loops comprise 15, 20, 25 or 30 nucleotides. In an embodiment, one or all of the loop sequences are longer than 20 nucleotides. In other embodiments, loops are larger, for example comprising 50, 100, 150, 200 or 300 ribonucleotides. In an embodiment, loops comprise 160 ribonucleotides. In another embodiment, less preferred, loops comprise 200, 500, 700 or 1,000 ribonucleotides provided that the loops do not
20 interfere with the hybridisation of the sense and antisense RNA sequences. In an embodiment, each of the loops have the same number of ribonucleotides. For example, loops can have between 100 and 1,000 ribonucleotides in length. For example, loops can have between 600 and 1,000 ribonucleotides in length. For example, loops can have between 4 and 1,000 ribonucleotides. For example, loops preferably have
25 between 4 and 50 ribonucleotides. In another embodiment, loops comprise differing numbers of ribonucleotides.

In another embodiment, one or more loops comprise an intron which can be spliced out of the RNA molecule. In an embodiment, the intron is from a plant gene. Exemplary introns include intron 3 of the maize alcohol dehydrogenase 1 (Adh1)
30 (GenBank: AF044293), intron 4 of the soya beta-conglycinin alpha subunit (GenBank: AB051865); one of the introns of the pea rbcS-3A gene for the ribulose-1,5-bisphosphate carboxylase (RBC) small subunit (GenBank: X04333). Other embodiments of suitable introns are discussed in (McCullough and Schuler, 1997; Smith et al., 2000).

In various embodiments, a loop may be at the end of at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 consecutive basepairs, which may be canonical basepairs or may include one or more non-canonical basepairs.

In another embodiment, the RNA molecule comprises two or more sense
5 ribonucleotide sequences, and antisense ribonucleotide sequences fully base paired thereto, which are each identical in sequence to a region of a target RNA molecule. For example, the RNA molecule can comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more sense ribonucleotide sequences, and antisense ribonucleotide sequences fully base paired thereto, which sense ribonucleotide sequences are each independently identical in
10 sequence to a region of a target RNA molecule. In this embodiment, any one or more or all of the sequences can be separated by a linking ribonucleotide sequence(s). In this embodiment, any one or more or all of the sequences can be separated by a loop.

In an embodiment, the two or more sense ribonucleotide sequences are identical in sequence to different regions of the same target RNA molecule. For example, the
15 sequences can be identical to at least 2, at least 3, at least 4, at least 5, at least 6 regions of the same target molecule. In another embodiment, the two or more sense ribonucleotide sequences are identical in sequence. In an embodiment, the two or more sense ribonucleotide sequences are identical in sequence to the same region of the same target RNA molecule. In another embodiment, the two or more sense ribonucleotide
20 sequences are identical in sequence to different target RNA molecules. For example, the sequences can be identical to at least 2, at least 3, at least 4, at least 5, at least 6 regions of different target molecules.

In another embodiment, the two or more sense ribonucleotide sequences have no intervening loop (spacer) sequences.

25 In an embodiment, the RNA molecule has a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully basepaired with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end. In this embodiment, the ribonucleotide at the 5'
30 end and the ribonucleotide at the 3' end are not directly covalently bonded but are rather positioned adjacent with each basepaired.

In another embodiment, consecutive basepairs of RNA components are interspaced by at least one gap. In an embodiment, the "gap" is provided by an unpaired ribonucleotide. In another embodiment, the "gap" is provided by un-ligated
35 5' leader sequence and/or 3' trailer sequence. In this embodiment, the gap can be referred to as an "unligated gap". Mismatches and unligated gap(s) can be located at

various position(s) of the RNA molecule. For embodiment, an unligated gap can immediately follow an antisense sequence. In another embodiment, an unligated gap can be close to a loop of the RNA molecule. In another embodiment, an unligated gap is positioned about equidistant between at least two loops.

5 In an embodiment, the RNA molecule is produced from a single strand of RNA. In an embodiment, the single strand is not circularly closed, for example, comprising an unligated gap. In another embodiment, the RNA molecule is a circularly closed molecule. Closed molecules can be produced by ligating an above referenced RNA molecule comprising an unligated gap, for example with an RNA ligase.

10 In another embodiment, the RNA molecule comprises a 5'- or 3'-, or both, extension sequence. For example, the RNA molecule can comprise a 5' extension sequence which is covalently linked to the first 5' ribonucleotide. In another embodiment, the RNA molecule comprises a 3' extension sequence which is covalently linked to the second 3' ribonucleotide. In another embodiment, the RNA molecule
15 comprises a 5' extension sequence which is covalently linked to the first 5' ribonucleotide and a 3' extension sequence which is covalently linked to the second 3' ribonucleotide.

In another embodiment, the RNA molecule comprises a 5' extension sequence which is covalently linked to the second 5' ribonucleotide. In another embodiment, the
20 RNA molecule comprises a 3' extension sequence which is covalently linked to the first 3' ribonucleotide. In another embodiment, the RNA molecule comprises a 5' extension sequence which is covalently linked to the second 5' ribonucleotide and a 3' extension sequence which is covalently linked to the first 3' ribonucleotide.

In another embodiment, the RNA molecule can comprise one or more of the
25 following:

- 5' extension sequence which is covalently linked to the first 5' ribonucleotide;
- 3' extension sequence which is covalently linked to the second 3' ribonucleotide;
- 30 - 5' extension sequence which is covalently linked to the first 5' ribonucleotide and a 3' extension sequence which is covalently linked to the second 3' ribonucleotide;
- 5' extension sequence which is covalently linked to the second 5' ribonucleotide;
- 35 - 3' extension sequence which is covalently linked to the first 3' ribonucleotide;

- a 5' extension sequence which is covalently linked to the second 5' ribonucleotide and a 3' extension sequence which is covalently linked to the first 3' ribonucleotide.

In an example, the RNA molecule comprises a nucleic acid sequence set forth in
5 SEQ ID NO:146 or SEQ ID NO: 147.

Non-Canonical Basepairing

In an embodiment, RNA molecules of the present invention comprise a sense ribonucleotide sequence and an antisense ribonucleotide sequence which are capable of
10 hybridising to each other to form a double stranded (ds)RNA region with some non-canonical basepairing i.e. with a combination of canonical and non-canonical basepairing. In an embodiment, RNA molecules of the present invention comprise two or more sense ribonucleotide sequences which are each capable of hybridising to regions of one (contiguous) antisense ribonucleotide sequence to form a dsRNA region
15 with some non-canonical basepairing. See for example, Figure 1B. In an embodiment, RNA molecules of the present invention comprise two or more antisense sense ribonucleotide sequences which are each capable of hybridising to regions of one (contiguous) sense ribonucleotide sequence to form a dsRNA region with some non-canonical basepairing. See for example, Figure 1A. In an embodiment, RNA
20 molecules of the present invention comprise two or more antisense sense ribonucleotide sequences and two or more sense ribonucleotide sequences wherein each antisense ribonucleotide sequence is capable of hybridising to an antisense ribonucleotide sequence to form two or more dsRNA regions, one or both comprising some non-canonical basepairing.

25 In the following embodiments, the full length of the dsRNA region (i.e. the whole dsRNA region) of the RNA molecule of the invention is considered as the context for the feature if there is only one (contiguous) dsRNA region, or for each of the dsRNA regions of the RNA molecule if there are two or more dsRNA regions in the RNA molecule. In an embodiment, at least 5% of the basepairs in a dsRNA region are
30 non-canonical basepairs. In an embodiment, at least 6% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 7% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 8% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 9% or 10% of the basepairs in a dsRNA region are non-canonical basepairs. In an
35 embodiment, at least 11% or 12% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 15% or about 15% of the basepairs in a

dsRNA region are non-canonical basepairs. In an embodiment, at least 20% or about 20% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 25% or about 25% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, at least 30% or about 30% of the basepairs in a dsRNA region are non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 40% of the basepairs in the dsRNA region are non-canonical basepairs, more preferably a maximum of 35% of the basepairs in the dsRNA region are non-canonical basepairs, still more preferably a maximum of 30% of the basepairs in the dsRNA region are non-canonical basepairs. In an embodiment, less preferred, about 35% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, even less preferred, about 40% of the basepairs in a dsRNA region are non-canonical basepairs. In each of the above embodiments, the dsRNA region may or may not comprise one or more non-basepaired ribonucleotides, in either the sense sequence or the antisense sequence, or both.

In an embodiment, between 10% and 40% of the basepairs in a dsRNA region of the RNA molecule of the invention are non-canonical basepairs. In an embodiment, between 10% and 35% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 10% and 30% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 10% and 25% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 10% and 20% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 10% and 15% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 15% and 30% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 15% and 25% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 15% and 20% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 5% and 30% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 5% and 25% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 5% and 20% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 5% and 15% of the basepairs in a dsRNA region are non-canonical basepairs. In an embodiment, between 5% and 10% of the basepairs in a dsRNA region are non-canonical basepairs. In each of the above embodiments, the dsRNA region may or may not comprise one or more non-basepaired ribonucleotides, in either the sense sequence or the antisense sequence, or both.

In an embodiment, the dsRNA region of the RNA molecule of the invention comprises 20 contiguous basepairs, wherein at least one basepair of the 20 contiguous basepairs is a non-canonical basepair. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 2 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 3 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 4 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 5 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 6 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 7 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 8 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs, wherein at least 9 basepairs of the 20 contiguous basepairs are non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 10 of the 20 contiguous basepairs in the dsRNA region are non-canonical basepairs, more preferably a maximum of 9 of the basepairs in the dsRNA region are non-canonical basepairs, still more preferably a maximum of 8 of the basepairs in the dsRNA region are non-canonical basepairs, even still more preferably a maximum of 7 of the basepairs in the dsRNA region are non-canonical basepairs, and most preferably a maximum of 6 of the basepairs in the dsRNA region are non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs are G:U basepairs. Preferably, the features of the above embodiments apply to each and every one of the 20 contiguous basepairs that are present in the RNA molecule of the invention.

In an embodiment, the dsRNA region of the RNA molecule of the invention comprises 21 contiguous basepairs, wherein at least one basepair of the 21 contiguous basepairs is a non-canonical basepair. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 2 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 3 basepairs of the 21 contiguous basepairs are

non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 4 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 5 basepairs of the 21 contiguous basepairs are
5 non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 6 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 7 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 21
10 contiguous basepairs, wherein at least 8 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 21 contiguous basepairs, wherein at least 9 basepairs of the 21 contiguous basepairs are non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 10 of the 21 contiguous basepairs in the dsRNA region are non-canonical basepairs,
15 more preferably a maximum of 9 of the basepairs in the dsRNA region are non-canonical basepairs, still more preferably a maximum of 8 of the basepairs in the dsRNA region are non-canonical basepairs, even still more preferably a maximum of 7 of the basepairs in the dsRNA region are non-canonical basepairs, and most preferably a maximum of 6 of the basepairs in the dsRNA region are non-canonical basepairs.
20 Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs are G:U basepairs. Preferably, the features of the above embodiments apply to each and every one of the 21 contiguous basepairs that are present in the RNA molecule of the invention.

25 In an embodiment, the dsRNA region of the RNA molecule of the invention comprises 22 contiguous basepairs, wherein at least one basepair of the 22 contiguous basepairs is a non-canonical basepair. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 2 basepairs of the 22 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 22
30 contiguous basepairs, wherein at least 3 basepairs of the 22 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 4 basepairs of the 22 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 5 basepairs of the 22 contiguous basepairs are
35 non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 6 basepairs of the 22 contiguous basepairs are

non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 7 basepairs of the 22 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 8 basepairs of the 22 contiguous basepairs are
5 non-canonical basepairs. In an embodiment, the dsRNA region comprises 22 contiguous basepairs, wherein at least 9 basepairs of the 22 contiguous basepairs are non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 10 of the 22 contiguous basepairs in the dsRNA region are non-canonical basepairs, more preferably a maximum of 9 of the basepairs in the dsRNA region are non-
10 canonical basepairs, still more preferably a maximum of 8 of the basepairs in the dsRNA region are non-canonical basepairs, even still more preferably a maximum of 7 of the basepairs in the dsRNA region are non-canonical basepairs, and most preferably a maximum of 6 of the basepairs in the dsRNA region are non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least
15 one G:U basepair, more preferably all of the non-canonical basepairs are G:U basepairs. Preferably, the features of the above embodiments apply to each and every one of the 22 contiguous basepairs that are present in the RNA molecule of the invention.

In an embodiment, the dsRNA region of the RNA molecule of the invention
20 comprises 23 contiguous basepairs, wherein at least one basepair of the 23 contiguous basepairs is a non-canonical basepair. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 2 basepairs of the 23 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 3 basepairs of the 23 contiguous basepairs are
25 non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 4 basepairs of the 23 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 5 basepairs of the 23 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 23
30 contiguous basepairs, wherein at least 6 basepairs of the 23 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 7 basepairs of the 23 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 8 basepairs of the 23 contiguous basepairs are
35 non-canonical basepairs. In an embodiment, the dsRNA region comprises 23 contiguous basepairs, wherein at least 9 basepairs of the 23 contiguous basepairs are

non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 10 of the 23 contiguous basepairs in the dsRNA region are non-canonical basepairs, more preferably a maximum of 9 of the basepairs in the dsRNA region are non-canonical basepairs, still more preferably a maximum of 8 of the basepairs in the dsRNA region are non-canonical basepairs, even still more preferably a maximum of 7 of the basepairs in the dsRNA region are non-canonical basepairs, and most preferably a maximum of 6 of the basepairs in the dsRNA region are non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs are G:U basepairs. Preferably, the features of the above embodiments apply to each and every one of the 23 contiguous basepairs that are present in the RNA molecule of the invention.

In an embodiment, the dsRNA region of the RNA molecule of the invention comprises 24 contiguous basepairs, wherein at least one basepair of the 24 contiguous basepairs is a non-canonical basepair. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 2 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 3 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 4 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 5 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 6 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 7 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 8 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 24 contiguous basepairs, wherein at least 9 basepairs of the 24 contiguous basepairs are non-canonical basepairs. In each of these embodiments, it is preferred that a maximum of 10 of the 24 contiguous basepairs in the dsRNA region are non-canonical basepairs, more preferably a maximum of 9 of the basepairs in the dsRNA region are non-canonical basepairs, still more preferably a maximum of 8 of the basepairs in the dsRNA region are non-canonical basepairs, even still more preferably a maximum of 7 of the basepairs in the dsRNA region are non-canonical basepairs, and most preferably

a maximum of 6 of the basepairs in the dsRNA region are non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs are G:U basepairs. Preferably, the features of the above embodiments apply to each and every
5 one of the 24 contiguous basepairs that are present in the RNA molecule of the invention.

In the following embodiments, the full length of the dsRNA region (i.e. the whole dsRNA region) of the RNA molecule of the invention is considered as the context for the feature if there is only one (contiguous) dsRNA region, or for each of
10 the dsRNA regions of the RNA molecule if there are two or more dsRNA regions in the RNA molecule. In an embodiment, the dsRNA region does not comprise 20 contiguous canonical basepairs i.e. every subregion of 20 contiguous basepairs includes at least one non-canonical basepair, preferably at least one G:U basepair. In an embodiment, the dsRNA region does not comprise 19 contiguous canonical basepairs.
15 In an embodiment, the dsRNA region does not comprise 18 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 17 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 16 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 15 contiguous canonical basepairs. In an embodiment, the dsRNA region
20 does not comprise 14 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 13 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 12 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 11 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 10 contiguous canonical
25 basepairs. In an embodiment, the dsRNA region does not comprise 9 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 8 contiguous canonical basepairs. In an embodiment, the dsRNA region does not comprise 7 contiguous canonical basepairs. In the above embodiments, it is preferred that the longest subregion of contiguous canonical basepairing in the dsRNA region of
30 the RNA molecule, or each and every dsRNA region in the RNA molecule, is 5, 6 or 7 contiguous canonical basepairs i.e. towards the shorter lengths mentioned. Each of the features of the above embodiments is preferably combined in the RNA molecule with the following features. In an embodiment, the dsRNA region comprises between 10 and 19 or 20 contiguous basepairs. In a preferred embodiment, the dsRNA region
35 comprises between 12 and 19 or 20 contiguous basepairs. In an embodiment, the dsRNA region comprises between 14 and 19 or 20 contiguous basepairs. In these

embodiments, the dsRNA region comprises 15 contiguous basepairs. In an embodiment, the dsRNA region comprises 16, 17, 18 or 19 contiguous basepairs. In an embodiment, the dsRNA region comprises 20 contiguous basepairs. Preferably, in the above embodiments, the contiguous basepairs comprise at least one non-canonical
5 basepair which comprises at least one G:U basepair, more preferably all of the non-canonical basepairs in the region of contiguous basepairs are G:U basepairs.

In an embodiment, the dsRNA region comprises a subregion of 4 canonical basepairs flanked by non-canonical basepairs, i.e. at least one, preferably one or two (not more than 2), non-canonical basepairs adjacent to each end of the 4 canonical
10 basepairs. In an embodiment, the dsRNA region comprises 2 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 3 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 4 or 5 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA
15 region comprises 6 or 7 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 8 to 10 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 11 to 15 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region
20 comprises between 2 and 50 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 40 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 30 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA
25 region comprises between 2 and 20 subregions each of 4 canonical basepairs flanked by non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs flanking the contiguous canonical basepairs in the subregions are G:U basepairs. In variations of the above embodiments, one or both of the flanking non-
30 canonical basepairs are replaced with a non-basepaired ribonucleotide in the sense sequence, the antisense sequence or in both sequences, for some or all of the subregions. It is readily understood that, in the above embodiments, the maximum number of subregions is determined by the length of the dsRNA region in the RNA molecule.

35 In an embodiment, the dsRNA region comprises a subregion of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region

comprises 2 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 3 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 4 or 5 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 6 or 7 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 8 to 10 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 11 to 15 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 50 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 50 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 30 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 20 subregions each of 5 canonical basepairs flanked by non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs flanking the contiguous canonical basepairs in the subregions are G:U basepairs. In variations of the above embodiments, one or both of the flanking non-canonical basepairs are replaced with a non-basepaired ribonucleotide in the sense sequence, the antisense sequence or in both sequences, for some or all of the subregions. It is readily understood that, in the above embodiments, the maximum number of subregions is determined by the length of the dsRNA region in the RNA molecule.

In an embodiment, the dsRNA region comprises a subregion of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 2 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 3 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 4 or 5 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 6 or 7 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 8 to 10 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises 11 to 16 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 60 subregions each of 6

canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 60 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 30 subregions each of 6 canonical basepairs flanked by non-canonical basepairs.

5 In an embodiment, the dsRNA region comprises between 2 and 20 subregions each of 6 canonical basepairs flanked by non-canonical basepairs. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs flanking the contiguous canonical basepairs in the subregions are G:U basepairs. In variations of the above embodiments,

10 one or both of the flanking non-canonical basepairs are replaced with a non-basepaired ribonucleotide in the sense sequence, the antisense sequence or in both sequences, for some or all of the subregions. It is readily understood that, in the above embodiments, the maximum number of subregions is determined by the length of the dsRNA region in the RNA molecule.

15 In an embodiment, the dsRNA region comprises a subregion of 10 contiguous basepairs wherein 2-4 of the basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 2 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 3 subregions each of 10 contiguous basepairs wherein 2-4 of

20 the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 4 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 5 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 10 subregions each of 10 contiguous basepairs wherein 2-4 of

25 the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises 4 subregions each of 15 contiguous basepairs wherein 2-6 of the 15 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 50 subregions each of 10 contiguous basepairs

30 wherein 2-4 of the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 40 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous basepairs are non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 30 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous basepairs

35 are non-canonical basepairs. In an embodiment, the dsRNA region comprises between 2 and 20 subregions each of 10 contiguous basepairs wherein 2-4 of the 10 contiguous

basepairs are non-canonical basepairs. In an embodiment, the non-canonical basepairs in one (contiguous) or more, or all dsRNA regions of the RNA molecule are not adjacent a non-base pair. In another embodiment, the non-canonical basepairs are at least 2 contiguous base pairs from a non-base pair. In another embodiment, the non-canonical basepairs are at least 3, 4, 5, 6, 7, 8, 9, 10 or more contiguous base pairs from a non-base pair. In an embodiment the non-canonical basepairs in one (contiguous) or more, or all dsRNA regions of the RNA molecule are not adjacent a loop sequence. In another embodiment, the non-canonical basepairs are at least 2 contiguous base pairs from a loop sequence. In another embodiment, the non-canonical basepairs are at least 3, 4, 5, 6, 7, 8, 9, 10 or more contiguous base pairs from a loop sequence. Preferably, in the above embodiments, the non-canonical basepairs comprise at least one G:U basepair, more preferably all of the non-canonical basepairs in the subregions are G:U basepairs. In variations of the above embodiments, one or more of the 2-4 or 2-6 non-canonical basepairs are replaced with a non-basepaired ribonucleotide in the sense sequence, the antisense sequence or in both sequences, for some or all of the subregions. It is readily understood that, in the above embodiments, the maximum number of subregions is determined by the length of the dsRNA region in the RNA molecule.

In an embodiment, the ratio of canonical to non-canonical basepairs in the dsRNA region is between 2.5:1 and 3.5:1, for example about 3:1. In an embodiment, the ratio of canonical to non-canonical basepairs in the dsRNA region is between 3.5:1 and 4.5:1, for example about 4:1. In an embodiment, the ratio of canonical to non-canonical basepairs in the dsRNA region is between 4.5:1 and 5.5:1, for example about 5:1. In an embodiment, the ratio of canonical to non-canonical basepairs in the dsRNA region is between 5.5:1 and 6.5:1, for example about 6:1. Different dsRNA regions in the RNA molecule may have different ratios.

In the above embodiments, the non-canonical basepairs in the dsRNA region(s) of the RNA molecule are preferably all G:U basepairs. In an embodiment, at least 99% of the non-canonical basepairs are G:U basepairs. In an embodiment, at least 98% of the non-canonical basepairs are G:U basepairs. In an embodiment, at least 97% of the non-canonical basepairs are G:U basepairs. In an embodiment, at least 95% of the non-canonical basepairs are G:U basepairs. In an embodiment, at least 90% of the non-canonical basepairs are G:U basepairs. In an embodiment, between 90 and 95% of the non-canonical basepairs are G:U basepairs. For example, if there are 10 non-canonical basepairs, at least 9 (90%) are G:U basepairs.

In another embodiment, between 3% and 50% of the non-canonical basepairs are G:U basepairs. In another embodiment, between 5% and 30% of the non-canonical basepairs are G:U basepairs. In another embodiment, between 10% and 30% of the non-canonical basepairs are G:U basepairs. In another embodiment, between 15% and 5 20% of the non-canonical basepairs are G:U basepairs.

In an example of the above embodiments, there are at least 3 G:U base pairings in one (contiguous) or more, or all dsRNA regions of the RNA molecule. In another example, there are at least 4, 5, 6, 7, 8, 9 or 10 G:U base pairings. In another example, there are at least between 3 and 10 G:U base pairings. In another example, there are at 10 least between 5 and 10 G:U base pairings.

The dsRNA region comprising non-canonical basepairing(s) comprises an antisense sequence of 20 contiguous nucleotides which acts as an antisense regulatory element. In an embodiment, the antisense regulatory element is at least 80%, preferably at least 90%, more preferably at least 95% or most preferably 100% 15 complementary to a target RNA molecule in a plant cell. In an embodiment, a dsRNA region comprises 2, 3, 4, or 5 antisense regulatory elements which either are complementary to the same target RNA molecule (i.e. to different regions of the same target RNA molecule) or are complementary to different target RNA molecules.

In an embodiment, one or more ribonucleotides of the sense ribonucleotide 20 sequence or one or more ribonucleotides of the antisense ribonucleotide sequence, or both, are not basepaired in the dsRNA region when the sense and antisense sequences hybridize. In this embodiment, the dsRNA region does not include any loop sequence which covalently joins the sense and antisense sequences. One or more ribonucleotides of a dsRNA region or subregion may not be basepaired. Accordingly, in this 25 embodiment, the sense strand of the dsRNA region does not fully basepair with its corresponding antisense strand.

In an embodiment, the chimeric RNA molecule does not comprise a non-canonical base pair at the base of a loop of the molecule. In another embodiment, one, two, three, four, five or more or all of the non-canonical base pairs are flanked by 30 canonical base pairs.

In an embodiment, the chimeric RNA molecule comprises at least one plant DCL-1 cleavage site.

In an embodiment, the target RNA molecule is not a viral RNA molecule.

In an embodiment, the target RNA molecule is not a South African cassava 35 mosaic virus RNA molecule.

In an embodiment, the chimeric RNA molecule comprises at least one non-basepair, or stretch of non-pasepairs, flanked by canonical base pairs, non-canonical base pairs, or a canonical base pair and a non-canonical base pair. For example, this may be a bulge as described herein.

5 In an embodiment, the chimeric RNA molecule does not comprise a double stranded region with greater than 11 canonical base pairs.

Moreover, in an embodiment and optionally in combination with any of the features of the above embodiments, the total number of ribonucleotides in the sense sequence(s) and the total number of ribonucleotides in the antisense sequence(s) may
10 not be identical, although preferably they are identical. In an embodiment, the total number of ribonucleotides in the sense ribonucleotide sequence(s) of the dsRNA region is between 90% and 110% of the total number of ribonucleotides in the antisense ribonucleotide sequence(s). In an embodiment, the total number of ribonucleotides in the sense ribonucleotide sequence(s) is between 95% and 105% of the total number of
15 ribonucleotides in the antisense ribonucleotide sequence(s). In an embodiment, chimeric RNA molecules of the present disclosure can comprise one or more structural elements such as internal or terminal bulges or loops. Various embodiments of bulges and loops are discussed above. In an embodiment, dsRNA regions are separated by a structural element such as a bulge or loop. In an embodiment, dsRNA regions are
20 separated by a intervening (spacer) sequence. Some of the ribonucleotides of the spacer sequence may be basepaired to other ribonucleotides in the RNA molecule, for example to other ribonucleotides within the spacer sequence, or they may not be basepaired in the RNA molecule, or some of each. In an embodiment, dsRNA regions are linked to a terminal loop. In an embodiment, dsRNA regions are flanked by terminal loops.

25 In an embodiment, where the dsRNA region of the RNA molecule of the invention has at least 3 non-canonical basepairs in any subregion of 5 contiguous basepairs, the non-canonical basepairs are not contiguous but are separated by one or more canonical basepairs i.e. the dsRNA region does not have 3 or more contiguous non-canonical basepairs. In an embodiment, the dsRNA region does not have 4 or
30 more contiguous non-canonical basepairs. For example, in an embodiment, the dsRNA region comprises at least 3 non-canonical basepairs in a subregion of 10 basepairs, wherein each non-canonical basepair is separated by 4 canonical basepairs.

In an embodiment, an RNA molecule of the invention comprises more than one dsRNA region. For example, the RNA molecule comprises 2, 3, 4, 5, 6, 7, 8, 9, 10 or
35 more dsRNA regions. In this example, one or more or all of the dsRNA regions can

comprise above exemplified properties such as non-canonical basepairing and/or number of antisense regulatory elements.

Silencing Activity

5 RNA molecules of the present disclosure have antisense activity as they comprise a sense ribonucleotide sequence that is essentially complementary to a region of a target RNA molecule. For example, the ribonucleotide sequence is essentially complementary to a region of a target RNA molecule in a plant cell. Such components of the RNA molecules defined herein can be referred to as an “antisense regulatory
10 element”. “Essentially complementary” means that the sense ribonucleotide sequence may have insertions, deletions and individual point mutations in comparison with the complement of the target RNA molecule in the plant cell. Preferably, the homology is at least 80%, preferably at least 90%, preferably at least 95%, most preferably 100%, between the sense ribonucleotide sequence with antisense activity and the target RNA
15 molecule. For example, the sense ribonucleotide sequence can comprise about 15, about 16, about 17, about 18, about 19 or more contiguous nucleotides that are identical in sequence to a first region of a target RNA molecule in a plant cell. In another example, the sense ribonucleotide sequence can comprise about 20 contiguous nucleotides that are identical in sequence to a first region of a target RNA molecule in a
20 plant cell.

“Antisense activity” is used in the context of the present disclosure to refer to an antisense regulatory element from an RNA molecule defined herein that modulates (increase or decrease) expression of a target RNA molecule.

In various examples, antisense regulatory elements according to the present
25 disclosure can comprise a plurality of monomeric subunits linked together by linking groups. Examples include primers, probes, antisense compounds, antisense oligonucleotides, external guide sequence (EGS) oligonucleotides, alternate splicers, gapmers, siRNAs and microRNAs. As such, RNA molecules according to the present disclosure can comprise antisense regulatory elements with single-stranded, double-
30 stranded, circular, branched or hairpin structures. In an example, the antisense sequence can contain structural elements such as internal or terminal bulges or loops.

In an example, RNA molecules of the present disclosure comprise chimeric oligomeric components such as chimeric oligonucleotides. For example, an RNA molecule can comprise differently modified nucleotides, mixed-backbone antisense
35 oligonucleotides or a combination thereof. In an example, chimeric oligomeric compounds can comprise at least one region modified so as to confer increased

resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target RNA molecule.

Antisense regulatory elements can have a variety of lengths. Across various examples, the present disclosure provides antisense regulatory elements consisting of

5 X-Y linked bases, where X and Y are each independently selected from 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50 (provided that $X < Y$). For example, in certain embodiments, the present disclosure provides antisense regulatory elements comprising: 8-9, 8-10, 8-11, 8-12, 8-13, 8-14, 8-15, 8-16, 8-17, 8-18, 8-19, 8-

10 20, 8-21, 8-22, 8-23, 8-24, 8-25, 8-26, 8-27, 8-28, 8-29, 8-30, 9-10, 9-11, 9-12, 9-13, 9-14, 9-15, 9-16, 9-17, 9-18, 9-19, 9-20, 9-21, 9-22, 9-23, 9-24, 9-25, 9-26, 9-27, 9-28, 9-29, 9-30, 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 10-21, 10-22, 10-23, 10-24, 10-25, 10-26, 10-27, 10-28, 10-29, 10-30, 11-12, 11-13, 11-14, 11-15, 11-16, 11-17, 11-18, 11-19, 11-20, 11-21, 11-22, 11-23, 11-24, 11-25, 11-26,

15 11-27, 11-28, 11-29, 11-30, 12-13, 12-14, 12-15, 12-16, 12-17, 12-18, 12-19, 12-20, 12-21, 12-22, 12-23, 12-24, 12-25, 12-26, 12-27, 12-28, 12-29, 12-30, 13-14, 13-15, 13-16, 13-17, 13-18, 13-19, 13-20, 13-21, 13-22, 13-23, 13-24, 13-25, 13-26, 13-27, 13-28, 13-29, 13-30, 14-15, 14-16, 14-17, 14-18, 14-19, 14-20, 14-21, 14-22, 14-23, 14-24, 14-25, 14-26, 14-27, 14-28, 14-29, 14-30, 15-16, 15-17, 15-18, 15-19, 15-20,

20 15-21, 15-22, 15-23, 15-24, 15-25, 15-26, 15-27, 15-28, 15-29, 15-30, 16-17, 16-18, 16-19, 16-20, 16-21, 16-22, 16-23, 16-24, 16-25, 16-26, 16-27, 16-28, 16-29, 16-30, 17-18, 17-19, 17-20, 17-21, 17-22, 17-23, 17-24, 17-25, 17-26, 17-27, 17-28, 17-29, 17-30, 18-19, 18-20, 18-21, 18-22, 18-23, 18-24, 18-25, 18-26, 18-27, 18-28, 18-29, 18-30, 19-20, 19-21, 19-22, 19-23, 19-24, 19-25, 19-26, 19-29, 19-28, 19-29, 19-30,

25 20-21, 20-22, 20-23, 20-24, 20-25, 20-26, 20-27, 20-28, 20-29, 20-30, 21-22, 21-23, 21-24, 21-25, 21-26, 21-27, 21-28, 21-29, 21-30, 22-23, 22-24, 22-25, 22-26, 22-27, 22-28, 22-29, 22-30, 23-24, 23-25, 23-26, 23-27, 23-28, 23-29, 23-30, 24-25, 24-26, 24-27, 24-28, 24-29, 24-30, 25-26, 25-27, 25-28, 25-29, 25-30, 26-27, 26-28, 26-29, 26-30, 27-28, 27-29, 27-30, 28-29, 28-30, or 29-30 linked bases.

30 RNA molecules according to the present disclosure can comprise multiple antisense regulatory elements. For example, RNA molecules can comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 antisense regulatory elements. In an example, the antisense regulatory elements are the same. In this example, the RNA molecule can comprise at least 2, at least 3, at least 4,

35 at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 copies of an antisense regulatory element. In another example, RNA molecules according to the present

disclosure can comprise different antisense regulatory elements. For example, antisense regulatory elements may be provided to target multiple genes in a pathway such as lipid biosynthesis. In this example, the RNA molecule can comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10
5 different antisense regulatory elements.

Antisense sequences according to the present disclosure can modulate, preferably decrease, expression or amount of various target RNA molecules. In an example, the target RNA molecule modulates flowering in a plant disclosed herein. Examples of such target RNA molecules are described in the art (e.g. Cockram et al.,
10 2007; Chen et al., 2009; Jung and Muller., 2009; Cho et al., 2017). In an example, the target RNA molecule modulates vernalisation in a plant disclosed herein. In an example, the target RNA molecule promotes early flowering. In another example, the target RNA molecule promotes late flowering. In an example, the target RNA molecule encodes a plant polycomb group (PcG) protein. In an example, the target
15 RNA molecule encodes VERNALIZATION1 (VRN1; UniProt accession number: Q8L3W1) or VERNALIZATION2 (VRN2; UniProt accession number: Q8W5B1) or homologous genes in other species. In an example, the target RNA molecule encodes a PcG from *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an example, the target RNA
20 molecule encodes a PcG from *Arabidopsis*, corn, canola, cotton, soybean, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an example, the target RNA molecule encodes VRN1 and/or VRN2 from wheat. In an example, the target RNA molecule encodes EMBRYONIC FLOWER2 (EMF2; UniProt accession number: Q8L6Y4) or FERTILIZATION INDEPENDENT SEED2 (FIS2; UniProt accession
25 number: P0DKJ7) or homologous genes in other species. In an example, the target RNA molecule encodes one or more or all of VRN1, VRN2, EMF2, FIS2. Other examples of target RNA molecules encode EARLYINSHORTDAYS4 (ESD4; UniProt accession number: Q94F30) and FLOWERING LOCUS T (FLT; UniProt accession number: Q9SXZ2) or homologous genes in other species.

30 Accordingly, in various examples, the target RNA molecules can be a gene transcript of one or more of *VRN1*, *VRN2*, *EMF2*, *FIS2*, *ESD4*, *FLT1*, *FLT2*. In an example, the target RNA molecule can be a gene transcript of one or more of the following from wheat/barley, *VRN1/VRN-A1* (KR422423.1); *VRN2* (ZCCT1, TaVRN-2B) (AAS58481.1); *FT* (AY705794.1). In another example, the target RNA
35 molecule can be a gene transcript of one or more of the following from canola, *BnFLC1* (AY036888, Bna.FLC.A10, BnaA10g22080D); *BnFLC2* (AY036889);

BnFLC3 (AY036890); BnFLC4 (AY036891); BnFLC5 (AY036892); BnFRI (BnaA03g13320D); BnFT (BnaA02g12130D). For example, the target RNA molecule can be a gene transcript of BnFLC1 (AY036888, Bna.FLC.A10, BnaA10g22080D). In an example, the target RNA molecule can be a gene transcript of a FRIGIDA orthologue such as BnaA3.FRI (Yi et al., 2018) or homologous genes in other species.

5 In another example, the target RNA molecule can be a gene transcript of one or more of the following from *Arabidopsis*, FRI (AT4G00650); FLC (AT5G10140); VRN1 (AT3G18990); VRN2 (AT4G16845); VIN3 (AT5G57380); FT (AT1G65480); SOC1 (AT2G45660); CO (constans) (AT5G15840); LFY (AT5G61850); AP1 (AT1G69120)

10 or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from Rice, OsPhyB (OSNPB_030309200); OsCol4 (Hd-1) (HC084637); RFT1 (OSNPB_070486100); OsSNB (OSNPB_070235800); OsIDS1 (Os03g0818800); OsGI (OSNPB_010182600) or homologous genes in other species. In another example, the target RNA molecule

15 can be a gene transcript of one or more of the following from *Medicago truncatula*, MtFTa1 (HQ721813); MtFTb1 (HQ721815) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of a homolog of one or more of the following from Legume, MtFTa1; MtFTb1. In another example, the target RNA molecule can be a gene transcript of one or more of the following from

20 Sugarbeet, chard, turnip, *BTC1* (HQ709091.); *BvFTI* (HM448909.1); *BvFLI* (DQ189214., DQ189215.) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from barley, HvVRN1 (AY896051); HvVRN2 (AY687931, AY485978); HvFT (DQ898519) or homologous genes in other species. In another example, the target RNA molecule

25 can be a gene transcript of one or more of the following from Maize, ZmMADS1/ZmM5 (LOC542042, HM993639); PHYA1 (AY234826); PHYA2 (AY260865); PHYB1 (AY234827); PHYB2 (AY234828); PHYC1 (AY234829); PHYC2 (AY234830); LD (AF166527); ZFL1 (AY179882); ZFL2 (AY179881); DWARF8 (AF413203); AN1 (L37750); ID1 (AF058757); ZCN8 (LOC100127519) or

30 homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from *Brassica rapa*, BrFLC2 (AH012704); BrFT (Bra004928); BrFRI (HQ615935) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of MsFRI-L (JX173068) from *Alfalfa (Medicago sativa)* or homologous genes in other

35 species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from Barrell medic, MtYFL (BT053010); MtSOC1a

(Medtr07g075870); MtSOC1b (Medtr08g033250); MtSOC1c (Medtr08g033220); MtFTa1 (HQ721813) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from cotton, GhCO (Gorai.008G059900); GhFLC (Gorai.013G069000); GhFRI
5 (Gorai.003G118000); GhFT (Gorai.004G264600); GhLFY (Gorai.001G053900); GhPHYA (Gorai.007G292800, Gorai.013G203900); GhPHYB (Gorai.011G200200); GhSOC1 (Gorai.008G115200); GhVRN1 (Gorai.002G006500, Gorai.005G240900, Gorai.012G150900, Gorai.013G040000); GhVRN2 (Gorai.003G176300); GhVRN5 (Gorai.009G023200) or homologous genes in other species. In another example, the
10 target RNA molecule can be a gene transcript of one or more of the following from onion, AcGI (GQ232756); AcFKF (GQ232754); AcZTL (GQ232755); AcCOL (GQ232751); AcFTL (CF438000); AcFT1 (KC485348); AcFT2 (KC485349); AcFT6 (KC485353); AcPHYA (GQ232753); AcCOP1 (CF451443) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of
15 one or more of the following from *Asparagus officinalis*, FPA (LOC109824259, LOC109840062); TWIN SISTER of FT-like (LOC109835987); MOTHER of FT (LOC109844838); FCA-like (LOC109841154, LOC109821266); PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (LOC109834006); FLOWERING LOCUS T-like (LOC109830558, LOC109825338, LOC109824462); Flowering locus K
20 (LOC109847537); Flowering time control protein FY (LOC109844014); flowering time control protein FCA-like (LOC109842562) or homologous genes in other species. In another example, the target RNA molecule can be a gene transcript of one or more of the following from lettuce, LsFT (LOC111907824); TFL1-like (LOC111903066); TFL1 homolog 1-like (LOC111903054); LsFLC (LOC111876490, JI588382); SOC1-like (LOC111912847, LOC111880753, LOC111878575); TsLFY (LC164345.1, XM_023888266.1) or homologous genes in other species. Those of skill in the art will appreciate that many of the above referenced gene transcripts and proteins encoded by the same are conserved amongst related crop species. Accordingly, in an example, the present disclosure extends to homologues thereof. Identifying homologues is
30 considered well within the purview of those skilled in the art using various online databases such as Genbank, EMBL-EBI, Ensembl Plants or performing online searches using tools such as nucleotide BLAST. Examples of homologues are provided above. Accordingly, in a preferred example, the target RNA molecule can be a gene transcript of BnFLC1 or a homolog thereof such as, for example BnFLC1 (AY036888), BnFLC1
35 (Bna.FLC.A10) or BnFLC1 (BnaA10g22080D).

In another example, the target RNA is a non-coding RNA that modulates flowering in plants. In an example, the non-coding RNA is a miRNA or precursor thereof. In an example, the target miRNA is a miRNA from the miR-156 family or a precursor thereof. For example, the target RNA can be any one or more of miR-156a, 5 miR-156b, miR-156c, miR-156d, miR-156e, miR-156f, miR-156g, miR-156h or a precursor thereof. In an example, the target RNA is one or more of miR-156a, miR-156b, miR-156c or a precursor thereof. In an example, the target RNA is miR-172 or a precursor thereof. Other exemplary target RNAs which are miRNAs or precursors thereof are described in Teotia and Tang., 2015). miRNA sequences are described in 10 the art and can be identified by for example miRBase: the microRNA database (Kozomara et al., 2019); www dot mirbase dot org).

In a preferred example, the target RNA molecule is a transcript from a VRN2 gene.

15 Nucleic Acids Encoding RNA Molecules

One of skill in the art will appreciate from the foregoing description that the present disclosure also provides an isolated nucleic acid encoding RNA molecules disclosed herein and the component parts thereof. For example, a nucleic acid comprising a sequence set forth in any one or more of SEQ ID NO:1, SEQ ID NO:2, 20 SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:150. The nucleic acid may be partially purified after expression in a host cell. The term "partially purified " is used to refer to an RNA molecule that has generally been separated from the lipids, nucleic acids, other peptides, and other contaminating molecules with which it is associated in a host cell. 25 Preferably, the partially purified polynucleotide is at least 60% free, more preferably at least 75% free, and more preferably at least 90% free from other components with which it is associated.

In another example, a polynucleotide according to the present disclosure is a heterologous polynucleotide. The term "heterologous polynucleotide" is well 30 understood in the art and refers to a polynucleotide which is not endogenous to a cell, or is a native polynucleotide in which the native sequence has been altered, or a native polypeptide whose expression is quantitatively altered as a result of a manipulation of the cell by recombinant DNA techniques.

In another example, a polynucleotide according to the present disclosure is a 35 synthetic polynucleotide. For example, the polynucleotide may be produced using techniques that do not require pre-existing nucleic acid sequences such as DNA

printing and oligonucleotide synthesis. In another example, the polynucleotide is produced from xeno nucleic acids.

In an example, a polynucleotide disclosed herein which encodes an RNA precursor molecule comprising an intron, preferably in a 5' extension sequence or in at least one loop sequence, wherein the intron is capable of being spliced out during transcription of the polynucleotide in a host cell or *in vitro*. In another example, the loop sequence comprises two, three, four, five or more introns. The present disclosure also provides an expression construct such as a DNA construct comprising an isolated nucleic acid of the disclosure operably linked to a promoter. In an example, such isolated nucleic acids and/or expression constructs are provided in a cell or plant. In an example isolated nucleic acids are stably integrated into the genome of the cell or plant organism. Various examples of suitable expression constructs, promoters and cells comprising the same are discussed below.

Synthesis of RNA molecules according to the present disclosure can be achieved using various methods known in the art. The Examples section provides an example of *in vitro* synthesis. In this example, constructs comprising RNA molecules disclosed herein are restricted at the 3' end, precipitated, purified and quantified. RNA synthesis can be achieved in bacterial culture following transformation of HT115 electro competent cells and induction of RNA synthesis using the T7, IPTG system.

20

Recombinant Vectors

One embodiment of the present invention includes a recombinant vector, which comprises at least one RNA molecule defined herein and is capable of delivering the RNA molecule into a host cell. Recombinant vectors include expression vectors. Recombinant vectors contain heterologous polynucleotide sequences, that is, polynucleotide sequences that are not naturally found adjacent to an RNA molecule defined herein, that preferably, are derived from a different species. The vector can be either RNA or DNA, and typically is a viral vector, derived from a virus, or a plasmid.

Various viral vectors can be used to deliver and mediate expression of an RNA molecule according to the present disclosure. The choice of viral vector will generally depend on various parameters, such as the cell or tissue targeted for delivery, transduction efficiency of the vector and pathogenicity. In an example, the viral vector integrates into host cellular chromatin (e.g. lentiviruses). In another example, the viral vector persists in the cell nucleus predominantly as an extrachromosomal episome (e.g. adenoviruses). Examples of these types of viral vectors include oncoretroviruses, lentiviruses, adeno-associated virus, adenoviruses, herpes viruses and retroviruses.

Plasmid vectors typically include additional nucleic acid sequences that provide for easy selection, amplification, and transformation of the expression cassette in prokaryotic cells, e.g., pUC-derived vectors, pGEM-derived vectors or binary vectors containing one or more T-DNA regions. Additional nucleic acid sequences include
5 origins of replication to provide for autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to insert nucleic acid sequences or genes encoded in the nucleic acid construct, and sequences that enhance transformation of plant cells.

10 "Operably linked" as used herein, refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory element (promoter) to a transcribed sequence. For example, a promoter is operably linked to a coding sequence of an RNA
15 molecule defined herein, if it stimulates or modulates the transcription of the coding sequence in an appropriate cell. Generally, promoter transcriptional regulatory elements that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory elements such as enhancers need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

20 When there are multiple promoters present, each promoter may independently be the same or different.

To facilitate identification of transformants, the recombinant vector desirably comprises a selectable or screenable marker gene. By "marker gene" is meant a gene that imparts a distinct phenotype to cells expressing the marker gene and thus, allows
25 such transformed cells to be distinguished from cells that do not have the marker. A selectable marker gene confers a trait for which one can "select" based on resistance to a selective agent (e.g., a herbicide, antibiotic). A screenable marker gene (or reporter gene) confers a trait that one can identify through observation or testing, that is, by "screening" (e.g., β -glucuronidase, luciferase, GFP or other enzyme activity not present
30 in untransformed cells). Exemplary selectable markers for selection of plant transformants include, but are not limited to, a *hyg* gene which encodes hygromycin B resistance; a neomycin phosphotransferase (*nptII*) gene conferring resistance to kanamycin, paromomycin; a glutathione-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides as for example, described in EP 256223; a
35 glutamine synthetase gene conferring, upon overexpression, resistance to glutamine synthetase inhibitors such as phosphinothricin as for example, described in WO

87/05327; an acetyltransferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin as for example, described in EP 275957; a gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine as for example, described by Hinchee et al. 5 (1988); a *bar* gene conferring resistance against bialaphos as for example, described in WO91/02071; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker et al., 1988); a dihydrofolate reductase (DHFR) gene conferring resistance to methotrexate (Thillet et al., 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea, or other 10 ALS-inhibiting chemicals (EP 154,204); a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan; or a dalapon dehalogenase gene that confers resistance to the herbicide.

Preferably, the recombinant vector is stably incorporated into the genome of the cell such as the plant cell. Accordingly, the recombinant vector may comprise 15 appropriate elements which allow the vector to be incorporated into the genome, or into a chromosome of the cell.

Expression Vector

As used herein, an "expression vector" is a DNA vector that is capable of 20 transforming a host cell and of effecting expression of an RNA molecule defined herein. Expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the host cell and that control the expression of RNA molecule according to the present disclosure. In particular, 25 expression vectors of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation such as promoter, enhancer, operator and repressor sequences. The choice of the regulatory sequences used may depends on 30 the target plant or part thereof. Such regulatory sequences may be obtained from any eukaryotic organism such as plants or plant viruses, or may be chemically synthesized.

Exemplary vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in for example, Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987, Weissbach and Weissbach, 35 Methods for Plant Molecular Biology, Academic Press, 1989, and Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990. Typically, plant

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
5 developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, a transcription termination site, and/or a polyadenylation signal.

Vectors of the invention can also be used to produce RNA molecules defined herein in a cell-free expression system, such systems are well known in the art.

10 In an example, a polynucleotide encoding an RNA molecule according to the present disclosure is operably linked to a promoter capable of directing expressing of the RNA molecule in a host cell. In an example, the promoter functions *in vitro*. In an example, the promoter is an RNA polymerase promoter. For example, the promoter can be an RNA polymerase III promoter. In another example, the promoter can be an
15 RNA polymerase II promoter. However, the choice of promoter may depend on the target plant or part thereof. Exemplary promoters which may be suitable for constitutive expression in plants include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter, the Figwort mosaic virus (FMV) 35S, the light-inducible promoter from the small subunit (SSU) of the ribulose-1,5-bis-phosphate carboxylase,
20 the rice cytosolic triosephosphate isomerase promoter, the adenine phosphoribosyltransferase promoter of *Arabidopsis*, the rice actin 1 gene promoter, the mannopine synthase and octopine synthase promoters, the Adh promoter, the sucrose synthase promoter, the R gene complex promoter, and the chlorophyll α/β binding protein gene promoter. These promoters have been used to create DNA vectors that
25 have been expressed in plants, see for example, WO 84/02913. All of these promoters have been used to create various types of plant-expressible recombinant DNA vectors.

For the purpose of expression in source tissues of the plant such as the leaf, seed, root or stem, it is preferred that the promoters utilized in the present invention have relatively high expression in these specific tissues. For this purpose, one may
30 choose from a number of promoters for genes with tissue- or cell-specific, or -enhanced expression. Examples of such promoters reported in the literature include, the chloroplast glutamine synthetase GS2 promoter from pea, the chloroplast fructose-1,6-biphosphatase promoter from wheat, the nuclear photosynthetic ST-LS1 promoter from potato, the serine/threonine kinase promoter and the glucoamylase (CHS) promoter
35 from *Arabidopsis thaliana*. Also reported to be active in photosynthetically active tissues are the ribulose-1,5-bisphosphate carboxylase promoter from eastern larch

(*Larix laricina*), the promoter for the Cab gene, Cab6, from pine, the promoter for the Cab-1 gene from wheat, the promoter for the Cab-1 gene from spinach, the promoter for the Cab 1R gene from rice, the pyruvate, orthophosphate dikinase (PPDK) promoter from *Zea mays*, the promoter for the tobacco Lhcb1*2 gene, the *Arabidopsis thaliana* Suc2 sucrose-H³⁰ symporter promoter, and the promoter for the thylakoid membrane protein genes from spinach (PsaD, PsaF, PsaE, PC, FNR, AtpC, AtpD, Cab, RbcS). Other promoters for the chlorophyll α/β -binding proteins may also be utilized in the present invention such as the promoters for LhcB gene and PsbP gene from white mustard (*Sinapis alba*).

10 A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of RNA-binding protein genes in plant cells, including promoters regulated by heat, light (e.g., pea RbcS-3A promoter, maize RbcS promoter), hormones such as abscisic acid, wounding (e.g., WunI), or chemicals such as methyl jasmonate, salicylic acid, steroid hormones, alcohol, Safeners (WO 97/06269), or it may also be
15 advantageous to employ organ-specific promoters.

As used herein, the term "plant storage organ specific promoter" refers to a promoter that preferentially, when compared to other plant tissues, directs gene transcription in a storage organ of a plant. For the purpose of expression in sink tissues
20 of the plant such as the tuber of the potato plant, the fruit of tomato, or the seed of soybean, canola, cotton, *Zea mays*, wheat, rice, and barley, it is preferred that the promoters utilized in the present invention have relatively high expression in these specific tissues. The promoter for β -conglycinin or other seed-specific promoters such as the napin, zein, linin and phaseolin promoters, can be used. Root specific promoters
25 may also be used. An example of such a promoter is the promoter for the acid chitinase gene. Expression in root tissue could also be accomplished by utilizing the root specific subdomains of the CaMV 35S promoter that have been identified.

In another embodiment, the plant storage organ specific promoter is a fruit specific promoter. Examples include, but are not limited to, the tomato
30 polygalacturonase, E8 and Pds promoters, as well as the apple ACC oxidase promoter (for review, see Potenza et al., 2004). In a preferred embodiment, the promoter preferentially directs expression in the edible parts of the fruit, for example the pith of the fruit, relative to the skin of the fruit or the seeds within the fruit.

In an embodiment, the inducible promoter is the *Aspergillus nidulans* alc
35 system. Examples of inducible expression systems which can be used instead of the *Aspergillus nidulans* alc system are described in a review by Padidam (2003) and

Corrado and Karali (2009). In another embodiment, the inducible promoter is a safener inducible promoter such as, for example, the maize *ln2-1* or *ln2-2* promoter (Hershey and Stoner, 1991), the safener inducible promoter is the maize GST-27 promoter (Jepson et al., 1994), or the soybean GH2/4 promoter (Ulmasov et al., 1995).

5 In another embodiment, the inducible promoter is a senescence inducible promoter such as, for example, senescence-inducible promoter SAG (senescence associated gene) 12 and SAG 13 from *Arabidopsis* (Gan, 1995; Gan and Amasino, 1995) and LSC54 from *Brassica napus* (Buchanan-Wollaston, 1994). Such promoters show increased expression at about the onset of senescence of plant tissues, in
10 particular the leaves.

For expression in vegetative tissue leaf-specific promoters, such as the ribulose biphosphate carboxylase (RBCS) promoters, can be used. For example, the tomato RBCS1, RBCS2 and RBCS3A genes are expressed in leaves and light grown seedlings (Meier et al., 1997). A ribulose bisphosphate carboxylase promoters expressed almost
15 exclusively in mesophyll cells in leaf blades and leaf sheaths at high levels, described by Matsuoka et al. (1994), can be used. Another leaf-specific promoter is the light harvesting chlorophyll a/b binding protein gene promoter (see, Shiina et al., 1997). The *Arabidopsis thaliana* myb-related gene promoter (Atmyb5) described by Li et al. (1996), is leaf-specific. The Atmyb5 promoter is expressed in developing leaf
20 trichomes, stipules, and epidermal cells on the margins of young rosette and cauline leaves, and in immature seeds. A leaf promoter identified in maize by Busk et al. (1997), can also be used.

In some instances, for example when LEC2 or BBM is recombinantly expressed, it may be desirable that the transgene is not expressed at high levels. An
25 example of a promoter which can be used in such circumstances is a truncated napin A promoter which retains the seed-specific expression pattern but with a reduced expression level (Tan et al., 2011).

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous gene sequence of an RNA molecule of the present
30 disclosure, or may be heterologous with respect to the coding region of the enzyme to be produced, and can be specifically modified if desired so as to increase translation of mRNA. For a review of optimizing expression of transgenes, see Koziel et al. (1996). The 5' non-translated regions can also be obtained from plant viral RNAs (Tobacco mosaic virus, Tobacco etch virus, Maize dwarf mosaic virus, Alfalfa mosaic virus,
35 among others), plant genes (wheat and maize chlorophyll a/b binding protein gene leader), or from a synthetic gene sequence. The present invention is not limited to

constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence. Leader sequences useful in context of the present invention comprise the maize Hsp70 leader (US 5,362,865 and 5 US 5,859,347), and the TMV omega element.

The termination of transcription is accomplished by a 3' non-translated DNA sequence operably linked in the expression vector to the RNA molecule of interest. The 3' non-translated region of a recombinant DNA molecule contains a polyadenylation signal that functions in plants to cause the addition of adenylate 10 nucleotides to the 3' end of the RNA. The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated region, the 3' untranslated region from pea small subunit Rubisco gene, the 3' untranslated region from soybean 7S seed storage protein gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate 15 signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

In an example, the expression vector comprises a nucleic acid sequence as shown in SEQ ID NO:150.

Transfer Nucleic Acids

20 Transfer nucleic acids can be used to deliver an exogenous polynucleotide to a cell and comprise one, preferably two, border sequences and one or more RNA molecules of interest. The transfer nucleic acid may or may not encode a selectable marker. Preferably, the transfer nucleic acid forms part of a binary vector in a bacterium, where the binary vector further comprises elements which allow replication 25 of the vector in the bacterium, selection, or maintenance of bacterial cells containing the binary vector. Upon transfer to a plant cell, the transfer nucleic acid component of the binary vector is capable of integration into the genome of the plant cell or, for transient expression experiments, merely of expression in the cell.

As used herein, the term "extrachromosomal transfer nucleic acid" refers to a 30 nucleic acid molecule that is capable of being transferred from a bacterium such as *Agrobacterium sp.*, to a plant cell such as a plant leaf cell. An extrachromosomal transfer nucleic acid is a genetic element that is well-known as an element capable of being transferred, with the subsequent integration of a nucleotide sequence contained within its borders into the genome of the recipient cell. In this respect, a transfer 35 nucleic acid is flanked, typically, by two "border" sequences, although in some instances a single border at one end can be used and the second end of the transferred

nucleic acid is generated randomly in the transfer process. An RNA molecule of interest is typically positioned between the left border-like sequence and the right border-like sequence of a transfer nucleic acid. The RNA molecule contained within the transfer nucleic acid may be operably linked to a variety of different promoter and terminator regulatory elements that facilitate its expression, that is, transcription and/or translation of the RNA molecule. Transfer DNAs (T-DNAs) from *Agrobacterium sp.* such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, and man made variants/mutants thereof are probably the best characterized examples of transfer nucleic acids. Another example is P-DNA ("plant-DNA") which comprises T-DNA border-like sequences from plants.

As used herein, "T-DNA" refers to a T-DNA of an *Agrobacterium tumefaciens* Ti plasmid or from an *Agrobacterium rhizogenes* Ri plasmid, or variants thereof which function for transfer of DNA into plant cells. The T-DNA may comprise an entire T-DNA including both right and left border sequences, but need only comprise the minimal sequences required in *cis* for transfer, that is, the right T-DNA border sequence. The T-DNAs of the invention have inserted into them, anywhere between the right and left border sequences (if present), the RNA molecule of interest. The sequences encoding factors required in *trans* for transfer of the T-DNA into a plant cell such as *vir* genes, may be inserted into the T-DNA, or may be present on the same replicon as the T-DNA, or preferably are in *trans* on a compatible replicon in the *Agrobacterium* host. Such "binary vector systems" are well known in the art. As used herein, "P-DNA" refers to a transfer nucleic acid isolated from a plant genome, or man made variants/mutants thereof, and comprises at each end, or at only one end, a T-DNA border-like sequence.

As used herein, a "border" sequence of a transfer nucleic acid can be isolated from a selected organism such as a plant or bacterium, or be a man made variant/mutant thereof. The border sequence promotes and facilitates the transfer of the RNA molecule to which it is linked and may facilitate its integration in the recipient cell genome. In an embodiment, a border-sequence is between 10-80 bp in length. Border sequences from T-DNA from *Agrobacterium sp.* are well known in the art and include those described in Lacroix et al. (2008).

Whilst traditionally only *Agrobacterium sp.* have been used to transfer genes to plants cells, there are now a large number of systems which have been identified/developed which act in a similar manner to *Agrobacterium sp.* Several non-*Agrobacterium* species have recently been genetically modified to be competent for

gene transfer (Chung et al., 2006; Broothaerts et al., 2005). These include *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mezorhizobium loti*.

Direct transfer of eukaryotic expression plasmids from bacteria to eukaryotic hosts was first achieved several decades ago by the fusion of mammalian cells and
5 protoplasts of plasmid-carrying *Escherichia coli* (Schaffner, 1980). Since then, the number of bacteria capable of delivering genes into mammalian cells has steadily increased (Weiss, 2003), being discovered by four groups independently (Sizemore et al. 1995; Courvalin et al., 1995; Powell et al., 1996; Darji et al., 1997).

As used herein, the terms "transfection", "transformation" and variations thereof
10 are generally used interchangeably. "Transfected" or "transformed" cells may have been manipulated to introduce the RNA molecule(s) of interest, or may be progeny cells derived therefrom. In an example, the transfer nucleic acid comprises a nucleic acid sequence as shown in SEQ ID NO:150.

15 Recombinant Cells

The invention also provides a recombinant cell, for example, a recombinant plant cell, which is a host cell transformed with one or more RNA molecules or vectors defined herein, or combination thereof. Suitable cells of the invention include any cell
20 that can be transformed with an RNA molecule or recombinant vector according to the present disclosure. Preferably, in an example, the host cell is a plant cell. The recombinant cell may be a cell in culture, a cell *in vitro*, or in an organism such as for example, a plant, or in an organ such as, for example, a seed or a leaf. Preferably, the cell is in a plant, more preferably in the seed of a plant.

Host cells into which the RNA molecules(s) are introduced can be either
25 untransformed cells or cells that are already transformed with at least one nucleic acid. Such nucleic acids may be related to lipid synthesis, or unrelated. Host cells of the present invention either can be endogenously (i.e., naturally) capable of expressing RNA molecule(s) defined herein, in which case the recombinant cell derived therefrom has an enhanced capability of producing the RNA molecule(s), or can be capable of
30 producing said RNA molecule(s) only after being transformed with at least one RNA molecule defined herein. In an example, the cell is a cell which is capable of being used for producing lipid. In an embodiment, a recombinant cell of the invention has an enhanced capacity to produce non-polar lipid such as TAG.

In a preferred embodiment, the plant cell is a seed cell, in particular, a cell in a
35 cotyledon or endosperm of a seed.

Transgenic Plants

The invention also provides a plant comprising one or more exogenous RNA molecules defined herein, a cell of according to the present disclosure, a vector according to the present disclosure, or a combination thereof. The term "plant" when
5 used as a noun refers to whole plants, whilst the term "part thereof" refers to plant organs (e.g., leaves, stems, roots, flowers, fruit), single cells (e.g., pollen), seed, seed parts such as an embryo, endosperm, scutellum or seed coat, plant tissue such as vascular tissue, plant cells and progeny of the same. As used herein, plant parts comprise plant cells.

10 As used herein, the terms "in a plant" and "in the plant" in the context of a modification to the plant means that the modification has occurred in at least one part of the plant, including where the modification has occurred throughout the plant, and does not exclude where the modification occurs in only one or more but not all parts of the plant. For example, a tissue-specific promoter is said to be expressed "in a plant",
15 even though it might be expressed only in certain parts of the plant. Analogously, "a transcription factor polypeptide that increases the expression of one or more glycolytic and/or fatty acid biosynthetic genes in the plant" means that the increased expression occurs in at least a part of the plant.

As used herein, the term "plant" is used in its broadest sense, including any
20 organism in the Kingdom Plantae. It also includes red and brown algae as well as green algae. It includes, but is not limited to, any species of flowering plant, grass, crop or cereal (e.g., oilseed, maize, soybean), fodder or forage, fruit or vegetable plant, herb plant, woody plant or tree. It is not meant to limit a plant to any particular structure. It also refers to a unicellular plant (e.g., microalga). The term "part thereof" in reference
25 to a plant refers to a plant cell and progeny of same, a plurality of plant cells, a structure that is present at any stage of a plant's development, or a plant tissue. Such structures include, but are not limited to, leaves, stems, flowers, fruits, nuts, roots, seed, seed coat, embryos. The term "plant tissue" includes differentiated and undifferentiated tissues of plants including those present in leaves, stems, flowers, fruits, nuts, roots,
30 seed, for example, embryonic tissue, endosperm, dermal tissue (e.g., epidermis, periderm), vascular tissue (e.g., xylem, phloem), or ground tissue (comprising parenchyma, collenchyma, and/or sclerenchyma cells), as well as cells in culture (e.g., single cells, protoplasts, callus, embryos, etc.). Plant tissue may be *in planta*, in organ culture, tissue culture, or cell culture.

35 As herein herein, a "seedling consists" refers to the stage of plant growth spanning emergence from the seed up until the formation of the first true leaves. In an

embodiment, the seedling comprises of three main parts: the radicle (embryonic root), the hypocotyl (embryonic shoot), and the cotyledon(s).

Different amounts of 18:3 and 16:3 fatty acids are found within the glycolipids of different plant species. This is used to distinguish between 18:3 plants whose fatty acids with 3 double bonds are generally always C₁₈ atoms long and the 16:3 plants that contain both C₁₆- and C₁₈-fatty acids. In 18:3 chloroplasts, enzymic activities catalyzing the conversion of phosphatidate to diacylglycerol and of diacylglycerol to monogalactosyl diacylglycerol (MGD) are significantly less active than in 16:3 chloroplasts. In leaves of 18:3 plants, chloroplasts synthesize stearyl-ACP2 in the stroma, introduce the first double bond into the saturated hydrocarbon chain, and then hydrolyze the thioester. Released oleate is exported across chloroplast envelopes into membranes of the eucaryotic part of the cell, probably the endoplasmic reticulum, where it is incorporated into PC. PC-linked oleoyl groups are desaturated in these membranes and subsequently move back into the chloroplast. The MGD-linked acyl groups are substrates for the introduction of the third double bond to yield MGD with two linolenoyl residues. This galactolipid is characteristic of 18:3 plants such as Asteraceae and Fabaceae, for example. In photosynthetically active cells of 16:3 plants which are represented, for example, by members of Apiaceae and Brassicaceae, two pathways operate in parallel to provide thylakoids with MGD. The cooperative 'eucaryotic' sequence is supplemented to various extents by a 'procaryotic' pathway. Its reactions are confined to the chloroplast and result in a typical arrangement of acyl groups as well as their complete desaturation once they are esterified to MGD. Procaryotic DAG backbones carry C₁₆:0 and its desaturation products at C-2 from which position C₁₈: fatty acids are excluded. The C-1 position is occupied by C₁₈ fatty acids and to a small extent by C₁₆ groups. The similarity in DAG backbones of lipids from blue-green algae with those synthesized by the chloroplast-confined pathway in 16:3 plants suggests a phylogenetic relation and justifies the term procaryotic.

As used herein, the term "vegetative tissue" or "vegetative plant part" is any plant tissue, organ or part other than organs for sexual reproduction of plants. The organs for sexual reproduction of plants are specifically seed bearing organs, flowers, pollen, fruits and seeds. Vegetative tissues and parts include at least plant leaves, stems (including bolts and tillers but excluding the heads), tubers and roots, but excludes flowers, pollen, seed including the seed coat, embryo and endosperm, fruit including mesocarp tissue, seed-bearing pods and seed-bearing heads. In one embodiment, the vegetative part of the plant is an aerial plant part. In another or further embodiment, the vegetative plant part is a green part such as a leaf or stem.

A "transgenic plant" or variations thereof refers to a plant that contains a transgene not found in a wild-type plant of the same species, variety or cultivar. Transgenic plants as defined in the context of the present invention include plants and their progeny which have been genetically modified using recombinant techniques to
5 cause production of at least one polypeptide defined herein in the desired plant or part thereof. Transgenic plant parts has a corresponding meaning.

The terms "seed" and "grain" are used interchangeably herein. "Grain" refers to mature grain such as harvested grain or grain which is still on a plant but ready for harvesting, but can also refer to grain after imbibition or germination, according to the
10 context. Mature grain commonly has a moisture content of less than about 18%. In a preferred embodiment, the moisture content of the grain is at a level which is generally regarded as safe for storage, preferably between 5% and 15%, between 6% and 8%, between 8% and 10%, or between 10% and 15%. "Developing seed" as used herein refers to a seed prior to maturity, typically found in the reproductive structures of the
15 plant after fertilisation or anthesis, but can also refer to such seeds prior to maturity which are isolated from a plant. Mature seed commonly has a moisture content of less than about 12%.

As used herein, the term "plant storage organ" refers to a part of a plant specialized to store energy in the form of for example, proteins, carbohydrates, lipid.
20 Examples of plant storage organs are seed, fruit, tuberous roots, and tubers. A preferred plant storage organ of the invention is seed.

As used herein, the term "phenotypically normal" refers to a genetically modified plant or part thereof, for example a transgenic plant, or a storage organ such as a seed, tuber or fruit of the invention not having a significantly reduced ability to
25 grow and reproduce when compared to an unmodified plant or part thereof. Preferably, the biomass, growth rate, germination rate, storage organ size, seed size and/or the number of viable seeds produced is not less than 90% of that of a plant lacking said recombinant polynucleotide when grown under identical conditions. This term does not encompass features of the plant which may be different to the wild-type plant but
30 which do not affect the usefulness of the plant for commercial purposes such as, for example, a ballerina phenotype of seedling leaves. In an embodiment, the genetically modified plant or part thereof which is phenotypically normal comprises a recombinant polynucleotide encoding a silencing suppressor operably linked to a plant storage organ specific promoter and has an ability to grow or reproduce which is essentially the same
35 as a corresponding plant or part thereof not comprising said polynucleotide.

Plants provided by or contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. In preferred embodiments, the plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, rice, sorghum, millet, cassava, barley) or legumes such as

5 soybean, beans or peas. The plants may be grown for production of edible roots, tubers, leaves, stems, flowers or fruit. The plants may be vegetable plants whose vegetative parts are used as food. The plants of the invention may be: *Acrocomia aculeata* (macauba palm), *Arabidopsis thaliana*, *Arachis hypogaea* (peanut), *Astrocaryum murumuru* (murumuru), *Astrocaryum vulgare* (tucumã), *Attalea geraensis*

10 (Indaiá-rateiro), *Attalea humilis* (American oil palm), *Attalea oleifera* (andaiá), *Attalea phalerata* (uricuri), *Attalea speciosa* (babassu), *Avena sativa* (oats), *Beta vulgaris* (sugar beet), *Brassica sp.* such as *Brassica carinata*, *Brassica juncea*, *Brassica napobrassica*, *Brassica napus* (canola), *Camelina sativa* (false flax), *Cannabis sativa* (hemp), *Carthamus tinctorius* (safflower), *Caryocar brasiliense* (pequi), *Cocos*

15 *nucifera* (Coconut), *Crambe abyssinica* (Abyssinian kale), *Cucumis melo* (melon), *Elaeis guineensis* (African palm), *Glycine max* (soybean), *Gossypium hirsutum* (cotton), *Helianthus sp.* such as *Helianthus annuus* (sunflower), *Hordeum vulgare* (barley), *Jatropha curcas* (physic nut), *Joannesia princeps* (arara nut-tree), *Lemna sp.* (duckweed) such as *Lemna aequinoctialis*, *Lemna disperma*, *Lemna ecuadoriensis*,

20 *Lemna gibba* (swollen duckweed), *Lemna japonica*, *Lemna minor*, *Lemna minuta*, *Lemna obscura*, *Lemna paucicostata*, *Lemna perpusilla*, *Lemna tenera*, *Lemna trisulca*, *Lemna turionifera*, *Lemna valdiviana*, *Lemna yungensis*, *Licania rigida* (oiticica), *Linum usitatissimum* (flax), *Lupinus angustifolius* (lupin), *Mauritia flexuosa* (buriti palm), *Maximiliana maripa* (inaja palm), *Miscanthus sp.* such as *Miscanthus x*

25 *giganteus* and *Miscanthus sinensis*, *Nicotiana sp.* (tobacco) such as *Nicotiana tabacum* or *Nicotiana benthamiana*, *Oenocarpus bacaba* (bacaba-do-azeite), *Oenocarpus bataua* (patauã), *Oenocarpus distichus* (bacaba-de-leque), *Oryza sp.* (rice) such as *Oryza sativa* and *Oryza glaberrima*, *Panicum virgatum* (switchgrass), *Paraqueiba paraensis* (mari), *Persea amencana* (avocado), *Pongamia pinnata* (Indian beech), *Populus trichocarpa*,

30 *Ricinus communis* (castor), *Saccharum sp.* (sugarcane), *Sesamum indicum* (sesame), *Solanum tuberosum* (potato), *Sorghum sp.* such as *Sorghum bicolor*, *Sorghum vulgare*, *Theobroma grandiflorum* (cupuassu), *Trifolium sp.*, *Trithrinax brasiliensis* (Brazilian needle palm), *Triticum sp.* (wheat) such as *Triticum aestivum*, *Zea mays* (corn), alfalfa (*Medicago sativa*), rye (*Secale cereale*), sweet potato (*Lopmoea batatus*), cassava

35 (*Manihot esculenta*), coffee (*Cofea spp.*), pineapple (*Anana comosus*), citrus tree (*Citrus spp.*), cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa spp.*),

avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*) and almond (*Prunus amygdalus*). For example, plants of the disclosure may be *Nicotiana benthamiana*. In
 5 preferred examples, plants of the disclosure are wheat, *Brassica* sp. or sugarbeet (*Beta vulgaris*).

Other preferred plants include C4 grasses such as, in addition to those mentioned above, *Andropogon gerardi*, *Bouteloua curtipendula*, *B. gracilis*, *Buchloe dactyloides*, *Schizachyrium scoparium*, *Sorghastrum nutans*, *Sporobolus cryptandrus*;
 10 C3 grasses such as *Elymus canadensis*, the legumes *Lespedeza capitata* and *Petalostemum villosum*, the forb *Aster azureus*; and woody plants such as *Quercus ellipsoidalis* and *Q. macrocarpa*. Other preferred plants include C3 grasses.

In a preferred embodiment, the plant is an angiosperm.

In an embodiment, the plant is an oilseed plant, preferably an oilseed crop plant.
 15 As used herein, an "oilseed plant" is a plant species used for the commercial production of lipid from the seeds of the plant. The oilseed plant may be, for example, oil-seed rape (such as canola), maize, sunflower, safflower, soybean, sorghum, flax (linseed) or sugar beet. Furthermore, the oilseed plant may be other *Brassic*as, cotton, peanut, poppy, rutabaga, mustard, castor bean, sesame, safflower, *Jatropha curcas* or nut
 20 producing plants. The plant may produce high levels of lipid in its fruit such as olive, oil palm or coconut. Horticultural plants to which the present invention may be applied are lettuce, endive, or vegetable *Brassic*as including cabbage, broccoli, or cauliflower. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, tomato, or pepper.

25 In a preferred embodiment, the plant is a non-transgenic plant.

In a preferred embodiment, the transgenic plant is homozygous for each and every gene that has been introduced (transgene) so that its progeny do not segregate for the desired phenotype. The transgenic plant may also be heterozygous for the introduced transgene(s), preferably uniformly heterozygous for the transgene such as
 30 for example, in F1 progeny which have been grown from hybrid seed. Such plants may provide advantages such as hybrid vigour, well known in the art.

Transformation

RNA molecules disclosed herein may be stably introduced to above referenced
 35 host cells and/or plants. For the avoidance of doubt, an example of the present disclosure encompasses an above referenced plant stably transformed with an RNA

molecule disclosed herein. As used herein, the terms "stably transforming", "stably transformed" and variations thereof refer to the integration of the RNA molecule or a nucleic acid encoding the same into the genome of the cell such that they are transferred to progeny cells during cell division without the need for positively
5 selecting for their presence. Stable transformants, or progeny thereof, can be identified by any means known in the art such as Southern blots on chromosomal DNA, or *in situ* hybridization of genomic DNA, enabling their selection.

Transgenic plants can be produced using techniques known in the art, such as those generally described in Slater et al., *Plant Biotechnology - The Genetic*
10 *Manipulation of Plants*, Oxford University Press (2003), and Christou and Klee, *Handbook of Plant Biotechnology*, John Wiley and Sons (2004).

In an embodiment, plants may be transformed by topically applying an RNA molecule according to the present disclosure to the plant or a part thereof. For example, the RNA molecule may be provided as a formulation with a suitable carrier
15 and sprayed, dusted or otherwise applied to the surface of a plant or part thereof. Accordingly, in an example, the methods of the present disclosure encompass introducing an RNA molecule disclosed herein to a plant, the method comprising topically applying a composition comprising the RNA molecule to the plant or a part thereof.

20 *Agrobacterium*-mediated transfer is a widely applicable system for introducing genes into plant cells because DNA can be introduced into cells in whole plant tissues, plant organs, or explants in tissue culture, for either transient expression, or for stable integration of the DNA in the plant cell genome. For example, floral-dip (*in planta*) methods may be used. The use of *Agrobacterium*-mediated plant integrating vectors to
25 introduce DNA into plant cells is well known in the art. The region of DNA to be transferred is defined by the border sequences, and the intervening DNA (T-DNA) is usually inserted into the plant genome. It is the method of choice because of the facile and defined nature of the gene transfer.

Acceleration methods that may be used include for example, microprojectile
30 bombardment and the like. One example of a method for delivering transforming nucleic acid molecules to plant cells is microprojectile bombardment. This method has been reviewed by Yang et al., *Particle Bombardment Technology for Gene Transfer*, Oxford Press, Oxford, England (1994). Non-biological particles (microprojectiles) that may be coated with nucleic acids and delivered into cells, for example of immature
35 embryos, by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

In another method, plastids can be stably transformed. Methods disclosed for plastid transformation in higher plants include particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination (US 5,451,513, US 5,545,818, US 5,877,402, US 5,932,479, 5 and WO 99/05265). Other methods of cell transformation can also be used and include but are not limited to the introduction of DNA into plants by direct DNA transfer into pollen, by direct injection of DNA into reproductive organs of a plant, or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos.

10 The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach et al., In: Methods for Plant Molecular Biology, Academic Press, San Diego, Calif., (1988)). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the 15 usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene is well known in the art. Preferably, the regenerated plants are self-pollinated to 20 provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polynucleotide is cultivated using methods well known to one skilled in the art.

25 To confirm the presence of the transgenes in transgenic cells and plants, a polymerase chain reaction (PCR) amplification or Southern blot analysis can be performed using methods known to those skilled in the art. Expression products of the transgenes can be detected in any of a variety of ways, depending upon the nature of the product, and include Northern blot hybridisation, Western blot and enzyme assay. 30 Once transgenic plants have been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts, may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics. Preferably, the vegetative plant parts are harvested at a time when the yield of non-polar lipids are at their highest. In 35 one embodiment, the vegetative plant parts are harvested about at the time of flowering,

or after flowering has initiated. Preferably, the plant parts are harvested at about the time senescence begins, usually indicated by yellowing and drying of leaves.

Transgenic plants formed using *Agrobacterium* or other transformation methods typically contain a single genetic locus on one chromosome. Such transgenic plants
5 can be referred to as being hemizygous for the added gene(s). More preferred is a transgenic plant that is homozygous for the added gene(s), that is, a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by self-fertilising a
10 hemizygous transgenic plant, germinating some of the seed produced and analysing the resulting plants for the gene of interest.

It is also to be understood that two different transgenic plants that contain two independently segregating exogenous genes or loci can also be crossed (mated) to produce offspring that contain both sets of genes or loci. Selfing of appropriate F1
15 progeny can produce plants that are homozygous for both exogenous genes or loci. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Similarly, a transgenic plant can be crossed with a second plant comprising a genetic modification such as a mutant gene and progeny containing both of the transgene and the genetic modification identified. Descriptions of other breeding methods that are commonly used for different traits and
20 crops can be found in Fehr, In: *Breeding Methods for Cultivar Development*, Wilcox J. ed., American Society of Agronomy, Madison Wis. (1987).

Formulations

RNA molecules of the invention can be provided as various formulations. For
25 example, RNA molecules may be in the form of a solid, ointment, gel, cream, powder, paste, suspension, colloid, foam or aerosol. Solid forms may include dusts, powders, granules, pellets, pills, pastilles, tablets, filled films (including seed coatings) and the like, which may be water-dispersible ("wetttable"). In one example, the composition is in the form of a concentrate.

30 In an example, RNA molecules may be provided as a topical formulation. In an example, the formulation stabilises the RNA molecule in formulation and/or *in-vivo*. For example, RNA molecules of the invention may be provided in a lipid formulation. In an example, the formulation comprises a transfection promoting agent.

The term "transfection promoting agent" as used herein refers to a composition
35 added to the RNA molecule for enhancing the uptake into a cell including, but not limited to, a plant cell or a fungal cell. Any transfection promoting agent known in the

art to be suitable for transfecting cells may be used. Examples include cationic lipid such as one or more of DOTMA (N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide),
5 DDAB (dimethyl dioctadecyl ammonium bromide). lipospermines, specifically DOSPA (2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamin-ium trifluoro-acetate) and DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, and the di- and tetra-alkyl-tetra-methyl spermines, including but not limited to TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS
10 (tetramethyltetraoleyl spermine), TMTLS (tetramethyltetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine) and TMDOS (tetramethyldioleyl spermine). Cationic lipids are optionally combined with non-cationic lipids, particularly neutral lipids, for example lipids such as DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol. Non-limiting examples of
15 suitable commercially available transfection reagents include Lipofectamine (Life Technologies) and Lipofectamine 2000 (Life Technologies).

In an example, RNA molecules of the invention can be incorporated into formulations suitable for application to a field. In an example, the field comprises plants. Suitable plants include crop plants (for example, cereals and pulses, maize,
20 wheat, potatoes, tapioca, rice, sorghum, soybean millet, cassava, barley, or pea), or legumes. The plants may be grown for production of edible roots, tubers, leaves, stems, flowers or fruit. In an example, the crop plant is a cereal plant. Examples of cereal plants include, but are not limited to, wheat, barley, sorghum oats, and rye. In these examples, the RNA molecule may be formulated for administration to the plant,
25 or to any part of the plant, in any suitable way. For example, the composition may be formulated for administration to the leaves, stem, roots, fruit vegetables, grains and/or pulses of the plant. In one example, the RNA molecule is formulated for administration to the leaves of the plant, and is sprayable onto the leaves of the plant.

Depending on the desired formulation, RNA molecules of the invention may be
30 formulated with a variety of other agents. Exemplary agents comprise one or more of suspension agents, agglomeration agents, bases, buffers, bittering agents, fragrances, preservatives, propellants, thixotropic agents, anti-freezing agents, and colouring agents.

In other examples, RNA molecule formulations can further comprise an
35 insecticide, a pesticide, a fungicide, an antibiotic, an insect repellent, an anti-parasitic agent, an anti-viral agent, or a nematicide.

RNA molecules according to the present disclosure can be provided in a kit or pack. For example, RNA molecules disclosed herein may be packaged in a suitable container with written instructions for producing an above referenced cell or plant.

5 Methods of Modulating Flowering

In an example, the RNA molecules according to the present disclosure can be delivered to plants, plant cells or plant parts, preferably to seed that will be used to produce plants, to modulate flowering. Such uses involve delivering RNA molecules according to the present disclosure using various methods such as those described
10 above for delivering RNA molecules. In an example, plants disclosed herein can be modified to express RNA molecules according to the present disclosure. In another example, RNA molecules can be sprayed onto plants as required. For example, RNA molecules can be sprayed onto a crop to promote flowering in the crop. In an example, the RNA molecules according to the present disclosure can be delivered to plants to
15 modulate vernalization. Exemplary crops include cotton, maize, tomato, chickpea, pigeon pea, alfalfa, rice, sorghum and cowpea. Other exemplary crops include corn, canola, cotton, soybean, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. Further examples of suitable plants and crops are discussed throughout the present disclosure. In an example, the methods of the present disclosure can be used to
20 modulate flowering in plants such as *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an example, the methods of the present disclosure can be used to modulate flowering in plants such as *Arabidopsis*, corn, canola, cotton, soybean, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. For example, the plant can be sugarbeet. In an
25 example, the plant is wheat or barley. In an example, the methods of the present disclosure are used to direct early flowering in plants such as *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. In an example, the methods of the present disclosure are used to direct early flowering in plants such as *Arabidopsis*, corn, canola, cotton, soybean,
30 wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye. For example, early flowering can be directed in sugarbeet. In another example, the early flowering is directed in wheat or barley. In another example, the methods of the present disclosure can be used to modulate flowering in grass such as turfgrasses. In an example, the methods of the present disclosure are used to direct late flowering in a grass. For
35 example, the late flowering is directed in turfgrasses.

In an example, RNA molecules of the disclosure are delivered to genetically unmodified plants.

The RNA molecules of the disclosure when delivered and/or expressed in a plant can have a wide range of desired properties which influence, for example, an
5 agronomic trait such as early flowering.

In a particular example, the plants produce increased levels of enzymes for oil production in plants such as Brassicas, for example oilseed rape or sunflower, safflower, flax, cotton, soybean or maize; enzymes involved in starch synthesis in
10 plants such as potato, maize, and cereals such as wheat barley or rice; enzymes which synthesize, or proteins which are themselves, natural medicaments, such as pharmaceuticals or veterinary products.

Other exemplary physical or phenotypic characteristics of plants produced from the plant cells or seeds contacted with the RNA molecules of the invention may be affected in addition to the modulated flowering time phenotype, such as reduced
15 chlorophyll content, stem elongation, advanced or retarded senescence, and increase or reduction of apical dominance which may result in an altered plant architecture, each of which are different from the plant phenotype when grown in the absence of the contact with the RNA molecules. According to the present invention, these phenotypes, if deleterious, are advantageously reduced or absent in the subsequent generation of
20 plants which may be used for producing grain, fruits, pods or vegetative parts such as leaves, stems, fibre, tubers or beets.

In the case of plants from which vegetative parts are to be harvested, the RNA molecules of the invention can be used in the previous generation to induce earlier flowering for seed production, in an otherwise later-flowering variety in the absence of
25 treatment with the RNA molecules. For example, sugarbeet which stores sugar in the beets in the vegetative state but mobilises that sugar at the onset of flowering, leading to reduction in sugar content in the beets. Since hybrid seed stock is sown for the cultivation of sugar beets it must be ensured that the parent plants still flower in order to produce the seed stock

30

EXAMPLES

Example 1. Materials and methods

Synthesis of genetic constructs

To design a typical ledRNA construct, a region of the target RNA of about 100-
35 1000 nucleotides in length, typically 400-600 nucleotides, was identified. In one example, the 5' half of the sequence and approximately 130 nt of the flanking region

and similarly the 3' half and 130 nt of flanking region were orientated in an antisense orientation relative to a promoter. These sequences were interrupted with the 400-600 nucleotide sense target sequence (Figure 1A). The 5' end of the resultant construct was preceded with a promoter such as a T7 or SP6 RNA polymerase promoter and the 3' end engineered to include a restriction enzyme cleavage site to allow for termination of transcription *in vitro*.

For transcription in cells such as bacterial cells, promoter and terminator sequences were incorporated to facilitate expression as a transgene, for example using an inducible promoter. The double-stranded region and loop sequence lengths can be varied. The constructs were made using standard cloning methods or ordered from commercial service providers.

Synthesis of RNA

Following digestion with restriction enzyme to linearize the DNA at the 3' end, transcription using RNA polymerase resulted in the 5' and 3' arms of the ledRNAi transcript annealing to the central target sequence, the molecule comprising a central stem or double-stranded region with a single nick and terminal loops. The central sequence can be orientated in sense or antisense orientation relative to the promoter (Figure 1A, 1B respectively).

For *in vitro* synthesis, DNA of the construct was digested at the 3' restriction site using the appropriate restriction enzyme, precipitated, purified and quantified. RNA synthesis was achieved using RNA polymerase according to the manufacturer's instructions. The ledRNA was resuspended in annealing buffer (25 mM Tris-HCL, pH 8.0, 10 mM MgCl₂) using DEPC-treated water to inactivate any traces of RNase. The yield and integrity of the RNA produced by this method was determined by nano-drop analysis and gel electrophoresis (Figure 2), respectively.

Synthesis of ledRNA was achieved in bacterial cells by introducing the constructs into *E. coli* strain HT115. Transformed cell cultures were induced with IPTG (0.4 mM) to express the T7 RNA polymerase, providing for transcription of the ledRNA constructs. RNA extraction from the bacterial cells and purification was performed essentially as described in Timmons et al. (2001).

For RNA transcription with Cy3 labelling, the ribonucleotide (rNTP) mix contained 10mM each of ATP, GTP, CTP, 1.625mM UTP and 8.74mM Cy3-UTP. The transcription reactions were incubated at 37°C for 2.5 hr. The transcription reactions (160µl) were transferred to Eppendorf tubes, 17.7µl turbo DNase buffer and 1µl turbo DNA added, and incubated at 37°C for 10 minutes to digest the DNA. Then,

17.7µl Turbo DNase inactivation solution was added, mixed and incubated at room temperature for 5 min. The mixture was centrifuged for 2 min and the supernatant transferred to a new RNase free Eppendorf tube. Samples of 1.5µl of each transcription reaction were electrophoresed on gels to test the quality of the RNA product. Generally, one RNA band was observed of 500 bp to 1000bp in size depending on the construct. The RNA was precipitated by adding to each tube: 88.5µl 7.5M Ammonium acetate and 665µl cold 100% ethanol. The tubes were cooled to -20°C for several hours or overnight, then centrifuged at 4°C for 30 min. The supernatant was removed carefully and the pellet of RNA washed with 1ml 70% ethanol (made with nuclease free water) at -20°C and centrifuged. The pellet was dried and the purified RNA resuspended in 50µl 1x RNAi annealing buffer. The RNA concentration was measured using nanodrop method and stored at -80°C until used.

Example 2. Design of ledRNA

As shown schematically in Figure 1A, a typical ledRNA molecule comprises a sense sequence which can be considered to be two adjacent sense sequences, covalently linked and having identity to the target RNA, an antisense sequence which is complementary to the sense sequence and which is divided into two regions, and two loops that separate the sense from the antisense sequences. A DNA construct which encodes this form of ledRNA therefore comprises, in 5' to 3' order, a promoter for transcription of the ledRNA coding region, a first antisense region having complementarity with a region towards the 5' end of the target RNA, a first loop sequence, the sense sequence, a second loop sequence, then the second antisense region having complementarity with a region towards the 3' end of the target RNA, and finally a means to terminate transcription. In this arrangement, the two antisense sequences flanked the sense sequence and loop sequences. When transcribed, the two regions of antisense sequence anneal with the sense sequence, forming a dsRNA stem with two flanking loops.

In another but related form of ledRNA, the sense sequence is split into two regions whilst the two antisense regions remain as a single sequence (Figure 1B). A DNA construct which encodes this second form of ledRNA therefore comprises, in 5' to 3' order, a promoter for transcription of the ledRNA coding region, a first sense region having identity with a region towards the 3' end of the target RNA, a first loop sequence, the antisense sequence, a second loop sequence, then the second sense region having identity towards the 5' end of the target RNA, and finally a means to terminate

transcription. In this arrangement, the two sense sequences flanked the antisense sequence and loop sequences.

Without wishing to be limited by theory, because of the closed loops at each end, these ledRNA structures would be more resistant to exonucleases than an open-ended dsRNA formed between single-stranded sense and antisense RNAs and not having loops, and also compared to a hairpin RNA having only a single loop. In addition, the inventors conceived that a loop at both ends of the dsRNA stem would allow Dicer to access both ends efficiently, thereby enhancing processing of the dsRNA into sRNAs and silencing efficiency.

10 As a first example, a genetic construct was made for *in vitro* transcription using T7 or SP6 RNA polymerase to form ledRNAs targeting genes encoding GFP or GUS. The ledGFP construct comprised the following regions in order: the first half of antisense sequence corresponded to nucleotides 358 to 131 of the GFP coding sequence (CDS) (SEQ ID NO:7), the first antisense loop corresponded to nucleotides 130 to 1 of
15 GFP CDS, the sense sequence corresponded of nucleotides 131 to 591 of GFP CDS, the second antisense loop corresponding to nucleotides 731 to 592 of GFP CDS, and the second half of the antisense sequence corresponded to nucleotides 591 to 359 of the GFP CDS.

The ledGUS construct comprised the following regions in order: the first half of
20 antisense sequence corresponded to nucleotides 609 to 357 of GUS CDS (SEQ ID NO:8); the first antisense loop corresponded to nucleotides 356 to 197 of GUS CDS, the sense sequence corresponded to nucleotides 357 to 860 of GUS CDS, the second antisense loop corresponding to nucleotides 1029 to 861 of GUS CDS; and the second half of antisense sequence corresponded to nucleotides 861 to 610 of GUS CDS.

25 For making the separate strand sense/antisense GUS dsRNA (conventional dsRNA), the same target sequence corresponding to nucleotides 357 to 860 of GUS CDS was ligated between the T7 and SP6 promoters in pGEM-T Easy vector. The sense and antisense strands were transcribed separately with T7 or SP6 polymerases, respectively, and annealed in annealing buffer after mixing the transcripts and heating
30 the mixture to denature the RNA strands.

Example 3. Stability of ledRNAs

The ability of ledRNA to form dsRNA structures was compared with open-ended dsRNA (i.e no loops, formed by annealing of separate single-stranded sense and
35 antisense RNA) and long hpRNA. ledRNA, long hpRNA, and the mixture of sense and antisense RNA, were denatured by boiling and allowed to anneal in annealing buffer

(250mM Tris-HCL, pH 8.0 and 100mM MgCl₂), and then subjected to electrophoresis in a 1.0% agarose gel under non-denaturing conditions.

As shown in Figure 2, both the GUS ledRNA and the GFP ledRNA gave a dominant RNA band of the mobility expected for a double-stranded molecule, 5 indicating the formation of the predicted ledRNA structure. This was in contrast to the mixture of sense and antisense RNA, which showed only a weak band for a dsRNA, indicating that most of the sense and antisense RNAs were not readily annealed to each other to form dsRNA. The hairpin RNA samples gave two prominent bands, indicating that only part of the transcript formed the predicted hairpin RNA structure. Thus, 10 ledRNA was the most efficient in forming the predicted dsRNA structure.

The ability of ledRNA to stay and spread on leaf surface was also compared with dsRNA. The GUS ledRNA (ledGUS), when applied to the lower part of tobacco leaf surface, could be readily detected in the untreated upper part of the leaf after 24 hrs (Figure 3). However, the separate strand GUS dsRNA (dsGUS) could not be detected 15 in the untreated upper part of the leaf (Figure 3). This result indicates that the ledRNA is more resistant to degradation than dsRNA and therefore able to spread inside plant leaf tissues.

Example 4. Testing of ledRNAs by topical delivery

20 The ability of the ledRNAs to induce RNAi after topical delivery was tested in *Nicotiana benthamiana* and *Nicotiana tabacum* plants expressing a GFP or GUS reporter gene, respectively. The sequences of the GFP and GUS target sequences and of the ledRNA encoding constructs are shown in SEQ ID NOs: 7, 8, 4 and 5, respectively. The ribonucleotide sequence of the encoded RNA molecules are provided 25 as SEQ ID NO's 1 (GFP ledRNA) and 2 (GUS ledRNA).

To facilitate reproducible and uniform application of ledRNA onto leaf surfaces, ledRNA at a concentration of 75-100 µg/ml, in 25 mM Tris-HCL, pH 8.0, 10 mM MgCl₂ and Silwet 77 (0.05%), was applied to the adaxial surface of leaves using a soft paint brush. At 6 hours and 3 days following ledRNA application, leaf samples were 30 taken for the analysis of targeted gene silencing.

Application of ledRNA against GFP in *N. benthamiana* leaves and against GUS in *N. tabacum* leaves resulted in clear reductions of 20-40% and 40-50% of the respective target gene activity at the mRNA (GFP) or protein activity (GUS) level at 6 hours post treatment. However, in this experiment the reduction did not persist at 3 35 days post treatment. The inventors considered that the observation at 3 days was likely due to some nonspecific responses of transgenes to dsRNA treatment or dissipating

amount of ledRNA. However, in a separate experiment, GUS silencing was detected in both the treated and distal untreated leaf areas at 24 hrs post ledRNA treatment (Figure 4).

5 **Example 5. ledRNA-induced silencing of an endogenous target gene**

In a further example, a ledRNA was designed to target an mRNA encoded by an endogenous gene, namely the FAD2.1 gene of *N. benthamiana*. The sequence of the target FAD2.1 mRNA and of the ledFAD2.1 encoding construct are shown in SEQ ID NOs: 9 and 6, respectively. The ribonucleotide sequence of the encoded RNA molecule is provided as SEQ ID NO:3 (*N. benthamiana* FAD2.1 ledRNA).

The FAD2.1 ledRNA construct was comprised of the following: the first half of antisense sequence corresponding to nucleotides 678 to 379 of FAD2.1 CDS (Niben101Scf09417g01008.1); the first antisense loop corresponding to nt. 378 to 242 of FAD2.1 CDS; the sense sequence corresponding of nt. 379 to 979; the second antisense loop corresponding to nt 1115 to 980; and the second half of antisense sequence corresponding to nt 979 to nt 679 of FAD2.1 CDS.

The ledGUS RNA from the previous example was used in parallel as a negative control. In the first experiment, target gene silencing was assayed for both the level of FAD2.1 mRNA and the accumulation C18:1 fatty acid (Figure 5). The level of activity of a related gene, FAD2.2, was also assayed. For each sample approximately 3 µg of total RNA was DNase treated and reverse transcribed at 50 °C for 50 minutes using oligo dT primers. The reactions were terminated at 85 °C for 5 minutes and diluted to 120 µl with water. Using a rotor gene PCR machine, 5µl of each sample, in triplicate, were analysed for their relative expression of FAD 2.1 and FAD 2.2 mRNA using gene specific primers with reference to the house keeping gene actin. In a subsequent experiment, northern blot hybridization was also used to confirm the silencing of the FAD2.1 gene by topically applied ledFAD2.1 RNA (Figure 6).

The FAD2.1 mRNA was reduced significantly, to a level which was barely detectable in leaf tissues treated with the ledRNA at the 2, 4 and 10 hour time points (Figure 5). In this experiment, it was unclear why the level of FAD2.1 mRNA was not reduced as much at the 6 hour time point. In the repeated experiment shown in Figure 6, strong FAD2.1 downregulation occurred at both 6 and 24 hrs, particularly at the 24 hr time point. The related FAD2.2 gene, with sequence homology to FAD2.1, also showed downregulation at the 2 and 4 hour time points by the ledRNA (Figure 5).

Since FAD2.1 and FAD2.2 encode fatty acid Δ 12 desaturases which desaturate oleic acid to linoleic acid, the levels of these fatty acids were assayed in leaf tissues

treated with the ledRNAs. There was a clear increase in oleic acid (18:1) accumulation in ledRNA-treated leaf tissues at the 2, 4 and 6 hour time points, which indicated a reduced amount of the FAD2 enzyme (Figure 5). Thus, both qRT-PCR and the fatty acid composition assay showed that the ledRNA induced silencing of the FAD2.1 gene.

5

Example 6. Design and testing of hairpin RNAs comprising G:U basepairs or mismatched nucleotides

Modified hairpin RNAs targeting GUS RNA

Reporter genes such as the gene encoding the enzyme β -glucuronidase (GUS) provide a simple and convenient assay system that can be used to measure gene silencing efficiency in a eukaryotic cell including in plant cells (Jefferson et al., 1987). The inventors therefore designed, produced and tested some modified hairpin RNAs for their ability to reduce the expression of a GUS gene as a target gene, using a gene-delivered approach to provide the hairpin RNAs to the cells, and compared the modified hairpins to a conventional hairpin RNA. The conventional hairpin RNA used as the control in the experiment had a double-stranded region of 200 contiguous basepairs in length in which all of the basepairs were canonical basepairs, i.e. G:C and A:U basepairs without any G:U basepairs, and without any non-basepaired nucleotides (mismatches) in the double-stranded region, targeting the same 200nt region of the GUS mRNA molecule as the modified hairpin RNAs. The sense and antisense sequences that formed the double-stranded region were covalently linked by a spacer sequence included a PDK intron (Helliwell et al., 2005; Smith et al., 2000), providing for an RNA loop of 39 or 45 nucleotides in length (depending on the cloning strategy used) after splicing of the intron from the primary transcript. The DNA fragment used for the antisense sequence was flanked by *XhoI*-*Bam*HI restriction sites at the 5' end and *Hind*III-*Kpn*I restriction sites at the 3' end for easy cloning into an expression cassette, and each sense sequence was flanked by *Xho*I and *Kpn*I restriction sites. The 200 bp dsRNA region of each hairpin RNA, both for the control hairpin and the modified hairpins, included an antisense sequence of 200 nucleotides which was fully complementary to a wild-type GUS sequence from within the protein coding region. This antisense sequence, corresponding to nucleotides 13-212 of SEQ ID NO:10, was the complement of nucleotides 804-1003 of the GUS open reading frame (ORF) (cDNA sequence provided as SEQ ID NO:8). The *GUS* target mRNA was therefore more than 1900nt long. The length of 200 nucleotides for the sense and antisense sequences was chosen as small enough to be reasonably convenient for synthesis of the DNA fragments using synthetic oligonucleotides, but also long enough to provide

multiple sRNA molecules upon processing by Dicer. Being part of an ORF, the sequence was unlikely to contain cryptic splice sites or transcription termination sites.

Preparation of genetic constructs

5 The 200 bp GUS ORF sequence was PCR-amplified using the oligonucleotide primer pair GUS-WT-F (SEQ ID NO:52) and GUS-WT-R (SEQ ID NO:53), containing *XhoI* and *BamHI* sites or *HindIII* and *KpnI* sites, respectively, to introduce these restriction enzyme sites 5' and 3' of the GUS sequence. The amplified fragment was inserted into the vector pGEM-T Easy and the correct nucleotide sequence
10 confirmed by sequencing. The GUS fragment was excised by digestion with *BamHI* and *HindIII* and inserted into the *BamHI/HindIII* site of pKannibal (Helliwell and Waterhouse, 2005), which inserted the GUS sequence in the antisense orientation relative to the operably linked CaMV e35S promoter (Grave, 1992) and *ocs* gene polyadenylation/transcription terminator (Ocs-T). The resultant vector was designated
15 pMBW606 and contained, in order 5' to 3', a 35S::PDK Intron::antisense GUS::Ocs-T expression cassette. This vector was the intermediate vector used as the base vector for assembling four hpRNA constructs.

Construct hpGUS[wt] having only canonical basepairs

20 To prepare the vector designated hpGUS[wt] encoding the hairpin RNA molecule used as a control in the experiment, having only canonical basepairs, the 200 bp GUS PCR fragment was excised from the pGEM-T Easy plasmid with *XhoI* and *KpnI*, and inserted into the *XhoI/KpnI* sites between the 35S promoter and the PDK intron in pMBW606. This produced the vector designated pMBW607, containing the
25 35S::Sense GUS[wt]::PDK Intron::antisense GUS::OCS-T expression cassette. This cassette was excised by digestion with *NotI* and inserted into the *NotI* site of pART27 (Gleave, 1992), resulting in the vector designated hpGUS[wt], encoding the canonically basepaired hairpin RNA targeting the GUS mRNA.

When self-annealed by hybridisation of the 200nt sense and antisense
30 sequences, this hairpin had a double-stranded region of 200 consecutive basepairs corresponding to GUS sequences. The sense and antisense sequences in the expression cassette were each flanked by *BamHI* and *HindIII* restrictions sites present at the 5' and 3' ends, respectively, relative to the GUS sense sequence. When transcribed, the nucleotides corresponding to these sites were also capable of hybridising, extending the
35 double-stranded region by 6bp at each end. After transcription of the expression cassette and splicing of the PDK intron from the primary transcript, the hairpin RNA

structure prior to any processing by Dicer or other RNAses was predicted to have a loop structure of 39 nucleotides. The nucleotide sequence of the hairpin RNA structure including its loop is provided as SEQ ID NO:15, and its free energy of folding was predicted to be -471.73 kcal/mol. This was therefore an energetically stable hairpin structure. The free energy was calculated using “RNAfold” (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) based on the nucleotide sequences after the splicing out of the PDK intron sequence.

When transcribed from the expression cassette having the 35S promoter and OCS-T terminator, the resultant hairpin RNAs were embedded in a larger RNA molecule with 8 nucleotides added to the 5' end and approximately 178 nucleotides added at the 3' end, without considering addition of any poly-A tail at the 3' end. Since the same promoter-terminator design was used for the modified hairpin RNAs, those molecules also had these extensions at the 5' and 3' ends. The length of the hairpin RNA molecules after splicing of the PDH intron was therefore approximately 630 nucleotides.

Construct hpGUS[G:U] comprising G:U basepairs

A DNA fragment comprising the same 200 nucleotide sense sequence, but in which all 52 cytidine nucleotides (C) of the corresponding wild-type GUS region were substituted with thymidine nucleotides (T), was assembled by annealing the overlapping oligonucleotides GUS-GU-F (SEQ ID NO:54) and GUS-GU-R (SEQ ID NO:55) and PCR extension of the 3' ends using the high-fidelity LongAmp Taq polymerase (New England Biolabs, catalogue number M0323). The amplified DNA fragment was inserted into the pGEM-T Easy vector and the correct nucleotide sequence (SEQ ID NO:11) was confirmed by sequencing. A DNA fragment comprising the modified sequence was then excised by digestion with *XhoI* and *KpnI* and inserted into the *XhoI/KpnI* sites of the base vector pMBW606. This produced the construct designated pMBW608, containing the expression cassette 35S::sense GUS[G:U]::PDK Intron::antisense GUS::OCS-T. This expression cassette was excised with *NotI* digestion and inserted into the *NotI* site of pART27, resulting in the vector designated hpGUS[G:U], encoding the G:U basepaired hairpin RNA molecule.

This cassette encoded a hairpin RNA targeting the GUS mRNA and which, when self-annealed by hybridisation of the 200nt sense and antisense sequences, had 52 G:U basepairs (instead of G:C basepairs in hpGUS[wt]) and 148 canonical basepairs, i.e. 26% of the nucleotides of the double-stranded region were involved in G:U basepairs. The 148 canonical basepairs in hpGUS[G:U] were the same as in the control

hairpin RNA, in the corresponding positions, including 49 U:A basepairs, 45 A:U basepairs and 54 G:C basepairs. The longest stretches of contiguous canonical basepairing in the double-stranded region was 9 basepairs. The antisense nucleotide sequence of hpGUS[G:U] was thereby identical in length (200nt) and sequence to the
5 antisense sequence of the control hairpin RNA hpGUS[wt]. After transcription of the expression cassette and splicing of the PDK intron from the primary transcript, the hairpin RNA structure prior to any processing by Dicer or other RNAses was predicted to have a loop structure of 45 nucleotides. The nucleotide sequence of the hairpin structure including its loop is provided as SEQ ID NO:16, and its free energy of folding
10 was predicted to be -331.73 kcal/mol. As for hpGUS[wt], this was therefore an energetically stable hairpin structure, despite the 52 G:U basepairs which individually are much weaker than the G:C basepairs in hpGUS[wt].

An alignment of the modified GUS sense sequence (nucleotides 9-208 of SEQ ID NO:11) with the corresponding region of the GUS target gene (SEQ ID NO:14) is
15 shown in Figure 7.

Construct hpGUS[1:4] comprising mismatched nucleotides every fourth nucleotide

A DNA fragment comprising the same 200 bp sense sequence, but in which every fourth nucleotide of the corresponding wild-type GUS sequence was substituted,
20 was designed and assembled. Every 4th nucleotide in each block of 4 nucleotides (nucleotides at positions 4, 8, 12, 16, 20 etc) was substituted by changing C's to G's, G's to C's, A's to T's and T's to A's, leaving the other nucleotides unchanged. These substitutions were all transversion substitutions, which were expected to have a greater destabilising effect on the resultant hairpin RNA structure than transition substitutions.
25 The DNA fragment was assembled by annealing the overlapping oligonucleotides GUS-4M-F (SEQ ID NO:56) and GUS-4M-R (SEQ ID NO:57) and PCR extension of 3' ends using LongAmp Taq polymerase. The amplified DNA fragment was inserted into the pGEM-T Easy vector and the correct nucleotide sequence (SEQ ID NO:12) was confirmed by sequencing. A DNA fragment comprising the modified sequence
30 was then excised by digestion with *XhoI* and *KpnI* and inserted into the *XhoI/KpnI* sites of the base vector pMBW606. This produced the construct designated pMBW609, containing the expression cassette 35S::sense GUS[1:4]::PDK Intron::antisense GUS::OCS-T. This expression cassette was excised with *NotI* digestion and inserted into the *NotI* site of pART27, resulting in the vector designated hpGUS[1:4], encoding
35 the 1:4 mismatched hairpin RNA molecule.

This cassette encoded a hairpin RNA targeting the GUS mRNA and which, when self-annealed by hybridisation of the sense and antisense sequences, had mismatches for 50 nucleotides of the 200nt antisense sequence, including the mismatch for the nucleotide at position 200. Excluding position 200, the double-stranded region of the hairpin RNA had 150 canonical basepairs and 49 mismatched nucleotide pairs over a length of 199nt sense and antisense sequences, i.e. 24.6% of the nucleotides of the double-stranded region were predicted to be mismatched (not involved in basepairs). After transcription of the expression cassette and splicing of the PDK intron from the primary transcript, the hairpin RNA structure prior to any processing by Dicer or other RNAses was predicted to have a loop structure of 45 nucleotides. The nucleotide sequence of the hairpin structure including its loop is provided as SEQ ID NO:17, and its free energy of folding was predicted to be -214.05 kcal/mol. As for hpGUS[wt], this was therefore an energetically stable hairpin structure, despite the mismatched nucleotides.

An alignment of the modified GUS sense sequence (nucleotides 9-208 of SEQ ID NO:12) with the corresponding region of the GUS target gene (SEQ ID NO:14) is shown in Figure 8.

Construct hpGUS[2:10] in which nucleotides 9 and 10 of 10 nucleotides was mismatched

A DNA fragment comprising the same 200 bp sense sequence, but in which every ninth and tenth nucleotide of the corresponding wild-type GUS sequence was substituted, was designed and assembled. Each 9th and 10th nucleotide in each block of 10 nucleotides (nucleotides at positions 9, 10, 19, 20, 29, 30 etc) was substituted by changing C's to G's, G's to C's, A's to T's and T's to A's, leaving the other nucleotides unchanged. The DNA fragment was assembled by annealing the overlapping oligonucleotides GUS-10M-F (SEQ ID NO:58) and GUS-10M-R (SEQ ID NO:59) and PCR extension of 3' ends using LongAmp Taq polymerase. The amplified DNA fragment was inserted into pGEM-T Easy and the correct nucleotide sequence (SEQ ID NO:13) was confirmed by sequencing. A DNA fragment comprising the modified sequence was then excised by digestion with *XhoI* and *KpnI* and inserted into the *XhoI/KpnI* sites of the base vector pMBW606. This produced the construct designated pMBW610, containing the expression cassette 35S::sense GUS[2:10]::PDK Intron::antisense GUS::OCS-T. This expression cassette was excised with *NotI* digestion and inserted into the *NotI* site of pART27, resulting in the vector designated hpGUS[2:10], encoding the 2:10 mismatched hairpin RNA molecule.

This cassette encoded a hairpin RNA targeting the GUS mRNA which, when self-annealed by hybridisation of the sense and antisense sequences, had mismatches for 50 nucleotides of the 200nt antisense sequence, including mismatches for the nucleotides at positions 199 and 200. Excluding positions 199 and 200, the double-stranded region of the hairpin RNA had 160 canonical basepairs and 19 di-nucleotide mismatches over a length of 198nt sense and antisense sequences, i.e. 19.2% of the nucleotides of the double-stranded region were predicted to be mismatched (not involved in basepairs). The 160 basepairs in hpGUS[2:10] were the same as in the control hairpin RNA, in the corresponding positions, including 41 U:A basepairs, 34 A:U basepairs, 42 G:C and 43 C:G basepairs. After transcription of the expression cassette and splicing of the PDK intron from the primary transcript, the hairpin RNA structure prior to any processing by Dicer or other RNAses was predicted to have a loop structure of 45 nucleotides. The nucleotide sequence of the hairpin structure including its loop is provided as SEQ ID NO:18, and its free energy of folding was predicted to be -302.78 kcal/mol. As for hpGUS[wt], this was therefore an energetically stable hairpin structure, despite the mismatched nucleotides which were expected to bulge out of the stem of the hairpin structure.

An alignment of the modified GUS sense sequence (nucleotides 9-208 of SEQ ID NO:13) with the corresponding region of the GUS target gene (SEQ ID NO:14) is shown in Figure 9.

The four genetic constructs for expression of the control and modified hairpin RNAs are shown schematically in Figure 10.

Example 7. Testing the modified hairpin RNAs in transgenic plants

Plants of the species *Nicotiana tabacum* (tobacco) transformed with a GUS target gene were used to test the efficacy of the four hairpin RNA constructs described above. Specifically, the target plants were from two homozygous, independent transgenic lines, PPGH11 and PPGH24, each containing a single-copy insertion of a GUS transgene from a vector pWBPPGH which is shown schematically in Figure 11. The GUS gene in the T-DNA of pWBPPGH had a GUS coding region (nucleotides 7-1812 of SEQ ID NO:8) operably linked to a 1.3 kb long promoter of the phloem protein 2 (PP2) gene from *Cucurbita pepo* L. cv. Autumn Gold (Wang et al., 1994; Wang, 1994). The construct pWBPPGH was made by excising the PP2 promoter plus the 5' UTR and 54 nucleotides of the PP2 protein coding region, encoding the first 18 amino acids of PP2, from the lambda genomic clone CPP1.3 (Wang, 1994), and fusing this fragment with the GUS coding sequence starting with the nucleotides encoding the 3rd

amino acid of GUS, generating an N-terminal fusion polypeptide having GUS activity. The pPP2::GUS:Nos-T cassette was inserted into pWBVec2a (Wang et al., 1998) to generate pWBPPGH, which was used to transform plants of *Nicotiana tabacum* cv. Wisconsin 38 using *Agrobacterium tumefaciens*-mediated leaf disk transformation
5 (Ellis et al., 1987), selecting for resistance to hygromycin. GUS activities in homozygous progeny plants of two transgenic lines PPGH11 and PPGH24 were similar. GUS expression in both transgenic plants was not restricted to phloem but present in most tissues of the plants. GUS expression from the PP2 promoter in these
10 PP2-GUS plants as the testing plants: i) they give constitutively high levels of GUS expression about the same as to a 35S-GUS plant; ii) the PP2 promoter is an endogenous PP2 gene promoter derived from *Cucurbita pepo* with a different sequence to the 35S promoter used to drive the expression of the hpRNA transgenes, which therefore would not be subject to transcriptional cosuppression by the incoming 35S
15 promoter.

All four hairpin RNA constructs (Example 6) were used to transform PPGH11 and PPGH24 plants using the *Agrobacterium*-mediated leaf-disk method (Ellis et al., 1987), using 50 mg/L kanamycin as the selective agent. This selection system with
20 kanamycin, a different agent to the previously used hygromycin used to introduce the T-DNA of pWBPPGH, was observed to yield only transformed plants, with no non-transformed plants being regenerated. Regenerated transgenic plants containing the T-DNAs from the hpGUS constructs were transferred to soil for growth in the greenhouse and maintained for about 4 weeks before assaying for GUS activity. When assayed, the transgenic plants were healthy and actively growing and in appearance were identical
25 to non-transformed control plants and the parental PPGH11 and PPGH24 plants. In total, 59 transgenic plants were obtained that were transformed with the T-DNA encoding hpGUS[wt], 74 plants were obtained that were transformed with the T-DNA encoding hpGUS[G:U], 33 plants were obtained that were transformed with the T-DNA encoding hpGUS[1:4] and 41 plants were obtained that were transformed with
30 the T-DNA encoding hpGUS[2:10].

GUS expression levels were measured using the fluorimetric 4-methylumbelliferyl β -D-glucuronide (MUG) assay (Jefferson et al., 1987) following the modified kinetic method described in Chen et al. (2005). Plants were assayed by taking
35 leaf samples of about 1cm diameter from three different leaves on each plant, choosing leaves which were well expanded, healthy and green. Care was taken that the test plants were at the same stage of growth and development as the control plants. Each assay

used 5 µg protein extracted per leaf sample and measured the rate of cleavage of MUG as described in Chen et al. (2005).

Representative data are shown in Figure 12, showing GUS activity (MUG units in the assay) for each independent transgenic plant. Since the data for the hpGUS[wt] construct showed that some plants exhibited strong silencing with a reduction in activity of at least 90% and others weaker silencing, 10% GUS activity relative to the control plants was chosen, in this context, as an activity level for classifying the plants into two categories and comparing the different constructs.

The genetic construct encoding the canonically basepaired hpGUS[wt] induced strong GUS silencing, using the 10% activity level as the benchmark for strong silencing, in 32 of the 59 transgenic plants tested (54.2%). The other 27 plants all showed reduced GUS activity but retained more than 10% of the enzyme activity relative to the control plants, and so were considered to exhibit weak silencing in this context. The transgenic plants with this construct showed a wide range in the extent of GUS gene silencing (Figure 12), from less than 1% to about 80% activity remaining, which was typical for conventional hairpin designs (Smith et al., 2000).

In clear contrast, the hpGUS[G:U] construct induced consistent and uniform silencing across the independent transgenic lines, with 71 of the 74 plants (95.9%) that were tested showing strong GUS silencing. Different again, all of the 33 hpGUS[1:4] plants tested showed reduced levels of GUS activity, with only 8 (24%) yielding <10% of the GUS activity relative to the control plants, and the other 25 classified as having weaker silencing. These results indicated that this construct induced weaker but more uniform levels of GUS down-regulation across the transgenic lines. The hpGUS[2:10] construct performed more like the hpGUS[wt] construct, inducing good levels of silencing in some lines (28 of 41, or 68.3%) and gave little or no GUS silencing in the remaining 13 plants.

When only the silenced lines (<10% remaining activity) were used for comparison and average GUS activities calculated, the hpGUS[wt] plants showed the highest average extent of silencing, followed in order by the hpGUS[G:U] plants and the hpGUS[2:10] plants (Figure 13). The hpGUS[1:4] plants showed the least average reduction in GUS activity. The extent of GUS silencing showed a good correlation with the thermodynamic stability of the predicted hpRNA structures derived from the four different hpRNA constructs (Example 6).

To test whether the differences would persist in progeny plants, representative transgenic plants containing both the target GUS gene, which was homozygous, and the hpGUS transgene (hemizygous) were self-fertilised. Kanamycin-resistant progeny

plants from the hpGUS lines were selected, so discarding any null segregants lacking the hpGUS transgenes. This ensured that the hpGUS transgenes were present in all of the progeny, in either the homozygous or heterozygous state. The progeny plants were assayed for GUS activity and representative data are presented in Figure 14. Progeny
5 containing the hpGUS[wt] transgenes obviously fell into two categories, namely those that had strong GUS silencing and others that showed weak or no silencing. These classes correlated well with the phenotype of the previous generation, showing that the extent of target gene silencing was heritable. All of the plants in the hpGUS[G:U] lines tested consistently showed strong silencing, whilst the plants in the hpGUS[1:4] lines
10 consistently showed weaker silencing. The inventors concluded that the phenotypes observed in the parental generation were generally maintained in the progeny plants.

Southern blot hybridisation experiments on transformed plants

The uniformity of the strong gene silencing observed in the large number of
15 independent transgenic plants generated with the hpGUS[G:U] construct was striking as well as surprising and unexpected. The inventors sought to establish whether any explanation other than an effect caused by the hpGUS[G:U] RNA was causing the uniformity of the silencing. To test whether the multiple transgenic plants arose from independent transformation events as intended, Southern blot hybridisation experiments
20 were carried out on DNA isolated from 18 representative transgenic plants containing the hpGUS[G:U] construct. DNA was isolated from leaf tissues using the hot-phenol method described by Wang et al. (2008). For Southern blot hybridization, approximately 10 µg of DNA from each plant sample was digested with *HindIII* enzyme, separated by gel electrophoresis in 1% agarose gels in TBE buffer, and blotted
25 onto Hybond-N+ membrane using the capillary method (Sambrook et al., 1989). The membrane was hybridized overnight at 42°C with a ³²P-labelled DNA fragment from the OCS-T terminator region. This probe was chosen as it hybridized to the hpGUS[G:U] transgene but not to the GUS target gene which did not have an OCS-T terminator sequence. The membrane was washed at high stringency and retained probe
30 visualized with a PhosphoImager.

An autoradiograph of a hybridised blot is shown in Figure 15. Each lane showed from one to five or six hybridising bands. No two lanes showed the same pattern i.e. the autoradiograph showed that the 16 representative hpGUS[G:U] plants each had different patterns of *HindIII* fragments that hybridized and therefore came
35 from different transgene insertions. The inventors concluded that the uniform GUS silencing observed for hpGUS[G:U] lines was not due to similar transgene insertion

patterns in the plants, and that the uniformity of silencing was caused by the structure of the hpGUS[G:U] RNA. The inventors also concluded that multiple copies of the hpGUS[G:U] transgene were not required in order to obtain strong gene silencing; a single copy of the transgene was sufficient.

5

Northern blot hybridisation experiments on transformed plants

To determine whether the hpGUS[G:U] RNA was processed in the same manner as the control hairpin RNA in the transgenic plants, Northern blot hybridisation experiments were carried out on RNA isolated from leaves of the transgenic plants. The Northern blot experiments were carried out to detect the shorter RNAs (sRNA, approx 10 21-24 nucleotides in length) which resulted from Dicer-processing of the hairpin RNAs. The experiment was carried out on small RNA isolated from transgenic hpGUS[wt] and hpGUS[G:U] plants which also containing the GUS target gene which was expressed as a (sense) mRNA. Nine plants for each construct were selected for 15 sRNA analysis. For the hpGUS[wt] transgenic population, plants showing weak GUS silencing were included as well as some exhibiting strong GUS silencing. The small RNA samples were isolated using the hot-phenol method (Wang et al., 2008), and Northern blot hybridization was performed according to Wang et al. (2008), with gel electrophoresis of the RNA samples carried out under denaturing conditions. The 20 probes used were ³²P-labelled RNAs corresponding to either the sense sequence or the antisense sequence corresponding to nucleotides 804-1003 of SEQ ID NO:8.

An autoradiograph of a Northern blot, hybridised with either the antisense probe (upper panel) to detect sense sRNA molecules derived from the hairpin RNAs, or hybridised with the sense probe to detect the antisense sRNAs (lower panel), is shown 25 in Figure 16. At the bottom, the Figure shows a qualitative score for the level of GUS expression relative to the control plants lacking the hpGUS constructs. Hybridisation to small RNAs of about 20-25 nucleotides was observed, based on the mobility of the sRNAs compared to RNAs of known length in other experiments. The hpGUS[wt] lines showed a range of variation in the amount of sRNA accumulation. This was 30 observed for both the sense and antisense sRNAs, although the antisense sRNA bands were not as clear as the sense bands. Since the hpGUS[wt] plants contained both the hpGUS transgene, expressing both sense and antisense sequences corresponding to the 200nt target region, and the GUS target gene expressing the full-length sense gene, the sense sRNAs could have been generated from either the hairpin RNA or the target 35 mRNA. There appeared to be negative correlation between the level of sRNA and the degree of GUS silencing in the hpGUS[wt] plants. For example, the two plants

represented in lanes 4 and 5 accumulated relatively more sRNA but showed only a moderate extent of GUS downregulation. In contrast, the two plants represented in lanes 7 and 8 had strong GUS silencing but accumulated relatively low levels of sRNA.

In contrast to the hpGUS[wt] plants and consistent with the relatively uniform
5 extent of silencing by the hpGUS[G:U] construct, the hpGUS[G:U] plants accumulated uniform amounts of antisense sRNAs across the lines. Furthermore, the degree of GUS silencing appeared to show good correlation with the amount of antisense sRNA. Almost no sense sRNAs were detected in these plants. This was expected since the
10 RNA probe used in the Northern blot hybridisation was transcribed from the wild-type GUS sequence and therefore had a lower level of complementarity to sense sRNAs from hpGUS[G:U] where all C nucleotides were replaced with U nucleotides, allowing only lower stringency hybridisation. However, this experiment did not exclude the possibility that the hpGUS[G:U] RNA was processed to produce less sense sRNAs or that they were degraded more quickly.

15 The Northern blot hybridisation experiment was repeated, this time using only a sense probe to detect antisense sRNAs; the autoradiograph is shown in Figure 17. Once again, the production of antisense sRNAs from the hpGUS[wt] construct correlated negatively with the GUS activity (upper panel of Figure 17). Plants which were strongly silenced yielded high levels of antisense sRNAs (lanes 1, 3, 5, 8 and 10)
20 whereas plants that showed only weak or no silencing did not produce a hybridisation signal in this experiment (lanes 2, 4, 6, 7 and 9). In very clear contrast, the plants expressing hpGUS[G:U] produced a much lower, but consistent, amount of antisense sRNAs. The observation that the strongly silenced plants expressing hpGUS[G:U] accumulated much lower levels of sRNAs than the strongly silenced plants expressing
25 hpGUS[wt] was intriguing and suggested to the inventors that the hpGUS[wt] was being processed by a different mechanism in the plants but was still about as effective as the hpGUS[wt] construct. A further observation in this experiment provided a clue in that the two, relatively faint antisense bands for the hpGUS[G:U] plants appeared to have the same mobility as the second and fourth bands observed for the antisense
30 sRNA bands from hpGUS[wt]. This was confirmed in further experiments described below. The inventors postulated that the four bands for the sRNAs from hpGUS[wt] represent 24-, 22-, 21- and 20-mers, and that the hpGUS[G:U] RNA was processed primarily to produce 22- and 20-mers antisense sRNAs.

An important, definite conclusion from the data described above was that the
35 hpGUS[G:U] RNA molecule was processed by one or more Dicer enzymes to produce sRNAs, in particular the production of antisense sRNAs which are thought to be

mediators of RNA interference in the presence of various proteins such as Argonaute. The observed production of antisense sRNAs implied that the sense sRNAs were also produced, but the experiments did not distinguish between degradation/instability of the sense sRNAs or the lack of detection of sense sRNAs due to insufficient hybridisation
5 with the probe that was used. From these experiments, the inventors also concluded that there were clear differences between the hpGUS[wt] and hpGUS[G:U] RNA molecules in their processing. This indicated that the molecules were recognised differently by one or more Dicers.

10 **Example 8. Analysis of sRNAs from transgenic plants expressing modified hairpin RNAs**

Another Northern blot hybridisation experiment was carried out to detect antisense sRNAs from hpGUS[G:U] plants and to compare their sizes to those produced from hpGUS[wt]. The autoradiograph is shown in Figure 18. This time, the
15 difference in size of the two antisense sRNA bands from hpGUS[G:U] compared to the main two bands from hpGUS[wt] was more distinct. This was best seen by comparing the mobility of the bands in adjacent lanes 9 and 10 of Figure 18. This result confirmed that the two hairpin RNAs were processed differently by one or more Dicers in the plants.

20 To further investigate this, the small RNA populations from the hpGUS[wt] and hpGUS[G:U] were analysed by deep sequencing of the total, linker-amplifiable sRNAs isolated from the plants. The frequency of sRNAs which mapped to the double-stranded regions of the hairpin RNAs was determined. The length distribution of such sRNAs was also determined. The results showed that there was an increase in the
25 frequency of 22-mer antisense RNAs from the hpGUS[G:U] construct relative to the hpGUS[wt] construct. The increase in the proportion of sRNAs of 22 nt in length indicated a shift in processing of the hpGUS[G:U] hairpin by Dicer-2 relative to hpGUS[wt].

30 **Example 9. DNA methylation analysis of transgenes in plants**

The observations on the variability in the extent of GUS silencing conferred by hpGUS[wt] and that antisense 24-mer sRNAs were detected in the hpGUS[wt] plants but apparently not in the hpGUS[G:U] plants led the inventors to question whether the two populations of plants differed in their level of DNA methylation of the target GUS
35 gene. Sequence-specific 24-mer sRNAs are thought to be involved in promoting DNA methylation of inverted repeat structures in plants (Dong et al., 2011). The inventors

therefore tested the levels of DNA methylation of the GUS transgene in the hpGUS plants, in particular of the 35S promoter region of the hairpin encoding gene (silencing gene).

To do this, the DNA-methylation dependent endonuclease McrBC was used.

5 McrBC is a commercially available endonuclease which cleaves DNA containing methylcytosine (^mC) bases on one or both strands of double-stranded DNA (Stewart et al., 2000). McrBC recognises sites on the DNA which consist of two half-sites of the form 5' (G or A)^mC 3', preferably G^mC. These half-sites may be separated by several hundred basepairs, but the optimal separation is from 55 to about 100 bp. Double-

10 stranded DNA having such linked G^mC dinucleotides on both strands serve as the best substrate. McrBC activity is dependent on either one or both of the GC dinucleotides being methylated. Since plant DNA can be methylated at the C in CG, CHG or CHH sequences where H stands for A, C or T (Zhang et al., 2018), digestion of DNA using McrBC with subsequent PCR amplification of gene-specific sequences can be used to

15 detect the presence or absence of ^mC in specific DNA sequences in plant genomes. In this assay, PCR amplification of McrBC-digested genomic DNA which is methylated yields reduced amounts of the amplification product compared to DNA which is not methylated, but will yield an equal amount of PCR product as untreated DNA if the DNA is not methylated.

20 Genomic DNA was isolated by standard methods from plants containing the hpGUS[wt], hpGUS[G:U] or hpGUS[1:4] construct in addition to the target GUS gene (Draper and Scott, 1988). Purified DNA samples were treated with McrBC (Catalog No. M0272; New England Biolabs, Massachusetts) according to the manufacturer's instructions, including the presence of Mg²⁺ ion and GTP required for endonuclease

25 activity. In summary, approximately 1 µg of genomic DNA was digested with McrBC overnight in a 30µl reaction volume. The digested DNA samples were diluted to 100µl and regions of interest were PCR-amplified as follows.

The treated DNA samples were used in PCR reactions using the following primers. For the 35S-GUS junction sequence for hpGUS[wt]: Forward primer (35S-

30 F3), 5'-TGGCTCCTACAAATGCCATC-3' (SEQ ID NO:60); Reverse primer (GUSwt-R2), 5'-CARRAACTRTRTCRCCCTTCAC-3' (SEQ ID NO:61). For the 35S-GUS junction sequence for hpGUS[G:U]: Forward primer (GUSgu-R2), 5'-CAAAAAC TATTCACCCTTCAC-3' (SEQ ID NO:62); reverse primer (GUS4m-R2), CACRAARTRTACRCRCCTTRAC (SEQ ID NO:63). For the 35S promoter sequence

35 for both constructs: Forward primer (35S-F2), 5'-GAGGATCTAACAGAACTCGC-3' (SEQ ID NO:64); reverse primer (35S-R1), 5'-CTCTCAAATGAAATGAACTTCC-

3' (SEQ ID NO:65). In each case, R=A or G, Y=C or T. PCR reactions were performed with the following cycling conditions: 94°C for 1 min, 35 cycles of 94°C for 30 sec, 55°C annealing for 45 sec, 68°C extension for 1 min, and final extension at 68°C for 5 min. PCR amplification products were electrophoresed and the intensity of the bands
5 quantitated.

Representative results are shown in Figures 19 and 20. For the 35S-GUS junction region which included 200bp of the 35S promoter sequence including the transcriptional start site, most of hpGUS[wt] plants showed significant levels of DNA methylation. Within the population of hpGUS[wt] plants, individual plants that retained
10 higher levels of GUS activity i.e. less silencing, appeared to have more methylation of the promoter-GUS sense junction region. The results were similar for the 35S promoter region. In contrast, most of the hpGUS[G:U] and hpGUS[1:4] plants showed weaker DNA methylation at the 35S-GUS junction. The inventors considered that this proximal promoter sequence was important for expression of the transgene and
15 methylation at this region would be likely to reduce expression of the silencing construct through transcriptional gene silencing (TGS) of the transgene. This is termed “self-silencing”.

General Discussion in Relation to Examples 6 to 9

20 *Disruption of inverted repeat DNA structure in a transgene enhances its stability*

Both of the populations of hpGUS[wt] and hpGUS[2:10] transgenic plants showed a wide range in the extent target gene silencing. In contrast, both of the populations containing hpGUS[G:U] and hpGUS[1:4] plants displayed relatively uniform GUS silencing in many independent lines, with strong silencing observed by
25 the former construct and relatively weaker but still substantial reduction in gene activity by the latter construct. In the hairpin RNAs from the [G:U] and [1:4] constructs, about 25% of the nucleotides in the sense and antisense sequences were either involved in G:U basepairs or in a sequence mismatch that were evenly distributed across the 200 nucleotide sense/antisense sequences. Because of the
30 sequence divergence between the sense and antisense sequences, the mismatches in the DNA constructs between the sense and antisense “arms” or the inverted repeat structure were considered to significantly disrupt that inverted-repeat DNA structure. Repetitive DNA structures may attract DNA methylation and silencing in various organisms (Hsieh and Fire, 2000). The hpGUS[2:10] construct also comprised
35 mismatches between the sense and antisense region, but each of the 2bp mismatches between the sense and antisense sequences were flanked by 8-bp consecutive matches,

so the mismatches may not have disrupted the inverted repeat DNA structure as much as in the [G:U] and [1:4] transgenes. The uniformity of the GUS silencing induced by the hpGUS[G:U] and hpRNA[1:4] might therefore have been due, at least in part, to disruption of the inverted-repeat DNA structure that resulted in less methylation and therefore reduced the self-silencing of the two transgenes. Another benefit of the mismatches between the sense and antisense DNA regions was that cloning of the inverted repeat in *E. coli* was aided since the bacteria tend to delete or re-arrange perfect inverted repeats.

10 *Thermodynamic stability of hpRNA is important for the degree of target gene silencing*

When only the strongly-silenced transgenic lines were compared, the hpGUS[wt] plants had the greatest extent of target gene downregulation, followed in order by hpGUS[G:U], hpGUS[2:10] and hpGUS[1:4]. RNAFold analysis predicted that the hpGUS[wt] hairpin RNA structure had the lowest free energy, i.e. the greatest stability, followed by hpGUS[G:U], hpGUS[2:10] and hpGUS[1:4] hairpins. The inventors considered that the more stable the hairpin RNA structure, the greater the extent of target gene silencing it could induce. This also favoured longer double-stranded RNA structures rather than shorter ones. Stable double-stranded RNA formation was thought to be required for efficient Dicer processing. The results of the experiments described here indicated another important advantage of the G:U basepaired construct over the constructs comprising mostly simple mismatched nucleotides such as hpGUS[1:4]: while both types of constructs had disrupted inverted repeat DNA structures which reduced self-silencing, at the RNA level the hpGUS[G:U] RNA was more stable due to the ability of G and U to form basepairs. A combination of the two types of modifications was also considered beneficial, including both G:U basepairs and some mismatched nucleotides in the double-stranded RNA structure but with relatively more nucleotides involved in G:U basepairs than in mismatches, by a factor of at least 2, 3, 4 or even 5.

30 *The hpGUS[G:U] RNA was efficiently processed by Dicer*

One important question that was answered in these experiments was whether the mismatched or G:U basepaired hpRNA could be processed by Dicer into small RNAs (sRNAs). The strong silencing in the hpGUS[G:U] plants and in the 1:4 and 2:10 mismatched hpRNA plants, implied that these hairpin RNA structures were processed by Dicer. This was confirmed for the [G:U] molecule by sRNA Northern blot hybridization, which readily detected antisense sRNAs. Furthermore, the degree of

GUS silencing in the hpGUS[G:U] plants showed a good correlation with the amount of antisense sRNAs that accumulated. Small RNA deep sequencing analysis of two selected lines from each (only one for hpGUS[wt]) confirmed that hpGUS[G:U] plants, like the hpGUS[wt] plants, generated abundant sRNAs, whereas the hpGUS[1:4] plants also generated sRNAs but with a much lower abundance (Figure 21). The lower level of sRNAs from the hpGUS[1:4] plants was consistent with the relatively low efficiency of GUS silencing and suggested that the low thermodynamic stability of the dsRNA stem in hpGUS[1:4] RNA reduced Dicer processing efficiency. It was noted that the extent of GUS silencing showed relatively poor correlation with the level of sRNA for the hpGUS[wt] construct, with some strongly silenced lines containing relatively low amounts of sRNA. This suggested that GUS silencing in some of the hpGUS[wt] lines was due at least in part to transcriptional silencing rather than sRNA-directed PTGS. The inventors recognised that the self-silencing of the hairpin-encoding gene, which involved methylation of the gene sequences such as the promoter region, was lessened by using the modified hairpin RNA constructs, particularly the G:U construct.

The G:U and 1:4 hpRNA transgenes showed reduced DNA methylation in the proximal 35S promoter region

McrBC digestion-PCR analysis showed that DNA methylation levels in the 240 bp 35S sequence near the transcription start site (TSS) was reduced in the hpGUS[G:U] and hpGUS[1:4] transgenic populations relative to the hpGUS[wt] population. This result indicated to the inventors that the disruption of the perfect inverted-repeat structure, due to the C to T modifications (in hpGUS[G:U]) or 25% nucleotide mismatches (in hpGUS[1:4]) in the sense sequence, minimized transcriptional self-silencing of the hpRNA transgenes. This was consistent with the uniformity of GUS gene silencing observed in the hpGUS[G:U] and hpGUS[1:4] populations relative to the hpGUS[wt] population. The inventors recognised that the hpGUS[G:U] construct was more ideal than the hpGUS[1:4] construct in reducing promoter methylation hence transcriptional self-silencing at least because it had a reduced number, or even lacked, cytosine nucleotides in the sense sequence and therefore did not attract DNA methylation that could spread to the promoter.

Example 10. Design and testing of hairpin RNAs comprising G:U basepairs targeting endogenous genes

Modified hairpin RNAs targeting EIN2 and CHS RNAs

Since the G:U modified hairpin RNA appeared to induce more consistent and uniform silencing of the target gene compared to the conventional hairpin RNA as described above, the inventors wanted to test whether the improved design would also reduce expression of endogenous genes. The inventors therefore designed, produced and tested several [G:U]- modified hairpin RNA constructs targeting either the *EIN2* or *CHS* genes, or both, which were endogenous genes in *Arabidopsis thaliana* chosen as exemplary target genes for attempted silencing. The *EIN2* gene (SEQ ID NO:19) encodes ethylene-insensitive protein 2 (EIN2) which is a central factor in signalling pathways regulated by the plant signalling molecule ethylene, i.e. a regulatory protein, and the *CHS* gene (SEQ ID NO:20) encodes the enzyme chalcone synthase (CHS) which is involved in anthocyanin production in the seedcoat in *A. thaliana*. Another G:U modified construct was produced which simultaneously targeted both of the *EIN2* and *CHS* genes, in which the *EIN2* and *CHS* sequences were transcriptionally fused to produce a single hairpin RNA. Furthermore, three additional constructs were made targeting either *EIN2*, *CHS* or both *EIN2* and *CHS*, in which cytidine bases in both the sense and antisense sequences were replaced with thymidine bases (herein designated a G:U/U:G construct), rather than in just the sense sequence as done for the modified hairpins targeting *GUS*. The modified hairpin RNA constructs were tested for their ability to reduce the expression of the endogenous *EIN2* gene or the *EIN2* and *CHS* genes using a gene-delivered approach to provide the hairpin RNAs to the cells. The conventional hairpin RNAs used as the controls in the experiment had a double-stranded RNA region of 200 basepairs in length for targeting the *EIN2* or *CHS* mRNAs, singly, or a chimeric double-stranded RNA region comprising 200 basepairs from each of the *EIN2* and *CHS* genes which were fused together as a single hairpin molecule. In the fused RNA, the *EIN2* double-stranded portion was adjacent to the loop of the hairpin and the *CHS* region was distal to the loop. All of the basepairs in the double-stranded region of the control hairpin RNAs were canonical basepairs.

Construct preparation

DNA fragments spanning the 200 bp regions of the wild-type *EIN2* (SEQ ID NO:19) and *CHS* cDNAs (SEQ ID NO:20) were PCR-amplified from *Arabidopsis thaliana* Col-0 cDNA using the oligonucleotide primer pairs *EIN2*wt-F (SEQ ID NO:66) and *EIN2*wt-R (SEQ ID NO:67) or *CHS*wt-F (SEQ ID NO:68) and *CHS*wt-R

(SEQ ID NO:69), respectively. The fragments were inserted into pGEMT-Easy as for the GUS hairpin constructs (Example 6). DNA fragments comprising the 200 bp modified sense EIN2[G:U] (SEQ ID NO:22) and CHS[G:U] (SEQ ID NO:24) fragments or the 200 bp modified antisense EIN2[G:U] (SEQ ID NO:25) and modified
 5 antisense CHS[G:U] (SEQ ID NO:26) fragments, each flanked by restriction enzyme sites, were assembled by annealing of the respective pairs of oligonucleotides, EIN2gu-F + EIN2gu-R, CHSgu-F + CHSgu-R, asEIN2gu-F + asEIN2gu-R, and asCHSgu-F + asCHSgu-R (SEQ ID NOs:70-77), followed by PCR extension of 3' ends using LongAmp Taq polymerase. All the G:U-modified PCR fragments were cloned into
 10 pGEM-T Easy vector and the intended nucleotide sequences confirmed by sequencing. The CHS[wt]::EIN2[wt], CHS[G:U]:EIN2[G:U], and asCHS[G:U]::asEIN2[G:U] fusion fragments were prepared by ligating the appropriate CHS and EIN2 DNA fragments at the common *Xba*I site in the pGEM-T Easy plasmid.

The 35S::sense fragment::PDK intron::antisense fragment::OCS-T cassettes
 15 were prepared in an analogous manner as for the hpGUS constructs. Essentially, the antisense fragments were excised from the respective pGEM-T Easy plasmids by digestion with *Hind*III and *Bam*HI, and inserted into pKannibal between the *Bam*HI and *Hind*III sites so they would be in the antisense orientation relative to the 35S promoter. The sense fragments were then excised from the respective pGEM-T Easy
 20 plasmid using *Xho*I and *Kpn*I and inserted into the same sites of the appropriate antisense-containing clone. All of the cassettes in the pGEM-T Easy plasmids were then excised with *Not*I and inserted into pART27 to form the final binary vectors for plant transformation.

The alignments of the modified sense[G:U] and antisense[G:U] nucleotide
 25 sequences with the corresponding wild-type sequences, showing the positions of the substituted nucleotides, are shown in Figures 22 to 25. The designs of the expression cassettes for the hairpin RNAs are shown schematically in Figure 26.

The predicted free energy of formation of the hairpin RNAs was estimated by using the FOLD program. These were calculated as (kcal/mol): hpEIN2[wt], -453.5;
 30 hpEIN2[G:U], -328.1; hpCHS[wt], -507.7; hpCHS[G:U] -328.5; hpEIN2[G:U/U:G], -173.5; hpCHS[G:Y/U:G], -186.0; hpCHS::EIN2[wt], -916.4; hpCHS::EIN2[G:U], -630.9; hpCHS::EIN2[G:U/U:G], -333.8.

Plant transformation

35 All of the EIN2, CHS and chimeric EIN2/CHS constructs were used to transform *Arabidopsis thaliana* race Col-0 plants using the floral dip method (Clough

and Bent, 1998). To select for transgenic plants, seeds collected from the *Agrobacterium*-dipped flowers were sterilized with chlorine gas and plated on MS medium containing 50 mg/L kanamycin. Multiple transgenic lines were obtained for all nine constructs (Table 1). These primary transformants (T1 generation) were transferred to soil, self-fertilised and grown to maturity. Seed collected from these plants (T2 seed) was used to establish T2 plants and screened for lines that were homozygous for the transgene. These were used for analysing EIN2 and CHS silencing.

Table 1. Summary of transgenic plants obtained in Col-0 background

Construct	Number of transgenic lines obtained
hpEIN2[wt]	46
hpCHS[wt]	34
hpEIN2[G:U]	23
hpCHS[G:U]	32
hpEIN2[G:U/U:G]	52
hpCHS[G:U/U:G]	13
hpCHS::EIN2[wt]	28
hpCHS::EIN2[G:U]	26
hpCHS::EIN2[G:U/U:G]	20

10

Analysis of the extent of EIN2 silencing

EIN2 is a gene in *A. thaliana* that encodes a receptor protein involved in ethylene perception. The gene is expressed in seedlings soon after germination of seeds as well as later in plant growth and development. *EIN2* mutant seedlings exhibit hypocotyl elongation relative to isogenic wild-type seedlings when germinated in the dark in the presence of 1-aminocyclopropane-1-carboxylic acid (ACC), an intermediate in the synthesis of ethylene in plants. *EIN2* gene expression and the extent of silencing in the transgenic plants was therefore assayed by germinating seed on MS medium containing 50 µg/L of ACC in total darkness and measuring their hypocotyl length, compared to the wild-type seedlings. The hypocotyl length was an easy phenotype to measure and was a good indicator of the extent of reduction in *EIN2* gene expression, indicating different levels of *EIN2* silencing. Plants with silenced *EIN2* gene expression were expected to have various degrees of hypocotyl elongation depending on the level of EIN2 silencing, somewhere in the range between wild-type seedlings (short hypocotyls) and null-mutant seedlings (long hypocotyls). Seeds from 20 randomly selected, independently transformed plants for each construct were assayed. Seeds from

one plant of the 20 containing the hpCHS::EIN2[G:U] construct did not germinate. The data for hypocotyl length are shown in Figure 27.

The hpEIN2[wt] lines showed a considerable range in the extent of *EIN2* silencing, with 7 lines (plant lines 2, 5, 9, 10, 12, 14, 16 in Figure 27) clearly showing low levels of silencing or the same hypocotyl length relative to the wild-type, and the other 13 lines having moderate to strong *EIN2* silencing. Individual plants within each independent line tended to exhibit a range in the extent of *EIN2* silencing, as indicated by differences in hypocotyl length. In contrast, only two lines (plant lines 5, 18 in Figure 27) comprising the hpEIN2[G:U] construct showed weak *EIN2* silencing, with the remaining 18 showing uniform, strong *EIN2* silencing. In addition, individual plants within each of the 18 lines appeared to have relatively uniform *EIN2* silencing compared to the plants transformed with the hpEIN2[wt] construct. The inventors concluded that the G:U modified hairpin RNA construct was able to confer more consistent, less variable gene silencing of an endogenous gene which was more uniform and more predictable than the conventional hairpin RNA targeting the same region of the endogenous RNA.

The transgenic hpEIN2[wt] and hpEIN2[G:U] populations also differed in the relationship between the extent of *EIN2* silencing and the transgene copy number. The transgene copy number was indicated by the segregation ratios for the kanamycin resistance marker gene in progeny plants— a 3:1 ratio of resistant:susceptible seedlings indicating a single locus insertion, whereas a ratio that was much higher indicated multi-loci transgene insertions. Several multiple copy-number lines transformed with the hpEIN2[wt] construct showed low levels of *EIN2* silencing, but this was not the case for the hpEIN2[G:U] lines where both the single and multi-copy loci lines showed strong *EIN2* silencing.

The *EIN2* gene was also silencing in the seedlings transformed with the CHS::EIN2 fusion hairpin RNA. Similar to the plants containing the single hpEIN2[G:U] construct, the hpCHS::EIN2[G:U] seedlings clearly showed more uniform *EIN2* silencing across the independent lines than the hpCHS::EIN2[wt] seedlings. The silencing among individual plants within an independent line also appeared to be more uniform for the hpCHS::EIN2[G:U] lines than the hpCHS::EIN2[wt] lines. At the same time, the extent of *EIN2* silencing was slightly stronger for the highly silenced hpCHS::EIN2[wt] plants than for the hpCHS::EIN2[G:U] plants, similar to the comparison between plants transformed with hpGUS[wt] and hpGUS[G:U]. Comparison of the extent of silencing indicated that the fusion constructs did not induce stronger *EIN2* silencing than the single hpEIN2[G:U]

construct, indeed, the fusion G:U hairpin construct appeared to induce slightly weaker *EIN2* silencing than the single gene-targeted hpEIN2[G:U] construct.

When the plants transformed with the G:U/U:G constructs were examined, where the cytidine (C) nucleotides of both the sense and antisense sequences were
5 modified to thymidine (T) nucleotides, little to no increase in hypocotyl length was observed for all 20 independent lines analysed compared to wild-type plants. This was observed for both the hpEIN2[G:U/U:G] and hpCHS::*EIN2*[G:U/U:G] constructs. These results indicated to the inventors that the G:U/U:G basepaired hairpin RNA constructs having about 46% substitutions were not effective at inducing target gene
10 silencing, perhaps because the basepairing of the hairpin RNAs had been destabilised too much. The inventors considered that two possible reasons might have contributed to the ineffectiveness. Firstly, the *EIN2* double-stranded region of the hairpin RNAs had 92 G:U basepairs of the 200 potential basepairs between the sense and antisense sequences. Secondly, the alignment of the modified antisense sequence with the
15 complement of the wild-type sense sequence showed that the 49 C to T replacements in the antisense sequence might have reduced the effectiveness of the antisense sequence to target the *EIN2* mRNA. The inventors concluded from this experiment that, at least for the *EIN2* target gene, there was an upper limit to the number of nucleotide substitutions that could be tolerated in the hairpin RNA and still maintain sufficient
20 effectiveness for silencing. For instance, $92/200=46\%$ substitutions was probably too high a percentage.

Analysis of the extent of CHS silencing

Transgenic plants were assayed for the level of CHS gene expression by
25 quantitative reverse transcription PCR (qRT-PCR) on RNA extracted from the whole plants, grown *in vitro* on tissue culture medium. The primers used for the CHS mRNA were: forward primer (CHS-200-F2), 5'-GACATGCCTGGTGCTGACTA-3' (SEQ ID NO:78); reverse primer (CHS-200-R2) 5'-CCTTAGCGATACGGAGGACA-3' (SEQ ID NO:79). The primers used for the reference gene *Actin2* used as a standard were:
30 Forward primer (*Actin2*-For) 5'-TCCCTCAGCACATTCCAGCA-3' (SEQ ID NO:80) and reverse primer (*Actin2*-Rev) 5'-GATCCCATTCATAAAACCCAG-3' (SEQ ID NO:81).

The data showed that the level of CHS mRNA the accumulated in the plants relative to the reference mRNA for the *Actin2* gene was decreased in the range of 50-
35 96% (Figure 28).

A. thaliana seed completely lacking CHS activity have a pale seed coat colour compared to the brown colour of wild-type seeds. Therefore, seed of the transgenic plants were examined visually for their seedcoat colour. An obvious reduction of seed coat colour was observed in seeds from several plants but not in other plants, despite the reduction in CHS mRNA in the leaves of those plants. It was considered, however, that the seed coat colour phenotype was exhibited only when CHS activity was almost completely abolished in the developing seed coat during growth of the plants. Moreover, the 35S promoter may not have been sufficiently active in the developing seed coat to provide the level of reduction in CHS activity to provide for the pale seed phenotype seen in null mutants. Improvement in the visual seed coat colour phenotype could be gained by using a promoter that is more active in the seed coat of the seed.

Reducing expression of PDS gene in *Arabidopsis thaliana*

Another *Arabidopsis* gene was selected as an exemplary target gene, namely the phytoene desaturase (*PDS*) gene which encodes the enzyme phytoene desaturase that catalyzes the desaturation of phytoene to zeta-carotene during carotenoid biosynthesis. Silencing of *PDS* was expected to result in photo-bleaching of *Arabidopsis* plants, which could easily be observed visually. A G:U-modified hpRNA construct was therefore made and tested in comparison to a traditional hpRNA constructs targeting a 450 nucleotide *PDS* mRNA sequence. The 450 nucleotide *PDS* sequence contained 82 cytosines (C) which were substituted with thymidines (T), resulting in 18.2% of the basepairs in the dsRNA region of the hpRNA hpPDS[G:U] being G:U base pairs. The genetic construct encoding hpPDS[G:U] and the control genetic construct encoding hpPDS[WT] were introduced into *Arabidopsis thaliana* Col-0 ecotype using *Agrobacterium*-mediated transformation.

For the hpPDS[WT] and hpPDS[G:U] constructs, 100 and 172 transgenic lines were identified, respectively. Strikingly, all these lines showed photo-bleaching in the cotyledons of young T1 seedlings that emerged on kanamycin-resistant selective medium, with no obvious difference between the two transgenic populations at this early stage of plant growth. These indicated that the two constructs were equally effective at inducing *PDS* silencing in cotyledons. However, some of the T1 plants developed true leaves that were no longer photo-bleached and looked green or pale green, indicating that *PDS* silencing was released or weakened in the true leaves. The proportion of transgenic lines showing green true leaves were much higher for the hpPDS[WT] population than for the hpPDS[G:U] population. The transgenic plants were grouped into three different categories based on strong *PDS* silencing (strong

photo-bleaching in whole plant), moderate *PDS* silencing (pale green or mottled leaves) and weak *PDS* silencing (fully green or weakly mottled leaves). The proportion of plants with weak *PDS* silencing was 43% for the hp*PDS*[WT] lines, compared to 7% for the hp*PDS*[G:U] lines. In fact, all the hp*PDS*[G:U] lines of the weak silencing group still showed mild mottling on true leaves, in contrast to the weakly silenced hp*PDS*[WT] plants that mostly had fully green leaves. These results indicated that the G:U-modified hpRNA construct gave more uniform *PDS* silencing across the independent transgenic population than the conventional (fully canonically basepaired) hp*PDS* construct, which was consistent with the results from *GUS* and *EIN2* silencing assays described above. More significantly, the *PDS* silencing results indicated a developmental variability of hpRNA transgene-induced gene silencing in plants that has not been noted before, and suggested that hpRNA transgene silencing was more efficient and stable in cotyledons than in true leaves. In accordance with the uniform gene silencing across independent lines, the *PDS* silencing result suggested that the G:U-modified hpRNA transgene was developmentally more stable than the conventional hpRNA construct, providing more stable and long-lasting silencing.

Example 11. Analysis of sRNAs from hairpin RNA constructs

Northern blot hybridisation was carried out on RNA samples to detect antisense sRNAs from hp*EIN2*[G:U] plants and to compare their amount and their sizes to sRNAs produced from hp*EIN2*[wt]. The probe was a ³²P-labelled RNA probe corresponding to the 200 nucleotide sense sequence in the hp*EIN2*[wt] construct and hybridisation was carried out under low stringency conditions to allow for the detection of shorter (20-24 nucleotides) sequences. The autoradiograph from the probed Northern blot is shown in Figure 29. This experiment showed that the hp*EIN2*[G:U] hairpin RNA was processed into sRNAs and the level of accumulation was relatively uniform across the 9 independently transformed hp*EIN2*[G:U] plants analysed compared to those of the hp*EIN2*[wt] lines. Similar to the analogous experiment for the *GUS* hairpin RNAs, a difference in mobility of the two antisense sRNA bands from hp*EIN2*[G:U] compared to the main two bands from hp*EIN2*[wt] was quite evident. This was best seen by comparing the mobility of the bands in adjacent lanes 10 and 11 of Figure 28.

To further investigate this, the small RNA populations from the hp*EIN2*[wt] and hp*EIN2*[G:U] are analysed by deep sequencing of the total sRNA populations isolated from whole plants. The proportion of each population that mapped to the double-stranded regions of the hp*EIN2*[wt] and hp*EIN2*[G:U] was determined. From about 16

million reads in each population, about 50,000 sRNAs mapped to the hpEIN2[wt] double-stranded region, whereas only about 700 mapped to hpEIN2[G:U]. This indicated that many fewer sRNAs were generated from the [G:U] hairpin. An increase in the proportion of *EIN2*-specific 22-mers was also observed.

5 Figure 29 showed that both the traditional (fully canonically basepaired) and the G:U-modified hpRNA lines accumulated two dominant size fractions of siRNAs. Consistent with previous reports, the dominant siRNAs from the traditional hpRNA lines migrated similarly to the 21 and 24-nt sRNA size markers. However, the two
10 dominant siRNA bands from both of the G:U modified transgenes migrated slightly faster on the gel, suggesting that they either had a smaller size than, or different terminal chemical modifications to, those from the traditional hpRNA transgenes.

To investigate if the size profile of siRNAs might differ between the two different types of constructs, small RNAs were isolated from one hpGUS[WT] line and two lines each of hpGUS[G:U], hpEIN2[WT] and hpEIN2[G:U] and sequenced using
15 the Illumina platform, resulting in approximately 16 million sRNA reads for each sample. Samples from two strongly silenced hpGUS[1:4] lines were also sequenced. The number of sRNAs which mapped to the double-stranded regions and the intron spacer region of the hairpin RNAs was determined. siRNAs were also mapped to the upstream and downstream regions in the target *GUS* mRNA and *ENI2* mRNA to detect
20 transitive siRNAs. The sequencing data confirmed that hpGUS[G:U] lines, like hpGUS[WT] lines, generated abundant siRNAs, whereas hpGUS[1:4] lines also generated siRNAs but with a much lower abundance. The lower levels of siRNAs from the hpGUS[1:4] lines were consistent with the relatively low efficiency of *GUS* silencing by hpGUS[1:4] and suggested that the low thermodynamic stability of the
25 dsRNA stem in hpGUS[1:4] RNA reduced Dicer processing efficiency relative to the traditional hairpin. There was no clear difference in size distribution of siRNAs between the traditional and mismatched hpRNA lines despite the clear shift in mobility of antisense siRNAs shown on the Northern blot, with all samples showing the 21-nt sRNA as the dominant size class. There were some subtle differences in the
30 proportional abundance of the 22 nt antisense siRNAs between the traditional and mismatched hpGUS lines: the hpGUS[G:U] and hpGUS[1:4] lines showed a higher proportion of the 22-nt size class than the hpGUS[WT] line. A distinct feature of the sequencing data for both the traditional and mis-matched hpRNA lines was that the 24-nt siRNAs showed much lower abundance than the 21-nt siRNAs in all samples,
35 namely about 3-21 fold less for the sense 24-nt siRNAs and about 4-35 fold less for the antisense 24-nt siRNAs. This differed markedly from the Northern blot result which

showed relatively equal amounts of the two dominant size classes. It was also interesting to note that the hpEIN2[WT]-7 and hpEIN2[G:U]-14/15 samples showed similar abundance of antisense siRNAs on the Northern blot, but in the sequencing data the hpEIN2[G:U] lines gave much smaller numbers of total 20-24 nt antisense siRNA reads (17,290 and 29,211) than the hpEIN2[WT]-7 line (134,112 reads).

For both the hpGUS[G:U] and hpEIN2[G:U] lines, almost all the sense siRNAs matched the G:U-modified sense sequence of the hpRNA, whereas most of the antisense siRNAs had the wild-type antisense sequence. This indicated that the great majority of these sense and antisense siRNAs were processed directly from the primary hpRNA[G:U] transcripts, but not due to RDR-mediated amplification from the hpRNA or target RNA transcripts, which would otherwise generate both sense and antisense siRNAs of the same template sequences. Consistent with this, only a small number of 20-24 nt sRNA reads (transitive siRNAs) were detected from the loop region (PDK intron) of the hpRNA transgenes or the untargeted downstream region of the *GUS* or *EIN2* mRNA. However, the two hpGUS[1:4] lines showed a relatively high proportion of wild-type sense siRNAs, suggesting that the strong *GUS* silencing in these two lines, a relatively rare case for the hpGUS[1:4] population, may involve RDR amplification. Indeed, a higher amount of siRNAs were detected from the target gene sequence downstream of the hpRNA target region than from the dsRNA stem in the hpGUS[1:4] lines, indicating the presence of transitive silencing in these lines.

Taken together, the sRNA sequencing data indicated that siRNAs from the traditional and mismatched hpRNA lines had a similar size profile, with the exception of the 22-nt size class, suggesting that the differential migration detected by Northern blot was due to different 5' or 3' chemical modifications. The discrepancy in relative sRNA abundance (eg. the hpEIN2[WT] vs. hpEIN2[G:U]-derived siRNAs and the 21-nt vs. 24-nt) between the Northern blot result and the sequencing data implied that the different siRNA populations and size classes may have different cloning efficiencies during sRNA library preparation.

Plant sRNAs are known to have a 2'-O-methyl group at the 3' terminal nucleotide that is thought to stabilize the sRNAs. This 3' methylation was previously shown to inhibit, but not prevent, 3' adaptor ligation reducing sRNA cloning efficiency (Ebhardt et al 2005). Therefore, hpRNA[WT] and hpRNA[G:U]-derived siRNAs were with sodium periodate in β -elimination assays. The treatment did not cause a shift in gel mobility for both hpRNA[WT] and hpRNA[G:U]-derived siRNAs, indicating that both siRNA populations were methylated at the 3' terminus and there was no difference

in 3' chemical modification between the hpRNA[WT] and hpRNA[G:U]-derived siRNAs.

The standard sRNA sequencing protocol is based on sRNAs having 5' monophosphate allowing 5' adaptor ligation (Lau et al., 2001). Dicer-processed sRNAs were assumed to have 5' monophosphate but in *C. elegans* many siRNAs are found to possess di- or tri-phosphate at the 5' terminus which changes gel mobility of sRNAs and prevents sRNA 5' adaptor ligation in the standard sRNA cloning procedure (Pak and Fire 2007). Whether plant sRNAs also have differential 5' phosphorylation was unknown. The 5' phosphorylation status of the hpRNA[WT] and hpRNA[G:U]-derived siRNAs was therefore examined by treating the total RNA with alkaline phosphatase followed by Northern blot hybridization. This treatment reduced the gel mobility for all hpRNA-derived sRNAs, indicating the presence of 5' phosphorylation. However, the hpRNA[G:U]-derived siRNAs showed greater mobility shift than the hpRNA[WT]-derived siRNAs after phosphatase treatment, resulting in the two groups of dephosphorylated siRNAs migrating at the same position on the gel. The 21 and 24-nt sRNA size markers were radio-actively labelled at the 5' end with ³²P using polynucleotide kinase reaction, and so should have a monophosphorylated 5' terminus. This suggested that the hpRNA[WT]-derived siRNAs, migrating at the same positions as the size markers, were likely to be monophosphorylated siRNAs, whereas the hpRNA[G:U]-derived siRNAs, migrating faster, have more than one phosphate at the 5' terminus. Thus, it was concluded that the siRNAs produced from the traditional and G:U-modified hpRNA transgenes in plant cells were phosphorylated differently.

Example 12. DNA methylation analysis of *EIN2* silenced plants

Both the GUS and the EIN2 silencing results indicated that the hpRNA constructs having unmodified sense sequences induced highly variable levels of target gene silencing compared to the constructs having modified sense sequences providing for G:U basepairs. As described above, the promoter region of the hpGUS[G:U] construct appeared to have less methylation compared to the hpGUS[wt] construct. To test for DNA methylation and compare the hpEIN2[wt] and hpEIN2[G:U] transgenic plants, 12 plants from each population were analysed for DNA methylation at the 35S promoter and the 35S-promoter-sense EIN2 junction region using the McrBC method. The primers used for the 35S promoter region: Forward primer (Top-35S-F2), 5'-AGAAAATYTTYGTYYAAYATGGTGG-3' (SEQ ID NO:82), reverse primer (Top-35S-R2), 5'-TCARTRRARATRTCACATCAATCC-3' (SEQ ID NO:83). The primers used for the 35S promoter-sense EIN2 junction region: Forward primer (Link-35S-F2),

5'-YYATYATTGYGATAAAGGAAAGG-3' (SEQ ID NO:84) and reverse primer (Link-EIN2-R2), 5'-TAATTRCCACCAARTCATACCC-3' (SEQ ID NO:85). In each of these primer sequences, Y=C or T and R=A or G.

Quantitation of the extent of DNA methylation was determined by carrying out
5 Real-Time PCR assays. For each plant, the quotient was calculated: rate of amplification of the DNA fragment after treatment of the genomic DNA with McrBC/ rate of amplification of the DNA fragment without treatment of the genomic DNA with McrBC.

Almost every hpEIN2[wt] plant showed significant levels of DNA methylation
10 at the 35S promoter, particularly at the 35S-EIN2 junction, but some more than others. As shown in Figures 30 and 31, the plant lines represented in lanes 1, 4, 7, 9, 11 and 12 all showed strong EIN2 silencing as shown by the longer hypocotyl lengths. In contrast, the other six lines represented in lanes 2, 3, 5, 6, 8, and 10 exhibited relatively weak EIN2 silencing, resulting in shorter hypocotyls. These weaker-silenced lines showed
15 more DNA methylation at the promoter and junction sequences as indicated by much lower PCR band intensity when the genomic DNA was pre-digested with McrBC. The quantitative RealTime PCR (qPCR) assays confirmed these observations (Figure 31). All 12 of the tested lines showed some extent of DNA methylation in both the 35S promoter region and in the 35S-sense junction region ("junction"). The greatest extent
20 of methylation i.e. the lowest quotient in the qPCR assays, was for hpEIN2[wt] lines 2, 3, 5, 6, 8 and 10, correlating perfectly with the reduced extent of silencing as measured by hypocotyl length. These results confirmed that the reduced EIN2 silencing in some of the hpEIN2[wt] lines was associated with increased promoter methylation. Even in the hpEIN2[wt] plant lines which were silenced for EIN2, there was still considerable
25 levels of DNA methylation, particularly of the 35S-sense EIN2 junction fragment region. When promoters are methylated, this is thought to cause transcriptional silencing. In the case of silencing constructs, as here, this is a form of "self-silencing".

In contrast to the hpEIN2[wt] lines, the hpEIN2[G:U] lines showed less DNA methylation at both the 35S promoter and the 35S-EIN2 junction. Indeed, four of these
30 12 G:U lines, corresponding to lanes 1, 2, 3 and 7 in Figure 30 (lanes 13, 14, 15 and 20 in Figure 31), had no obvious DNA methylation as indicated by the equal strength of PCR bands between McrBC-treated and untreated samples. When these amplifications were quantitated by qPCR, six of the 12 lines showed little to no reduction in the fragment from the McrBC treatment and therefore little to no DNA methylation – see
35 lower panel of Figure 31, lines 13, 14, 15, 18, 19 and 20. These results indicated that the relatively uniform *EIN2* silencing by the hpEIN2[G:U] construct, at least in some

lines, was due to significantly less promoter methylation and therefore less transcriptional self-silencing compared to hpEIN2[wt].

These conclusions were further confirmed by analysis of the genomic DNA from the transgenic plant lines with bisulfite sequencing. This assay made use of the fact that treatment of DNA with bisulfite converted unmethylated cytosine bases in the DNA to uracil (U), but left 5-methylcytosine bases (^mC) unaffected. Following the bisulfite treatment, the defined segment of DNA of interest was amplified in PCR reactions in a way whereby only the sense strand of the treated DNA was amplified. The PCR product was then subjected to bulk sequencing, revealing the positions and extent of methylation of individual cytosine bases in the segment of DNA. Therefore, the assay yielded single-nucleotide resolution information about the methylation status of a segment of DNA.

The three plant lines showing the strongest levels of EIN2 silencing for each of hpEIN2[wt] and hpEIN2[G:U] were analysed by bisulfite sequencing, corresponding to hpEIN2[wt] lines 1, 7 and 9 and hpEIN2[G:U] lines 13, 15 and 18 in Figure 31. These plant lines showed the longest hypocotyl lengths and therefore were expected to have the lowest levels of DNA methylation out of the 20 lines for each construct. The results are presented in Figures 32 and 33 for hpEIN2[wt] and hpEIN2[G:U], respectively. When compared, it was clear that numerous cytosines in the 35S promoter region and the EIN2 sense region in the hpEIN2[wt] plants were extensively methylated. In clear contrast, the three hpEIN2[G:U] plant lines showed much lower levels of cytosine methylation in the 35S promoter region.

Example 13. DNA methylation levels in promoter of the hpGUS[1:4] construct

When genomic DNA isolated from the hpGUS[1:4] plants was analysed for DNA methylation using the McrBC and bisulfite methods as described above, it was similarly observed that there was less methylation of cytosine bases in the 35S promoter and 35S promoter-GUS sense sequence regions relative to the hpGUS[wt] plants.

General Discussion relating to Examples 10 to 13

Double-stranded RNA having G:U basepairs induce more uniform gene silencing than conventional dsRNA

Like the GUS constructs, both hpEIN2[G:U] and hpCHS:EIN2[G:U] induced more consistent and uniform EIN2 silencing than the respective hpRNA[wt] constructs encoding a conventional hairpin RNA. The uniformity not only occurred across many

independent transgenic lines, but also across sibling plants within a transgenic line each having the same transgenic insertion. In addition to the uniformity, the extent of EIN2 silencing induced by hpEIN2[G:U] was close to that of strongly silenced hpEIN2[wt] lines. Analysis of *CHS* gene silencing indicated that the hpCHS[G:U] construct was effective at reducing CHS mRNA levels by 50-97% but few plants showed a clearly visible phenotype in reduced seed coat colour. The likely explanation for not seeing more visible phenotypes in seed coat colour was that even low levels of CHS activity might be sufficient for producing the flavonoid pigments. Other possible explanations were that the 35S promoter was not sufficiently active in the developing seedcoat to produce the phenotype, or that the hpCHS[G:U] construct sequence contained 65 cytosine substitutions (32.5%), compared to only 43 (21.5%) for the EIN2 sequence and 52 (26%) for the GUS sequence. Furthermore, many of these cytosine bases in the CHS sequence occurred in sets of two or three consecutive cytosines, so not all of those need be substituted. When all of the cytosines in the sense strand were substituted, this resulted in more G:U basepairs in the hpCHS[G:U] RNA than in the hpEIN2[G:U] and hpGUS[G:U] RNAs, perhaps more than optimal. To verify this, another set of CHS constructs are made using a sequence containing a range of cytosine substitutions, from about 5%, 10%, 15%, 20% or 25% cytosine bases substituted. These constructs are tested and an optimal level determined.

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The hpEIN2[G:U] lines express more uniform levels of siRNAs

Consistent with the relatively uniform EIN2 gene silencing, the hpEIN2[G:U] lines accumulated sRNAs with a more uniform level across the independent lines. This confirmed the conclusion with the hpGUS constructs that [G:U] modified hpRNA was efficiently processed by Dicer and capable of inducing effective target gene silencing.

25

Fusion constructs also provide for gene silencing

The purpose of including the CHS:EIN2 fusion constructs in the experiment was to test if two target genes could be silenced with a single hairpin-encoding construct. The GUS experiment suggested that the free energy and therefore stability of the hairpin RNA correlated positively with the extent of target gene silencing. The results showed that the CHS:EIN2 fusion construct did result in silencing of both genes – for CHS at least at the mRNA level.

The two hpRNA constructs, hpEIN2[G:U/U:G] and hpCHS:EIN2[G:U/U:G], in which both the sense and antisense sequences were modified from C to T so that 46% of basepairs were converted from canonical basepairs to G:U basepairs, induced only

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weak or no EIN2 silencing in most of the transgenic plants. Possible explanations include i) there were too many G:U basepairs which resulted in inefficient Dicer processing, and ii) sRNAs binding to target mRNA including too many G:U basepairs did not induce efficient mRNA cleavage, or a combination of factors.

5

Increased uniformity in target gene silencing by the G:U basepaired constructs is associated with reduced promoter methylation

DNA methylation analysis using both McrBC-digestion PCR and bisulfite sequencing showed that all hpEIN2[wt] plant lines showed DNA methylation at the promoter region, and the degree of methylation correlated negatively to the level of EIN2 silencing. Even the three least methylated lines, as judged by McrBC-digestion PCR, showed around 40% DNA methylation levels in the 35S promoter, relative to all cytosines being methylated. The widespread promoter methylation was thought to be due to sRNA-directed DNA methylation at the EIN2 repeat sequence that spread to the adjacent promoter region. In contrast to the hpRNA[wt] plant lines, a number of the hpEIN2[G:U] lines showed little to no promoter methylation and most of the plants analysed showed less methylated cytosines. As discussed for the hpGUS lines, several factors may contribute to the reduced methylation: i) the inverted-repeat DNA structure was disrupted by changing C bases to T bases in the sense sequence, and ii) the sense EIN2 sequence lacked cytosines so could not be methylated by sRNA-directed DNA methylation, and iii) a reduced level of production of 24-mer RNAs due to the change in the structure of the dsRNA region with the G:U basepairs, resulting in changes in the recognition by some Dicers and so a decrease in Dicer 3 and/or Dicer 4 activity and relatively more Dicer 2 activity. Thus, the hpEIN2[G:U] transgene may behave like a normal, non-RNAi transgene (such as an over-expression transgene) and the promoter methylation observed in some of the lines was due to T-DNA insertion patterns rather than the inherent inverted-repeat DNA structure of a hpRNA transgene.

Example 14. Modified hairpins for reducing expression of another endogenous gene

Genetic constructs for production of modified silencing RNAs, either for hairpin RNAs or ledRNAs, targeting other endogenous genes were designed and synthesized. These included the following.

The FANCM gene in *A. thaliana* and in *Brassica napus* encodes a Fanconi Anemia Complementation Group M (FANCM) protein, which is a DEAD/DEAH box RNA helicase protein, Accession Nos and NM_001333162 and XM_018659358. The

nucleotide sequence of the protein coding region of the cDNA corresponding to the *FANCM* gene of *A. thaliana* is provided in SEQ ID NO:31, and for *B. napus* in SEQ ID NO:32.

Genetic constructs were designed and made to express hairpin RNAs with or without C to T substitutions and an ledRNA targeting the *FANCM* gene in *A. thaliana* and in *Brassica napus*. A target region in the *A. thaliana* gene was selected: nucleotides 675-1174 (500 nucleotides) of SEQ ID NO:31. A target region in the *B. napus* gene was selected: nucleotides 896-1395 (500 bp) of SEQ ID NO:32. The constructs encoding the hairpin RNAs, using a wild-type sense sequence or a modified (G:U) sense sequence, were designed and assembled. Nucleotide sequences of the hpFANCM-At[wt], hpFANCM-At[G:U], hpFANCM-Bn[wt] and hpFANCM-Bn[G:U] constructs are provided in SEQ ID NOs:33-36. To make the G:U constructs, all cytosine bases in the sense sequences were replaced with thymine bases – 102/500 (providing 20.4% G:U basepairs) in the *A. thaliana* construct and 109/500 (21.8% G:U basepairs) in the *B. napus* construct. The longest stretch of contiguous canonical basepairing in the double-stranded region of the *B. napus* G:U modified hairpin was 17 basepairs, and the second longest 16 contiguous basepairs.

The *DDMI* gene in *B. napus* encodes a methyltransferase which methylates cytosine bases in DNA (Zhang et al., 2018). The nucleotide sequence of the protein coding region of the cDNA corresponding to the *DDMI* gene of *B. napus* in SEQ ID NO:37.

Genetic constructs were designed and made to express hairpin RNAs with or without C to T substitutions and an ledRNA targeting the *DDMI* gene in *Brassica napus*. Two non-contiguous target regions of the *B. napus* gene were selected: nucleotides 504-815 and 1885-2074 of SEQ ID NO:37, and were directly joined to make a chimeric sense sequence. The total length of the sense sequence was therefore 502 nucleotides. The constructs encoding the hairpin RNAs, using a wild-type sense sequence or a modified (G:U) sense sequence, were designed and assembled. Nucleotide sequences of the hpDDM1-Bn[wt] and hpDDM1-Bn[G:U] constructs are provided in SEQ ID NOs:38-39. To make the G:U construct, cytosines in the sense sequences were replaced with thymines – 106/502 (21.1% G:U basepairs) in the *B. napus* construct. The longest stretch of contiguous canonical basepairing in the double-stranded region of the G:U modified hairpin was 20 basepairs, and the second longest 15 contiguous basepairs.

For another construct targeting an endogenous gene, a genetic construct was designed to express a hairpin RNA with 95 C to T substitutions in the sense sequence,

out of 104 C's in the sense sequence of 350 nucleotides, providing for 95/350=27.1% G:U basepairs in the double-stranded region of the hairpin RNA. That is, not all of the C's in the sense sequence were replaced with T's. In particular, where a run of 3, 4 or 5 contiguous C's occurred in the sense sequence, only 1 or 2 of the three C's, or only 2 or 3 of four C's, or only 2, 3 or 4 of 5 contiguous C's, were replaced with T's. This provided for a more even distribution of G:U basepairs in the double-stranded RNA region. The longest stretch of contiguous canonical basepairing in the double-stranded region was 15 basepairs, and the second longest 13 contiguous basepairs.

A further construct was designed where one or two basepairs in every block of 4, 5, 6 or 7 nucleotides was modified with C to T or A to G substitutions. Where the wild-type sense sequence had a stretch of 8 or more nucleotides consisting of T's or G's, one or more nucleotides were substituted either in the sense strand to create a mismatched nucleotide within that block or a C to T or A to G substitution was made in the antisense strand, so as to avoid a double-stranded stretch of 8 or more contiguous canonical basepairs in the double-stranded region of the resultant hairpin RNA transcribed from the construct.

Example 15. Modified hairpins for reducing expression of genes in animal cells

To test modified silencing RNAs in animal cells, of the G:U basepaired form, the ledRNA form or combining the two modifications, a gene encoding an enhanced green fluorescent protein (EGFP) was used in the following experiments as a model target gene. The nucleotide sequence of the coding region for EGFP is shown in SEQ ID NO:40. A target region of 460 nucleotides was selected, corresponding to nucleotides 131-591 of SEQ ID NO:40.

A genetic construct designated hpEGFP[wt] was designed and made which expressed a hairpin RNA comprising, in order 5' to 3' with respect to the promoter for expression, an antisense EGFP sequence of 460 nucleotides which was fully complementary to the corresponding region (nucleotides 131-590) of the EGFP coding region, a loop sequence of 312 nucleotides derived in part from a GUS coding region (corresponding to nucleotides 802-1042 of the GUS ORF), and a sense EGFP sequence of 460 nucleotides which was identical in sequence to nucleotides 131-590 of the EGFP coding region. The sequence of the DNA encoding the hairpin RNA hpEGFP[wt] (SEQ ID NO:41) included a *NheI* restriction enzyme site at the 5' end and a *SalI* site at the 3' end to provide for cloning into the vector pCI (Promega Corporation). This vector was suitable for mammalian cell transfection experiments and would provide for expression from the strong CMV promoter/enhancer. The construct also had a T7 promoter

sequence inserted between the *NheI* site and the beginning of the antisense sequence to provide for *in vitro* transcription to produce the hairpin RNA using T7 RNA polymerase. The hairpin encoding cassette was inserted into the *NheI* to *SalI* site in the expression vector pCI whereby the RNA coding region was operably linked to the
5 CMV promoter and the SV40-late polyadenylation/transcription termination region.

A corresponding hairpin construct which had 157 C to T substitutions in the sense sequence and no substitutions in the antisense sequence was designed and made, designated hpEGFP[G:U] (SEQ ID NO:42). The target region in the EGFP coding region was nucleotides 131-590. The percentage of C to T substitutions and therefore
10 G:U basepairs in the stem of the hairpin RNA was $157/460 = 34.1\%$. The sense and antisense sequences were identical in length at 460 nucleotides. In the art of gene silencing, long double-stranded RNAs are generally avoided because of the potential for activating cellular response including interferon activation.

An ledRNA construct designated ledEGFP[wt] was designed and made to
15 express an ledRNA comprising, in order 5' to 3' with respect to the promoter for expression, an antisense EGFP sequence of 228 nucleotides which was fully complementary to nucleotides 131-358 of the EGFP coding sequence, a loop sequence of 150 nucleotides, a sense EGFP sequence of 460 nucleotides which was identical in sequence to nucleotides 131-590 of the EGFP coding region (SEQ ID NO:40), a loop
20 sequence of 144 nucleotides, and an antisense sequence of 232 nucleotides which was fully complementary to nucleotides 359-590 of the EGFP coding sequence, flanked by *NheI* and *SalI* restriction sites (SEQ ID NO:43). The encoded ledRNA was therefore of the type shown in Figure 1A. The ledRNA structure, when self-annealed by basepairing between the one sense and two antisense sequences, had a double-stranded region of
25 460 basepairs corresponding to the EGFP target region, with the two antisense sequences not directly joined covalently to each other but having a "gap" or "nick" between the ends corresponding to nucleotides 358 and 359. The ledRNA structure was embedded in a larger RNA transcript including 5'- and 3'-regions coming from sequences in the CMV promoter and SV40-late polyadenylation/transcription
30 termination regions.

A corresponding ledRNA construct which had 162 C to T substitutions in the sense sequence and no substitutions in the antisense sequence was also designed and made, designated ledEGFP[G:U] (SEQ ID NO:44). In each case, the target region in the EGFP coding region was nucleotides 131-590 relative to the protein coding region
35 starting with the ATG start codon (SEQ ID NO:40). The percentage of C to T

substitutions and therefore G:U basepairs in the stem of the ledRNA was 162/460= 35.2%.

Plasmids encoding the hpEGFP[wt], hpEGFP[G:U], ledEGFP[wt] and ledEGFP[G:U] silencing RNAs were tested for gene silencing activity in CHO, HeLa and VERO cells by transfection of the vectors into the cells. The assays were conducted by co-transfection of the test plasmids with a GFP expressing plasmid. All assays were conducted in triplicate. CHO cells (Chinese Hamster Ovary cells) and VERO cells (African Green monkey kidney cells) were seeded into 24 well plates at a density of 1×10^5 cells per well. CHO cells were grown in MEM α modification (Sigma, USA), and HeLa and VERO cells were grown in DMEM (Invitrogen, USA). Both base media were supplemented with 10% foetal bovine serum, 2 mM glutamine, 10 mM Hepes, 1.5 g/L sodium bicarbonate, 0.01% penicillin and 0.01% streptomycin. Cells were grown at 37 °C with 5% CO₂. Cells were then transfected with 1 μ g per well with plasmid DNA, or siRNA as a control for EGFP silencing, using Lipofectamine 2000. Briefly, the test siRNA or plasmid was combined with the GFP reporter plasmid (pGFP N1) and then mixed with 1 μ l of Lipofectamine 2000, both diluted in 50 μ l OPTI-MEM (Invitrogen, USA) and incubated at room temperature for 20 mins. The complex was then added to cells and incubated for 4 hr. Cell media was replaced and the cells incubated for 72 hr. Cells were next subjected to flow cytometry to measure GFP silencing. Briefly, cells to be analysed were trypsinized, washed in PBSA, resuspended in 200 μ L of 0.01% sodium azide and 2% FCS in PBSA and analysed using a FACScalibur (Becton Dickinson) flow cytometer. Data analysis was performed using CELLQuest software (Becton Dickinson) and reported as mean fluorescence intensity (MFI) as a percentage of control cells with reporter and non-related (negative control) shRNA.

The anti-GFP siRNA referred to as si22 was obtained from Qiagen (USA). The anti-GFP siRNA sequence of si22 was sense 5'-GCAAGCUGACCCUGAAGUUCAU-3' (SEQ ID NO:86) and antisense 5'-GAACUUCAGGGUCAGCUUGCCG-3' (SEQ ID NO:87). A positive control genetic construct designated as pshGFP was created via a one-step PCR reaction using the mouse U6 sequence as the template. Forward primer was 5'-TTTTAGTATATGTGCTGCCG-3' (SEQ ID NO:88) and reverse primer was 5'-

CTCGAGTTCCAAAAAAGCTGACCCTGAAGTTCATCTCTCTTGAAGATGAAC TTCAGGGTCAGCCAAACAAGGCTTTTCTCCAA -3' (SEQ ID NO:89). An amplification product which included the full-length expression cassette was ligated into pGEM-T Easy. A non-related shRNA control plasmid was also constructed via the same PCR method. For that construction, the forward primer was 5'-

TTTTAGTATATGTGCTGCCG -3' (SEQ ID NO:90) and the reverse primer was 5'-ctcgagttccaaaaaataagtcgcagcagtagacaatctcttgaattgtactgctgcgacttatgaataccgcttctctctgag-3' (SEQ ID NO:91).

The resultant data from one experiment are shown in Figure 34. Clear reduction
5 in EGFP activity (RNA silencing) was observed in both VERO and CHO cells for both si22 and pshGFP positive controls when compared to the irrelevant shRNA control. These positive controls were a well validated small dsRNA molecule (si22) or encoded a shRNA (pshGFP) that were known to have very strong silencing activity in mammalian cells. The control RNA molecules have double-stranded regions of 20
10 contiguous basepairs and 21 contiguous basepairs, respectively, using only canonical basepairs and without any mismatched nucleotides in the double-stranded regions, and within the range of 20-30 basepairs long generally used for mammalian cells. In contrast, the hpRNA and ledRNA constructs express molecules having long dsRNA regions. All four constructs were observed to specifically silence EGFP expression to
15 significant extents in both cell types (Figure 34). The inclusion of the G:U substitutions gave a pronounced improvement in silencing for both constructs in CHO cells. In VERO cells, a pronounced improvement in silencing was only observed with the ledEGFP[G:U] construct relative to ledEGFP[wt].

In a second experiment using HeLa (human) cells and assaying EGFP activity
20 at 48 hr post-transfection, similar results were obtained (Figure 35).

It was significant to note that the gene silencing was observed in mammalian cells using the hpRNA and ledRNA effector molecules given that they had longer double-stranded regions than the conventional 20 to 30 bp size range. It was also clear that the modification to substitute nucleotides to create the G:U basepairs significantly
25 enhanced the gene silencing effect of these longer dsRNA molecules. This effect may be due to these structures more closely resembling endogenous priRNAs, the precursors of miRNAs, observed in eukaryotic cells and thus improving the processing of the longer dsRNA for loading into the RNA induced silencing complex (RISC) effector proteins.

30

Example 16. RNA constructs targeting *DDMI* and *FANCM* genes in plants

The inventors considered ways to increase the rate by which novel genetic profiles and diversity (genetic gain) could be generated and explored for desirable performance traits in plants. One way that was considered was to find a way to increase
35 the rate of recombination that occurs during sexual reproduction of plants. Plant breeders rely on recombination events to create different genetic (allelic) combinations

that they can search through for the desired genetic profile associated with performance gains. However, the number of recombination events in each breeding step is extremely low relative to the number of possible genetic profiles that could be explored. In addition, the elements that control where these events occur in the genome are not well understood. The inventors therefore considered whether ledRNA delivered either exogenously or endogenously through a transgenic approach could be used to modify recombination rates in plants to allow rapid increases in genetic diversity and make possible faster genetic gain within breeding populations.

The epigenome of plants is influenced by a range of different chemical modifications on the DNA and associated proteins that organize, package and stabilize the genome. These modifications also regulate where recombination takes place, with tight genome packaging being a strong inhibitor of recombination (Yelina et al, 2012; Melamed-Bessudo et al., 2012). DECREASED DNA METHYLATION 1 (DDM1) is an enzyme which regulates methylation of DNA and genome packaging. Mutation of this gene can alter the position of recombination events (Yelina et al, 2012; Melamed-Bessudo et al., 2012).

Recombination events during meiosis are tightly regulated with only 1-2 events occurring on each chromosome to ensure proper chromosome segregation at metaphase 1. Recombination events are initiated through double stranded breaks (DSB) of the DNA through the enzyme SPO11 (Wijnker et al, 2008). This results in hundreds of DSB along the chromosome. While a few of these DSB result in crossovers, the majority are repaired by DNA repair enzymes, before a recombination event can take place. Furthermore there are a number of negative regulators which inhibit DSB developing into crossovers. In an initial approach contemplated by the inventors, genetic constructs encoding ledRNA molecules or conventional hairpin RNA molecules as a comparison were to be introduced into *A. thaliana* plants, targeting a gene encoding a protein factor which could potentially impact recombination rates such as FANCONI ANEMIA COMPLEMENTATION GROUP M (FANCM).

The nucleotide sequence of the *DDM1* gene of *A. thaliana* was provided by Accession No. AF143940 (Jeddeloh et al., 1999). Reduction of *DDM1* gene expression has been shown to decrease DNA methylation and increase the number and position of cross over events in *A. thaliana* (Melamed-Bessudo and Levy, 2012).

Brassica napus is an allotetraploid species and has two *DDM1* genes on each of the A and C subgenomes, on chromosomes A7, A9, C7 and C9, therefore having a total of four *DDM1* genes. These genes are designated BnaA07g37430D-1, BnaC07g16550D-1, BnaA09g52610D-1 and BnaC09g07810D-1. The nucleotide

sequence of the *DDMI* gene BnaA07g37430D-1 of *B. napus* is provided by Accession No. XR_001278527 (SEQ ID NO:93). A hairpin RNA construct was designed and made targeting a 500 nucleotide region of the four genes, corresponding to nucleotides 650-959 and 2029-2218 of SEQ ID NO:93. The nucleotide region used to design the
5 hpRNA and ledRNA constructs targeted all four of the *DDMI* genes BnaA07g37430D-1, BnaC07g16550D-1, BnaA09g52610D-1 and BnaC09g07810D-1 present in *B. napus*, based on sequence conservation between the genes. The order of elements in the hpRNA construct was promoter-sense sequence-loop sequence comprising an intron from Hellsgate vector-antisense sequence-transcription terminator/polyadenylation
10 region. The nucleotide sequence of the chimeric DNA encoding the hpRNA is provided as SEQ ID NO:94.

A second hairpin RNA construct was made encoding a hairpin RNA targeting the same 500 nucleotide region and having the same structure except that 97 cytosine nucleotides (C) of the sense sequence were replaced with thymidine nucleotides (T).
15 When the chimeric DNA was transcribed and the G:U substituted hpRNA was self-annealed, this provided for $97/500 = 19.4\%$ of the nucleotides in the dsRNA region being basepaired in a G:U basepair. The nucleotide sequence of the chimeric DNA encoding the G:U-modified hpRNA is provided as SEQ ID NO:95. Further, a chimeric DNA encoding a ledRNA targeting the same region of the *DDMI* gene of *B. napus* was
20 made. The nucleotide sequence of this chimeric DNA encoding the ledRNA is provided as SEQ ID NO:96.

For production of the RNAs by *in vitro* transcription, DNA preparations were cleaved with the restriction enzyme *HincII* which cleaved immediately after the coding region, transcribed *in vitro* with RNA polymerase T7, the RNA purified and then
25 concentrated in an aqueous buffer solution. LedRNA was used to target endogenous *DDMI* transcripts in *B. napus* (canola) cotyledons. Cotyledons from five-day-old seedlings grown aseptically on tissue culture medium were carefully excised and placed in a petri dish containing 2ml MS liquid media, comprising 2% (w/v) sucrose, with 113µg of ledRNA or 100ul of aqueous buffer solution as a control. MS liquid media
30 used for the treatments contained Silwett-77, a surfactant (0.5µl in 60ml). The petri dishes were incubated on a shaker with gentle shaking, so that the cotyledons soaked in the solution containing the ledRNA. Samples were harvested 5hr and 7hr after application of the ledRNA. In a parallel experiment, the upper surface of cotyledons was coated either with 10µg of ledRNA or buffer solution and incubated on a wet
35 tissue paper. Samples were collected 7hr after ledRNA application.

Furthermore, in order to target the *DDMI* endogenous transcripts in reproductive tissue of *B. napus*, canola floral buds were exposed to ledRNA either in the presence or absence of an aliquot of an *Agrobacterium tumefaciens* strain AGL1 cell suspension, i.e. living AGL1 cells. Aqueous buffer solution with or without the AGL1 cells served as respective controls. The AGL1 was grown in 10ml of LB liquid media containing 25mg/ml rifampicin for two days at 28°C. The cells were harvested by centrifugation at 3000rpm for 5 minutes. The cell pellet was washed and the cells resuspended in 2ml liquid MS media. Floral buds were incubated in a petri dish containing 2ml of MS liquid media, including 0.5µl of Silwett-77 in 50ml of MS liquid media, with 62µg of ledRNA or 62µg+50µl of AGL1 culture. As controls, 50µl of buffer solution or 50µl of buffer solution+50µl of AGL1 culture was used. Samples were incubated on a shaker with gentle shaking for 7hr. Three biological replicates were used for each of the treatments.

The treated and control cotyledons and floral buds were washed twice in sterile distilled water, the surface water removed using a tissue paper and flash frozen with liquid nitrogen. RNA was isolated from the treated and control tissues, treated with DNase to remove genomic DNA and quantified. First strand cDNA was synthesized using equal amounts of total RNA from ledRNA-treated samples and their respective controls. Expression of *DDMI* was analysed using quantitative real-time PCR (qRT-PCR).

In the treated cotyledons that were soaked with the ledRNA, *DDMI* transcript abundance was decreased by approximately 83-86% at 5hr, which decreased further with a reduction of 91% at 7hr compared to the controls. Similarly, a reduction of approximately 78-85% in the *DDMI* mRNA level compared to the control was observed in cotyledons that were coated with ledRNA. No difference in *DDMI* mRNA abundance was detected in the floral buds that were treated with ledRNA compared to control in the absence of *Agrobacterium* cells. However, a reduction of approximately 60-75% in *DDMI* transcript levels was observed in floral buds that were treated with ledRNA in presence of *Agrobacterium* compared to its respective control. No significant difference in *DDMI* transcript levels was detected when the control without *Agrobacterium* was compared with the control that had *Agrobacterium* showing that the *Agrobacterium* cells themselves were not causing the decrease in *DDMI* transcript. Taken together, these results indicated that the ledRNA was able to reduce endogenous *DDMI* transcript levels in both cotyledons and floral buds, while living *Agrobacterium* cells appeared to facilitate the ledRNA entry into the floral buds. Such accessibility of the ledRNA might also be achieved by physical means such as piercing the outer layers

of the floral buds, centrifugation or vacuum infiltration, or a combination of such methods.

Certain *Arabidopsis thaliana* mutants such as *zip4* mutants lack meiotic crossovers, causing mis-segregation of chromosome homologs and thus reduced fertility and leading to shorter siliques (fruit) that can be visually discriminated from that of the wild-type. The phenotype in *zip4* mutants can be reversed by reducing *FANCM* gene expression.

The nucleotide sequence of the *FANCM* gene of *A. thaliana* was provided by Accession No. NM_001333162 (SEQ ID NO:97). A hairpin RNA construct was designed and made targeting a 500 nucleotide region of the gene, corresponding to nucleotides 853-1352 of SEQ ID NO:97. The order of elements in the construct was promoter-sense sequence-loop sequence comprising an intron from Hellsgate vector-antisense sequence-transcription terminator/polyadenylation region. The nucleotide sequence of the chimeric DNA encoding the hpRNA is provided as SEQ ID NO:98. A second hairpin RNA construct was made encoding a similar hairpin RNA targeting the same 500 nucleotide region except that 102 cytosine nucleotides (C) of the sense sequence were replaced with thymidine nucleotides (T). When the chimeric DNA was transcribed and the resultant G:U substituted hpRNA self-annealed, this provided for 102/500 = 20.4% of the nucleotides in the dsRNA region being basepaired in a G:U basepair. The nucleotide sequence of the chimeric DNA encoding the G:U-modified hpRNA is provided as SEQ ID NO:99. Further, a chimeric DNA encoding a ledRNA targeting the same region of the *FANCM* gene of *A. thaliana* was made. The nucleotide sequence of this chimeric DNA encoding the ledRNA is provided as SEQ ID NO:100.

B. napus has one *FANCM* gene on each of its A and C subgenomes, designated BnaA05g18180D-1 and BnaC05g27760D-1. The nucleotide sequence of one of the *FANCM* genes of *B. napus* is provided by Accession No. XM_022719486.1; SEQ ID NO:101). A chimeric DNA encoding the hairpin RNA was designed and made targeting a 503 nucleotide region of the genes, corresponding to nucleotides 2847-3349 of SEQ ID NO:101. The order of elements in the construct was promoter-sense sequence-loop sequence comprising an intron from Hellsgate vector-antisense sequence-transcription terminator/polyadenylation region. The nucleotide sequence of the chimeric DNA encoding the hpRNA is provided as SEQ ID NO:102. A second hairpin RNA construct was made encoding a similar hairpin RNA targeting the same 503 nucleotide region except that 107 cytosine nucleotides (C) of the sense sequence were replaced with thymidine nucleotides (T). When the chimeric DNA was transcribed and the G:U substituted hpRNA self-annealed, this provided for 107/500 =

21.4% of the nucleotides in the dsRNA region being basepaired in a G:U basepair. The nucleotide sequence of the chimeric DNA encoding the G:U-modified hpRNA is provided as SEQ ID NO:103. Further, a chimeric DNA encoding a ledRNA targeting the same region of the *FANCM* gene of *B. napus* was made. The nucleotide sequence of this chimeric DNA encoding the ledRNA is provided as SEQ ID NO:104.

For production of the RNAs by *in vitro* transcription, DNA preparations were cleaved with the restriction enzyme *HincII* which cleaved immediately after the coding region, transcribed *in vitro* with RNA polymerase T7, the RNA purified and then concentrated in an aqueous buffer solution. LedRNA was used together with *Agrobacterium tumefaciens* AGL1 to target *FANCM* transcripts in pre-meiotic buds of a *zip4* mutant of *A. thaliana*. Siliques of the *zip4* mutant were shorter, readily observed visually, relative to wild-type siliques due to attenuated crossover formation, thus causing reduced fertility. Repressing *FANCM* in the *zip4* mutant has been shown to restore the fertility and restore silique length.

The *A. thaliana zip4* inflorescences containing the pre-meiotic buds were contacted with ledRNA targeting *FANCM* together with AGL1 or buffer solution with AGL1 as control, in each case in the presence of a surfactant, in this case Silwett-77. Once the seed setting was complete, the siliques developed from pre-meiotic buds were excised to determine the seed numbers. Among the 15 siliques from ledRNA-treated samples, two siliques displayed 10 seeds, one silique had 9 seeds, while the number of seeds in control siliques ranged from 3 to 6. These results indicated that the observed increase in seed number was due to the repression of *FANCM* transcript levels by the ledRNA, thereby resulting in an increased number of meiotic crossovers and increased fertility.

25

Example 17. RNA constructs for resistance to fungal disease

LedRNA targeting Mlo genes of barley and wheat

The fungal disease of cereal plants, powdery mildew, is caused by the ascomycete *Blumeria graminis* f. sp. *hordei* in barley and the related *Blumeria graminis* f. sp. *tritici* in wheat. *B. graminis* is an obligate biotrophic fungal pathogen of the order Erysiphales (Glawe, 2008) which requires a plant host for reproduction, involving a close interaction between fungal and host cells in order for the fungus to acquire nutrient from the plant. The fungus initially infects the epidermal layer of leaves, leaf sheaths or ears after fungal ascospores or conidia contact the surface. Leaves remain green and active for some time following infection, then powdery, mycelial masses grow and the leaves gradually become chlorotic and die off. As the

disease progresses, the fungal mycelium may become dotted with tiny black points which are the sexual fruiting bodies of the fungus. Powdery mildew disease has a worldwide distribution and is most damaging in cool, wet climates. The disease impacts grain yield mainly by reducing the number of heads as well as reducing kernel
5 size and weight. Currently, disease control is by spraying crops with fungicide which needs to be applied frequently when conditions are cool and damp, and is expensive, or by growing resistant cultivars. Moreover, fungicide resistance has emerged for powdery mildew in wheat in Australia.

The *Mlo* genes of barley and wheat encode Mlo polypeptides which confer
10 susceptibility to *B. graminis* by an unknown mechanism. There are multiple, closely related MLO proteins encoded by a *Mlo* gene family which are unique to plants. Each gene encodes a seven-transmembrane domain protein of unknown biochemical activity localized in the plasma membrane. Significantly, only specific *Mlo* genes within the family are capable of acting as powdery mildew susceptibility genes and these encode
15 polypeptides with conserved motifs within the cytoplasmic C-terminal domain of the Mlo proteins. The mechanism by which Mlo polypeptides act as powdery mildew susceptibility factors is unknown. Occurrence of natural wheat *mlo* mutants has not been reported, presumably because of the polyploid nature of wheat. However, artificially generated *mlo* mutants show some resistance to the disease but often exhibit
20 substantially reduced grain yield or premature leaf senescence (Wang et al., 2014; Acevedo-Garcia et al., 2017).

Hexaploid wheat has three homoeologs of Mlo genes, designated as *TaMlo-A1*, *TaMlo-B1* and *TaMlo-D1* located on chromosomes 5AL, 4BL and 4DL respectively (Elliott et al., 2002). Nucleotide sequences of cDNAs corresponding to the genes are
25 available as Accession Nos: *TaMlo-A1*, AF361933 and AX063298; *TaMlo-B1*, AF361932, AX063294 and AF384145; and *TaMlo-D1*, AX063296. The nucleotide sequences of the genes on the A, B and D genomes and the amino acid sequences of the encoded polypeptides are approximately 95-97% and 98% identical, respectively. All three genes are expressed in leaves of the plants with the expression levels increasing
30 as the plants grow and mature. The inventors therefore designed and made a ledRNA construct which would be capable of reducing expression of all three genes, taking advantage of the degree of sequence identity between the genes and targeting a gene region with high degree of sequence conservation.

A chimeric DNA encoding a ledRNA construct targeting all three of the *TaMlo-*
35 *A1*, *TaMlo-B1* and *TaMlo-D1* genes was made. The genetic construct was made using

the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the contiguous sequence being the sense sequence (Figure 1A).

A 500 bp nucleotide sequence of a *TaMlo* target gene was selected, corresponding to nucleotides 916-1248 fused with 1403-1569 of SEQ ID NO:136. The dsRNA region of each ledRNA was 500 bp in length; the sense sequence in the dsRNA region was an uninterrupted, contiguous sequence, for example corresponded to nucleotides 916-1248 fused with 1403-1569 of SEQ ID NO:136. The nucleotide sequence encoding the ledRNA is provided herein as SEQ ID NO:137.

The ledRNA was prepared by *in vitro* transcription using T7 RNA polymerase, purified and resuspended in buffer. 10µg of ledRNA per leaf was applied using a paint brush to a zone of leaves in wheat plants at the Zadoks 23 stage of growth. As controls, some leaves were mock-treated using buffer alone. Treated and control leaf samples were harvested and RNA extracted. QPCR assays on the extracted RNAs showed that *TaMlo* mRNA levels, being a combination of the three *TaMlo* mRNAs, were reduced by 95.7%. Plants at the Z73 stage of growth were also treated and assayed. They showed a 91% reduction in *TaMlo* gene expression by QPCR relative to the control leaf samples. The reduction in *TaMlo* gene expression observed in the treated leaf areas was specific to the treated zones – there was no reduction in *TaMlo* mRNA levels in distal, untreated parts of the leaves.

In barley *mlo* mutants, expression of a variety of disease defence-related genes was observed to be increased. Therefore, the ledRNA-treated wheat leaves were assayed by QPCR for the levels of defence related genes encoding PR4, PR10, β-1,3-glucanase, chitinase, germin and ADP-ribosylation factor. None of these genes were altered significantly in expression level in the treated leaf areas relative to the control leaf areas.

To test for ability of the ledRNA to increase disease resistance by reducing *Mlo* gene expression, spores of the powdery mildew fungus were applied to the treated and untreated zones of the leaves. Leaves were detached from wheat plants, treated with the ledRNA as before and maintained on medium (50mg Benzimidazole and 10g agar per Litre of water) to prevent the leaves from senescencing, under light. Twenty-four hours later, the leaves were inoculated with powdery mildew spores and disease progression followed for 5 to 24 days. Treated leaves showed little to no fungal mycelium growth and no leaf chlorosis relative to control leaves, not having received the ledRNA, which showed extensive mycelial growth surrounded by chlorotic zones.

In further experiments, lower levels of the ledRNA were applied to identify the minimal level of the ledRNA that was effective. Application of RNA in concentrations

as low as 200ng/μl (2μg per leaf total) showed significant suppression of powdery mildew lesions in the current formulations, suggesting the amount of inhibitory RNA could be substantially reduced while still providing suppression of fungal growth and development. Further, leaves were inoculated 1, 2, 4, 7 and 14 days after the ledRNA
5 treatment to see how long the protective effect remained. Effective silencing of the endogenous gene was observed throughout the time course from the first time point at 24 hours after treatment until the last time point at 14 days after treatment when the endogenous genes still showed 91% reduction in expression. Whole plants will also be sprayed with ledRNA preparations and tested for disease resistance after being
10 inoculated with the fungal disease agent.

LedRNA targeting VvMLO genes of Vitis vinifera

The *MLO* genes of *Vitis vinifera* and *Vitis pseudoreticulata* encode *MLO* polypeptides which confer susceptibility to the fungal disease powdery mildew, caused
15 by the ascomycete fungus, *Erysiphe necator*. *E. necator* is an obligate biotrophic fungal pathogen which requires a plant host for reproduction, involving a close interaction between fungal and host cells in order for the fungus to acquire nutrient from the plant. There are multiple, closely related *MLO* proteins encoded by a gene family all of which are unique to plants and encode seven-transmembrane domain proteins of unknown
20 biochemical activity localized in the plasma membrane. Significantly, only specific *MLO* genes within the family are capable of acting as powdery mildew susceptibility genes and these encode polypeptides with conserved motifs within the cytoplasmic C-terminal domain of the *MLO* proteins. The mechanism by which *MLO* polypeptides act as powdery mildew susceptibility factors is unknown.

25 LedRNA constructs targeting three different but related *MLO* genes of *Vitis* species, namely *VvMLO3*, *VvMLO4* and *VvMLO17* (nomenclature according to Feechan et al., Functional Plant Biology, 2008, 35: 1255-1266) were designed and made as follows. For the first one, for example, a 860 nucleotide sequence of a *VvMLO3* target gene was selected, corresponding to nucleotides 297-1156 of SEQ ID
30 NO:138. Chimeric DNAs encoding three ledRNA constructs targeting *VvMLO3*, *VvMLO4* and *VvMLO17* genes were made. The genetic constructs were made using the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the contiguous sequence being the sense sequence (Figure 1A). The dsRNA region of each ledRNA was 600bp in length; the sense sequence in the
35 dsRNA region was an uninterrupted, contiguous sequence, for example corresponded

to nucleotides 427-1156 of SEQ ID NO:138. The nucleotide sequence encoding one of the ledRNAs is provided herein as SEQ ID NO:139.

The ledRNAs are prepared by *in vitro* transcription and applied, separately or as a mixture of all three, to leaves of *Vitis vinifera* plants, variety Cabernet Sauvignon.

- 5 Subsequently, spores of the powdery mildew fungus are applied to the treated and untreated zones of the leaves. Reduction in the levels of the target mRNAs was observed using quantitative RT-PCR. Disease progression is followed over time. Substantial down-regulation of VvMlo4 was observed from application of ledRNA solution at 1µg/ml targeting VvMlo3, VvMlo4 or VvMlo11.

10

LedRNA targeting fungal genes

- LedRNA constructs were designed against the coding region of the Cyp51 gene of the fungal pathogen *Rhizoctonia solani*, a gene which is required for synthesis of ergosterol and survival and growth of the fungus. The genetic constructs were made using the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the contiguous sequence being the sense sequence (Figure 1A). A single ledRNA construct was designed to target two genes from *R. solani* with the dsRNA region of the ledRNA containing 350bp from each gene; the sense sequence in the dsRNA region was an uninterrupted, contiguous sequence, for example corresponded to nucleotides 884-1233 of SEQ ID NO:140 and nucleotides 174-523 of SEQ ID NO:141. The nucleotide sequence encoding one of the ledRNAs is provided herein as SEQ ID NO:142. The ledRNAs were prepared by *in vitro* transcription and applied to culture medium at a concentration of 5µg per 100µl culture with an inoculum of *R. solani* mycelium. Growth of the fungus was measured at time zero and each day over the following week by reading the optical density of the culture at 600nm. The growth of *R. solani* in cultures containing the ledRsCyp51 was significantly less than the control cultures containing either RNA buffer or the control ledGFP for which there is no corresponding target in *R. solani*.

- A ledRNA-encoding construct was also designed and made against the coding region of the *CesA3* cellulose synthase gene in *Phytophthora cinnamomi* isolate 94.48. The genetic constructs were made using the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the contiguous sequence being the sense sequence (Figure 1A). A ledRNA construct was designed to target the *CesA3* gene of *Phytophthora cinnamomi* with the dsRNA region of the ledRNA containing 500bp from the coding region of the gene; the sense sequence in the dsRNA region was an uninterrupted, contiguous sequence, for example

corresponded to nucleotides 884-1233 of SEQ ID NO:143. The nucleotide sequence encoding one of the ledRNAs is provided herein as SEQ ID NO:144. The ledRNA was transcribed *in vitro* and applied to culture media at a rate of 3µg per 100µl culture. Substantial loss of directional mycelial growth was observed in cultures with the
5 ledRNA targeting PcCesA3 compared to mock treated (RNA buffer only) or ledGFP treated cultures. The loss of directional growth and resulting amorphous, bulbous growth pattern was reminiscent of cells with disruption of cell wall biosynthesis and thus was consistent with silencing of the PcCesA3 gene.

10 **Example 18. RNA constructs targeting other genes in plants**

LedRNAs targeting Tor genes of *A. thaliana* and *N. benthamiana*

The Target of Rapamycin (*TOR*) gene encodes a serine-threonine protein kinase polypeptide that controls many cellular functions in eukaryotic cells, for example in response to various hormones, stress and nutrient availability. It is known as a master
15 regulator that regulates the translational machinery to optimise cellular resources for growth (Abraham, 2002). At least in animals and yeast, TOR polypeptide is inactivated by the antifungal agent rapamycin, leading to its designation as Target of Rapamycin. In plants, TOR is essential for embryonic development in the developing seed, as shown by the lethality of homozygous mutants in *TOR* (Mahfouz et al., 2006), as well
20 as being involved in the coupling of growth cues to cellular metabolism. Down-regulation of *TOR* gene expression was thought to result in an increase in fatty acid synthesis resulting in increased lipid content in plant tissues.

LedRNA constructs targeting a *TOR* gene of *Nicotiana benthamiana*, the nucleotide sequence of the cDNA protein coding region is provided as SEQ ID
25 NO:105, were designed and made using the design principles for ledRNAs with the split sequence being the sense sequence and the contiguous sequence being the antisense sequence (Figure 1B). The target region was 603 nucleotides in length, corresponding to nucleotides 2595-3197 of SEQ ID NO:105. The dsRNA region of the ledRNA was 603bp in length; the antisense sequence in the dsRNA region was an
30 uninterrupted, contiguous sequence corresponded to the complement of nucleotides 2595-3197 of SEQ ID NO:105. The nucleotide sequences encoding the ledRNA is provided herein as SEQ ID NO:106. DNA preparations of the genetic constructs encoding the ledRNA constructs were cleaved with the restriction enzyme *MlyI* which cleaved the DNA immediately after the coding region, transcribed *in vitro* with RNA
35 polymerase SP6 and the RNA purified and then concentrated in an aqueous buffer solution. Samples of the ledRNA were applied to the upper surface of *N. benthamiana*

leaves. After 2 days and 4 days, the treated leaf samples were harvested, dried, and the total fatty acid content measured by quantitative gas chromatography (GC). The leaf samples treated with the TOR ledRNAs showed an increase in total fatty acid (TFA) content from 2.5-3.0% (weight of TFA/dry weight) observed in the control (untreated) samples to between 3.5-4.0% for the ledRNA treated samples. That represented an increase of between 17% and 60% in the TFA content relative to the control, indicating that the *TOR* gene expression had been reduced in the ledRNA treated tissues.

LedRNA targeting *ALS* gene of *H. vulgare*

10 Acetolactate synthase (*ALS*) genes encode an enzyme (EC 2.2.1.6) found in plants and microorganisms which catalyse the first step in the synthesis of the branched chain amino acids leucine, valine and isoleucine. The *ALS* enzyme catalyses the conversion of pyruvate to acetolactate which is then further converted to the branched chain amino acids by other enzymes. Inhibitors of *ALS* are used as herbicides such as the sulfonylurea, imidazolinone, triazolopyrimidine, pyrimidinyl oxybenzoate and sulfonylamino carbonyl triazolinones classes of herbicides.

To test whether a ledRNA could reduce *ALS* gene expression by exogenous delivery of the RNA to plants, a genetic construct encoding a ledRNA was designed and made that targeted an *ALS* gene in barley, *Hordeum vulgare*. The *H. vulgare ALS* gene sequence is provided herein as SEQ ID NO:107 (Accession No. LT601589). The genetic construct was made using the design principles for ledRNAs, with the split sequence being the sense sequence and the contiguous sequence being the antisense sequence (Figure 1B). The target region was 606 nucleotides in length, corresponding to nucleotides 1333-1938 of SEQ ID NO:107. The dsRNA region of the ledRNA was 606bp in length; the antisense sequence in the dsRNA region was an uninterrupted, contiguous sequence corresponded to the complement of nucleotides 1333-1938 of SEQ ID NO:107. The nucleotide sequences encoding the ledRNA is provided herein as SEQ ID NO:108. The coding region was under the control of a SP6 RNA polymerase promoter for *in vitro* transcription.

30 The genetic construct encoding the ledRNA was digested with the restriction enzyme *MlyI*, which cleaved downstream of the ledRNA coding region, and transcribed *in vitro* with RNA polymerase SP6 according to the instructions with the transcription kit. The RNA was applied on the upper surface of leaves of barley plants. RNA was extracted from the treated leaf samples (after 24 hours). Quantitative reverse transcription-PCR (QPCR) assays were carried out on the RNA samples. The assays showed that the level of *ALS* mRNA was reduced in the ledRNA treated tissues.

(Total RNA was extracted for treated and untreated plants, DNase treated, quantified and 2 ug reverse transcribed using primer CTTGCCAATCTCAGCTGGATC (SEQ ID NO:229). The cDNA was used as template for quantitative PCR using the forward primer TAAGGCTGACCTGTTGCTTGC (SEQ ID NO:230) and reverse primer
 5 CTTGCCAATCTCAGCTGGATC (SEQ ID NO:229). ALS mRNA expression was normalised against the *Hordeum chilense* isolate H1 lycopene-cyclase gene. ALS expression was reduced by 82% in LED treated plants.

LedRNAs targeting *NCED1* and *NCED2* genes of wheat and barley

10 In plants, the plant hormone abscisic acid (ABA) is synthesized from carotenoid precursors with the first committed step in the synthesis pathway being catalyzed by the enzyme 9-cis epoxy-carotenoid dioxygenase (NCED) which cleaves 9-cis xanthophylls to xanthoxin (Schwartz et al., 1997). The hormone ABA is known to promote dormancy in seeds (Millar et al., 2006) as well as being involved in other processes
 15 such as stress responses. Increased expression of an NCED gene was thought to increase ABA concentration and thereby promote dormancy. There are two NCED isoenzymes in cereals such as wheat and barley, designated NCED1 and NCED2, encoded by separate, homologous genes.

For breakdown of ABA, the enzyme ABA-8-hydroxylase (ABA8OH-2, also
 20 known as CYP707A2) hydroxylates ABA as a step in its catabolism, resulting in the breaking of dormancy and seed germination.

LedRNA constructs targeting genes encoding HvNCED1 (Accession No. AK361999, SEQ ID NO:109) or HvNCED2 (Accession No. AB239298; SEQ ID NO:110) in barley *Hordeum vulgare* and the corresponding homologous genes in wheat
 25 were designed for transgenic expression in barley and wheat plants. These constructs used a highly conserved region of the wheat and barley *NCED1* and *NCED2* genes, the wheat and barley nucleotide sequences being about 97% identical in the conserved region. The genetic constructs were made using the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the
 30 contiguous sequence being the sense sequence (Figure 1A). The target region was 602 nucleotides in length, corresponding to nucleotides 435-1035 of SEQ ID NO:109. The dsRNA region of the ledRNA was 602bp in length; the sense sequence in the dsRNA region was an uninterrupted, contiguous sequence corresponded to nucleotides 435-1035 of SEQ ID NO:110. The nucleotide sequences encoding the NCED1 and NCED2
 35 ledRNAs are provided herein as SEQ ID NO:111 and 112.

In similar fashion, an ledRNA construct was made targeting an ABA-OH-2 gene of wheat *T. aestivum* and barley *H. vulgare* (Accession No. DQ145933, SEQ ID NO:113). The target region was 600 nucleotides in length, corresponding to nucleotides 639-1238 of SEQ ID NO:113. The dsRNA region of the ledRNA was 600bp in length; 5 the sense sequence in the dsRNA region was an uninterrupted, contiguous sequence corresponded to nucleotides 639-1238 of SEQ ID NO:113. The nucleotide sequence of the chimeric DNA encoding the ledRNA is provided as SEQ ID NO:114.

The chimeric DNAs encoding the ledRNAs were inserted into an expression vector under the control of a *Ubi* gene promoter that is expressed constitutively in most 10 tissues including in developing seed. The expression cassettes were excised and inserted into a binary vector. These were used to produce transformed wheat plants.

The transgenic wheat plants are grown to maturity, seed obtained from them and analysed for decreased expression of the *NCED* or *ABA-OH-2* genes and for effects on grain dormancy corresponding to decreased gene expression. A range of phenotypes in 15 the extent of altered dormancy is expected. To modulate the extent of the altered phenotypes, modified genetic constructs are produced for expression of ledRNAs having G:U basepairs in the double-stranded RNA regions, particularly for ledRNAs where between 15-25% of the nucleotides in the double-stranded region of the ledRNA are involved in a G:U basepair, as a percentage of the total number of nucleotides in the 20 double-stranded region.

LedRNA targeting EIN2 gene of *A. thaliana*

As described in Example 10, the *EIN2* gene of *Arabidopsis thaliana* encodes a receptor protein involved in ethylene perception. *EIN2* mutant seedlings exhibit 25 hypocotyl elongation relative to wild-type seedlings when germinated on ACC. Since the gene is expressed in seedlings soon after germination of seeds, delivery of a ledRNA by transgenic means was considered the most suitable approach for tested the extent of down-regulation of *EIN2*, relative to exogenous delivery of preformed RNA.

An ledRNA construct targeting the *EIN2* gene of *Arabidopsis thaliana* (SEQ ID 30 NO:115) was designed, targeting a 400 nucleotide region of the target gene mRNA. The construct is made by inserting a sequence (SEQ ID NO:116) encoding the ledRNA into a vector comprising a 35S promoter to express the ledRNA in *A. thaliana* plants. Transgenic *A. thaliana* plants are produced and tested for reduction of expression of the *EIN2* gene by QPCR and for the hypocotyl length assay in the presence of ACC. 35 Reduction in *EIN2* expression levels and increased hypocotyl lengths are observed in plants of some transgenic lines.

LedRNA targeting CHS gene of *A. thaliana*

The chalcone synthase (CHS) gene in plants encodes an enzyme that catalyzes the conversion of 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone which is the first committed enzyme in flavonoid biosynthesis. Flavonoids are a class of organic compounds found mainly in plants, involved in defense mechanisms and stress tolerance.

An ledRNA construct targeting the *CHS* gene of *Arabidopsis thaliana* (SEQ ID NO:117) was designed, targeting a 338 nucleotide region of the target gene mRNA. The construct is made by inserting a DNA sequence (SEQ ID NO:118) encoding the ledRNA into a vector comprising a 35S promoter to express the ledRNA in *A. thaliana* plants. Transgenic *A. thaliana* plants are produced by transformation with the genetic construct in a binary vector and tested for reduction of expression of the *CHS* gene by QPCR and for the reduced flavonoid production. Reduction in *CHS* expression levels and reduced levels of flavonoids are observed in plants of some transgenic lines, for example in the seed coat of transgenic seeds.

LedRNA targeting LanR gene of *Lupinus angustifolius*

The *LanR* gene of narrow-leafed lupin, *Lupinus angustifolius* L., encodes a polypeptide that is related in sequence to the tobacco *N* gene, which confers resistance to viral disease caused by tobacco mosaic virus (TMV).

A chimeric DNA for producing ledRNA molecules targeting the *LanR* gene of *L. angustifolius* (Accession No. XM_019604347, SEQ ID NO:119) was designed and made. The genetic construct was made using the design principles for ledRNAs described above, with the split sequence being the antisense sequence and the contiguous sequence being the sense sequence (Figure 1A). The nucleotide sequence encoding the ledRNA is provided herein as SEQ ID NO:120. The ledRNA was produced by *in vitro* transcription, purified and concentrated, and aliquots of the RNA are applied to leaves of *L. angustifolius* plants which contain the *LanR* gene. Samples of virus are applied to treated and non-treated plants, and disease symptoms compared after several days.

Example 19. RNA constructs targeting an insect gene

Introduction

Aphids are sap-sucking insects that cause substantial and at times severe damage to plants directly through feeding of plant sap and, in some cases, indirectly through

transmitting various viruses that cause disease in the plants. While Bt toxin has in some instances been effective in protecting crop plants from chewing insects, it generally hasn't been effective for sap-sucking insects. Use of plant cultivars that contain resistance genes can be an effective way to control aphids. However, most resistance

5 genes are highly specific to certain aphid species or biotypes and resistance is frequently over-come due to rapid evolution of new biotypes through genetic or epigenetic changes. Moreover, resistance genes are not accessible in many crops or may not exist for certain generalist aphid species such as green peach aphid which

10 application of pesticides which has led to pesticide resistance in aphids. For example, only one pesticide mode of action group remains effective in Australia against the green peach aphid as it has managed to gain resistance to all the other registered insecticides.

RNAi-mediated gene silencing has been shown in a few studies to be useful as a

15 research tool in a number of aphid species, for reviews see Scott et al., 2013; Yu et al., 2016, but has not been shown to effectively protect plants from aphid attack. In those studies, dsRNAs targeting key genes involved in aphid growth and development, infestation or feeding processes were delivered through direct injection to the aphids or by feeding the aphids on artificial diets containing the dsRNA.

20 To test the potential of modified RNAi molecules such as the ledRNA molecules described herein for the control of sap-sucking insects, the inventors selected green peach aphid (*Myzus persicae*) as a model sap-sucking insect, for several reasons. Firstly, green peach aphid is a polyphagous insect which infests a broad range of host plant species including major grain and horticultural crops worldwide. Secondly, green

25 peach aphid is responsible for the transmission of some devastating viruses, such as Beet Western Yellows Virus which has been highly damaging in some canola growing areas. Two aphid genes were initially selected for this study as target genes for down-regulation, one encoding a key effector protein (C002) and the second encoding a receptor of activated protein kinase C (Rack-1). The C002 protein is an aphid salivary

30 gland protein which is essential for aphid feeding on its host plant (Mutti et al., 2006; Mutti et al., 2008). Rack1 is an intracellular receptor that binds activated protein kinase C, an enzyme primarily involved in signal transduction cascades (McCahill et al., 2002; Seddas et al., 2004). MpC002 is predominantly expressed in the aphid salivary gland and MpRack1 is predominantly expressed in the gut. In previous studies, use of RNAi

35 via direct injection or artificial diet feeding led to the death of several aphid species tested (Pitino et al., 2011; Pitino and Hogenhout, 2012; Yu et al., 2016).

Materials and Methods: Aphid culture and plant materials

Green peach aphids (*Myzus persicae*) were collected in Western Australia. Before each experiment, aphids were reared on radish plants (*Raphanus sativus* L.)
5 under ambient light in an insectary room. Aphids were transferred to experimental artificial diet cages with a fine paintbrush.

The components of the artificial diet for the aphid feeding were the same as described in Dadd and Mittler (1966). The apparatus used for the aphid artificial diet used a plastic tube with 1cm diameter and 1cm height. The artificial aphid diet, 100 μ l
10 with or without ledRNA, was enclosed between two layers of parafilm to create a diet sachet. On top of that sachet, there was a chamber for the aphids to move around and feed from the diet by piercing their stylets through the top layer of the stretched parafilm. Eight first- or second-instar nymphs were gently transferred to the aphid chamber using a fine paint brush. The experiment was carried out in a growth cabinet at
15 20°C.

The tobacco and radish leaves used in one experiment were collected from plants grown in soil under 16hr light/8hr dark cycle at 22°C. With the experiments involving excised radish leaves, a small radish leaf (2-4cm²) attached to a fragment of stem (~2cm long) was excised. To keep the leaf fresh, the stem was inserted into
20 medium comprising 1.5g Bacto Agar and 1.16g Aquasol per 100ml water in a petri dish of 5cm diameter. Aphids were transferred to the leaves with a fine painting brush. The petri dishes with the leaves and aphids were kept in a growth cabinet under 16hr light/8hr dark cycle at 20 °C.

Double strand RNA (dsRNA) was prepared by *in vitro* RNA transcription of
25 DNA templates comprising one or more T7 promoters and T7 RNA polymerase using standard methods.

MpC002 and MpRack-1 genes and LedRNA constructs

The green peach aphid *MpC002* and *MpRack-1* genes tested as target genes
30 were the same as described by Pitino et al. (2011; 2012). The DNA sequences of both genes were obtained from the NCBI website, *MpC002* (>MYZPE13164_0_v1.0_000024990.1 | 894 nt) and *MpRack-1* (>MYZPE13164_0_v1.0_000198310.1 | 960 nt). The cDNA sequences of the two genes are provided herein as SEQ ID NOs: 123 and 124. LedRNA constructs were
35 designed in the same manner as described in earlier Examples. The DNA sequences encoding the ledRNA molecules are provided herein as SEQ ID NOs:125 and 126 were

used as transcription templates to synthesize the ledRNA. The vector DNAs encoding the ledRNA molecules targeting the *MpC002* and *MpRack-1* genes were introduced into *E. coli* strain DH5 α for preparing plasmid DNA for *in vitro* RNA transcription and into *E. coli* strain HT115 for *in vivo* (in bacteria) transcription.

5

Efficacy of ledRNA molecules on the reduction of aphid performance

To examine if the ledRNAs targeting the *MpC002* or *MpRack-1* genes affected aphid performance, each ledRNA was delivered to the aphids through the artificial diet means as described in Example 1. In each experiment, ten biological replicates were set up; each biological replicate had eight one- to two-instar nymphs of green peach aphid. The controls in each experiment used equivalent concentrations of an unrelated ledRNA, namely ledGFP.

At a lower concentration of 50 ng/ μ l of each ledRNA molecule, aphid survival after feeding from the artificial diet containing either *MpC002* or *MpRack-1* ledRNA was not significantly different from the control ledGFP. However, the ledRNA targeting the *MpC002* gene significantly ($P < 0.05$) reduced the reproduction rate of green peach aphids (Figure 37A). The average number of nymphs produced per adult aphid was reduced by about 75% compared to the number of nymphs produced from adults maintained on the control diet having the control ledRNA. At a higher concentration of 200 ng/ μ l, the ledRNAs targeting either *MpC002* or *MpRack-1* increased adult aphid mortality (Figure 37B). The reduction of aphid survival on the diets including the *MpC002* or *MpRack-1* ledRNAs was also observed after 24 hours and continued over the five-day period of the experiment. The results indicated that use of the ledRNAs targeting the essential aphid genes was able to cause the death of aphids and reduce aphid reproduction. The efficacy of each ledRNA was compared to double-stranded RNA molecules (dsRNAi) comprised of separate but annealed sense and antisense RNA strands targeting the same region of the target gene.

Uptake of ledRNA molecules by aphids

To track the uptake and distribution of the ledRNAs inside the aphids, the ledRNAs targeting the *MpC002* or *MpRack-1* genes were labelled with Cy3 (Cyanine-dye labelled nucleotide triphosphates) during the synthesis process as described in Example 1. The Cy3 labelling has been reported to have no effect on the biological function of conventional dsRNA molecules and so could be used as a label for detection by fluorescence. Aphids which had been fed the labelled ledRNAs were examined using confocal microscopy using a Leica EL 6000 microsystems instrument.

The Cy3-labelled ledRNA targeting *MpC002* or *MpRack-1* was detectable in aphid guts within hours of feeding on the artificial diet and subsequently in the reproduction system and even in newborn nymphs which were the progeny of the adults that had been fed. The results indicated that aphid genes critical for digestive system function or
5 reproduction could be effective targets for the ledRNA molecules through feeding.

LedRNA stability

To examine the stability of ledRNA in the diet and as recovered from the fed aphids, RNA was recovered from the artificial diet and from aphid honeydew after
10 feeding on the diets containing the labelled ledRNA molecules. The RNA samples were electrophoresed on gels and examined by fluorescence detection. The ledMpC002 RNA prior to feeding clearly displayed a single product of about 700bp on the agarose gel. The RNA recovered from the artificial diet showed a smear of RNA from 100-700bp in size, indicating some degradation after being exposed to the diet at room temperature
15 for 25 days, but still largely intact. RNA recovered from the aphid honeydew showed fluorescence in the RNA range from 350 to 700bp, so again was largely intact. Despite the degradation of some ledRNA, a large proportion of the ledRNA molecules was able to stay intact in the artificial diet and also in the aphid honeydew for a considerable period of time. This degree of stability of the ledRNA molecules should allow the
20 ledRNA to be active and retain activity when applied exogenously.

Absorbance of labelled ledRNA by plant leaves

The Cy3-labelled ledMpC002 RNA was painted on the upper surface of tobacco leaves in order to see if it was able to penetrate the leaf tissues. Ten microliters of Cy3-
25 labelled ledMpC002 (1 μ g/ μ l concentration) was painted in a circle of 2cm diameter and the applied region marked with a black marker pen. Images of leaf fluorescence at an excitation of 525nm were captured over a five hour period using a Leica EL 6000 microsystems instrument, comparing the painted tissues with those not painted. The Cy3 label was clearly detectable in mesophyll tissue within one hour after application,
30 so had clearly penetrated through the waxy cuticle layer on the leaf surface. The level of fluorescence increased at 2 hours and was maintained to the 5hr time point. It was not clear if the ledRNA molecules got into the cells or into the nuclei of the cells. However, as sap-sucking insects feed specifically from the phloem sieve elements of plant leaves and stems, RNA transmission into the plant cells was not required for the
35 silencing of aphid genes. The experiment indicated that the ledRNA molecules were found in the plant tissues through topical application.

Uptake of topical LedRNA by aphids

The Cy3-labelled ledGFP RNA was painted on radish leaves in order to see if aphids were able to uptake topically applied ledRNA from plants. Ten microliters of each Cy3-labelled ledGFP (10µg/µl concentration) was painted on a small excised radish leaf (~2cm²). The control leaf was painted with an equal amount of unlabelled ledGFP. The labelled and control radish leaves were each infested with eight aphids of various developmental stages. Images of leaf and aphid fluorescence were captured using the method described above for the tobacco leaves. While there was no detectable fluorescence in the control leaves and aphids, the leaf painted with Cy3 labelled ledGFP was highly fluorescent. Within 24 hours after feeding on the leaf with Cy3-labelled ledRNA, aphids showed strong fluorescence in the whole body but more pronounced in the guts and legs than other body parts. The experiment indicated that aphids were able to uptake the ledRNA molecules from plants through topical application.

Screening additional aphid RNAi target genes

In order to identify more aphid target genes, in total 16 aphid genes were evaluated for their suitability as RNAi targets. The candidate genes selected were involved in aphid development, reproduction, feeding or detoxification. Conventional dsRNA (dsRNAi) targeting each gene by comprising sense and antisense sequences corresponding to a region of target gene mRNA was supplemented to the aphid artificial diet at a concentration of 2µg RNA per µl diet. Impact on aphid survival and reproduction rates was used to determine the suitability of the aphid RNAi target genes. Of the 16 genes investigated, nine genes showed the reduction of aphid survival and/or reproduction rates. In addition to *MpC002* and *MpRack-1*, other suitable target genes were genes encoding the following polypeptides and the type of function they had in aphids: tubulin (Accession No. XM_022321900.1, cellular structure), Insulin-related peptide (XM_022313196.1, embryo development), V-type ATPase E subunit (XM_022312248.1, energy metabolism), gap hunchback (XM_022313819.1, growth and development), Ecdysis triggering hormone (XM_022323100.1, development - moulting), short neuropeptide F (XM_022314068.1, nervous system) and leucokinin (XM_022308286.1, water balance and food intake). For most genes, the impact of the RNAi appeared more robust and stronger on the aphid reproduction than on the survival, i.e. there was greater effects on reproduction.

Trans-generation effect of exogenous RNAi on aphids

To examine how long the RNAi effect could last, aphids at the two or three instars developmental stage were fed on an artificial diet supplemented with dsRNAi targeting MpC002, MpRack-1, MpGhb or with control dsGFP for 10 days. The aphids that survived were then transferred to excised radish leaves without RNA application. For all three genes, up to 6 days, the number of nymphs produced per survived aphid was significantly lower than the number for aphids fed on the control dsGFP RNA molecules or water. For the MpC002 and MpRack-1 dsRNAs, the lower reproduction rate on the radish leaves was maintained for at least 9 days. To investigate if the dsRNAi affected the following generations, the aphids which were born within three days on the radish leaves and which did not feed directly on RNA-containing diet were removed onto fresh excised radish leaves and their survival and production rate were monitored for 15 days. While there was no significant difference in the survival rate, the aphids which had been born from the mother aphids fed on the diet with MpC002, MpRack-1 or MpGh dsRNA, all produced a significantly lower number of aphids compared to the mother aphids fed on the diet with the control dsGFP or water. It was concluded that the effects caused by feeding dsRNA molecules to the parent aphid persisted in the progeny aphids.

20 *Conclusions*

The aims of this study were to test the application of exogenous RNAi using the ledRNA design for the control of aphids, a major group of sap-sucking insect pests that are a problem throughout the world, and to identify suitable target genes. Aphids are known to possess the RNAi machinery to process exogenous RNA (Scott et al., 2013; Yu et al., 2016). Here, oral delivery through an artificial diet containing ledRNA molecules targeting the *MpC002* or *MpRack-1* genes was able to cause aphid mortality and reduce the reproduction of the aphids. The molecules were tested against two different target genes, one encoding effector protein C002 and the other a receptor of activated protein kinase (Rack-1), which are essential for feeding and development of green peach aphid (*Myzus persicae*). When added to the artificial diet with a concentration as low as 50 ng/μl, the ledRNA molecules targeting these genes significantly reduced aphid reproduction. At a higher concentration of 200 ng/μl, the ledRNAs also increased aphid mortality. When ledRNA uptake was investigated using Cy3 labelling, ledRNA molecules were observed in aphid guts within hours of feeding on the artificial diet and subsequently in the reproduction system and even in newborn nymphs that were progeny of fed adults. The ledRNA effect on aphid reproduction

could last for at least two generations as indicated in the results with the traditional dsRNA.

It was also shown that the ledRNA molecules stayed largely intact in the artificial diet for at least three and half weeks. Largely intact ledRNA molecules were also found in the aphid honeydew, an excretion product from the aphids. When labelled ledRNA was applied onto plant leaves, it could get into the phloem where the aphids feed and was detected in the aphids. Together these results indicated the strong potential for ledRNA to be used for the control of aphids and other sap-sucking insects, including by exogenous delivery through the diet, providing a practical approach for management of aphids and other sap-sucking insects. These RNA molecules can also be expressed in transgenic plants, using promoters that favour synthesis of the RNA in phloem tissues, to control aphids and other sap-sucking insects. Furthermore, use of ledRNA[G:U] or hairpin[G:U] RNA comprising 10-30% G:U basepairs in the dsRNA region of the molecules is expected to provide even better control, based on the increased levels of accumulation of these dsRNA molecules through reduced self-silencing of the transgenes encoding these molecules.

Example 20. RNA constructs targeting other insect genes

LedRNA targeting genes of insect pests

Helicoverpa armigera is an insect pest in the order Lepidoptera, also known as the cotton bollworm or corn earworm. The larvae of *H. armigera* feed on a wide range of plants including many important cultivated crops and cause considerable crop damage worth billions of dollars per year. The larvae are polyphagous and cosmopolitan pests which can feed on a wide range of plant species including cotton, maize, tomato, chickpea, pigeon pea, alfalfa, rice, sorghum and cowpea.

The *H. armigera* ABC transporter white gene (ABCwhite) was selected as a target gene with a readily detected phenotype to test ledRNA and ledRNA(G:U) constructs in an insect larva. ABC transporters belong to the ATP Binding Cassette transporter superfamily – for example, 54 different ABC transporter genes were identified in the *Helicoverpa* genome. ABC transporters encode membrane-bound proteins that carry any one or more of a wide range of molecules across membranes. The proteins use energy released by ATP hydrolysis to transport the molecules across the membrane. Some ABC transporters were implicated in the degradation of plant secondary metabolites in the cotton bollworm, *H. armigera* (Khan et al., 2017). The ABCwhite protein transports ommochrome and pteridine pathway precursors into pigment granules in the eye and knockout mutants exhibit white eyes.

The nucleotide sequence of the *ABCwhite* gene is provided as SEQ ID NO:127 (Accession No. KU754476). To test whether a ledRNA could reduce *ABCwhite* gene expression by exogenous delivery of the RNA in the larval diet, a genetic construct encoding a ledRNA was designed and made that targeted the gene. The genetic
5 construct was made using the design principles for ledRNAs, with the split sequence being the sense sequence and the contiguous sequence being the antisense sequence (Figure 1B). The target region was 603 nucleotides in length, corresponding to nucleotides 496-1097 of SEQ ID NO:127. The dsRNA region of the ledRNA was 603bp in length; the antisense sequence in the dsRNA region was an uninterrupted,
10 contiguous sequence corresponded to the complement of nucleotides 496-1097 of SEQ ID NO:127. The nucleotide sequences encoding the ledRNA is provided herein as SEQ ID NO:128. The coding region was under the control of a T7 RNA polymerase promoter for *in vitro* transcription.

The genetic construct encoding the ledRNA was digested with the restriction
15 enzyme *Sna*BI, which cleaved downstream of the ledRNA coding region, and transcribed *in vitro* with RNA polymerase T7 according to the instructions with the transcription kit. The RNA is added to an artificial diet and provided to *H. armigera* larvae.

A corresponding ledRNA construct having G:U basepairs in the double-stranded
20 stem is made and compared to the canonically basepaired ledRNA.

LedRNAs targeting a gene in ants

Linepithema humile, commonly known as the Argentine ant, is an insect pest that has spread widely in several continents. The *L. humile* gene encoding pheromone
25 biosynthesis activating neuropeptide (PBAN) neuropeptides-like (LOC105673224) was selected as a target gene, involved in communication between the insects by pheromones.

The nucleotide sequence of the *PBAN* gene is provided as SEQ ID NO:129 (Accession No. XM_012368710). To test whether a ledRNA could reduce *PBAN* gene
30 expression by exogenous delivery of the RNA in the diet in the form of a bait, a genetic construct encoding a ledRNA was designed and made that targeted the gene. The genetic construct was made using the design principles for ledRNAs, with the split sequence being the sense sequence and the contiguous sequence being the antisense sequence (Figure 1B). The target region was 540 nucleotides in length, corresponding
35 to nucleotides 136-675 of SEQ ID NO:129. The dsRNA region of the ledRNA was 540bp in length; the antisense sequence in the dsRNA region was an uninterrupted,

contiguous sequence corresponded to the complement of nucleotides 136-675 of SEQ ID NO:129. The nucleotide sequences encoding the ledRNA is provided herein as SEQ ID NO:130. The coding region was under the control of a T7 RNA polymerase promoter for *in vitro* transcription.

5 The genetic construct encoding the ledRNA was digested with the restriction enzyme *Sna*BI, which cleaved downstream of the ledRNA coding region, and transcribed *in vitro* with RNA polymerase T7 according to the instructions with the transcription kit. The RNA is coated onto corn powder for oral delivery into *L. humile* ants.

10

LedRNA targeting genes of *L. cuprina*

Lucilia cuprina is an insect pest more commonly known as the Australian sheep blowfly. It belongs to the blowfly family, Calliphoridae, and is a member of the insect order Diptera. Five target genes were selected for testing with ledRNA constructs, 15 namely genes encoding V-type proton ATPase catalytic subunit A (Accession No. XM_023443547), RNase 1/2 (Accession No. XM_023448015), chitin synthase (Accession No. XM_023449557), ecdysone receptor (*EcR*; Accession No. U75355) and gamma-tubulin 1/1-like (Accession No. XM_023449717) of *L. cuprina*. Each of the genetic constructs was made using the design principles for ledRNAs, with the split 20 sequence being the sense sequence and the contiguous sequence being the antisense sequence (Figure 1B). In each case, the target region was about 600 nucleotides in length and the antisense sequence in the dsRNA region was an uninterrupted, contiguous sequence. The nucleotide sequence encoding the ledRNA targeting the *ATPase-A* gene is provided herein as SEQ ID NO:131. The nucleotide sequence 25 encoding the ledRNA targeting the *RNase 1/2* gene is provided herein as SEQ ID NO:132. The nucleotide sequence encoding the ledRNA targeting the chitin synthase gene is provided herein as SEQ ID NO:133. The nucleotide sequence encoding the ledRNA targeting the *EcR* gene is provided herein as SEQ ID NO:134. The nucleotide sequence encoding the ledRNA targeting the gamma-tubulin 1/1-like gene is provided 30 herein as SEQ ID NO:135. In each construct, the coding region was under the control of a T7 RNA polymerase promoter for *in vitro* transcription.

Example 21. Transgene-derived ledRNA accumulates at high levels in stably transformed plants

35 The DNA fragments encoding ledRNA sequences targeting the mRNAs from a GUS reporter gene or the *A. thaliana EIN2* gene were synthesized and cloned into

pART7 to form p35S:ledRNA:Ocs3' polyadenylation region/terminator expression cassettes for expression in plant cells. The fragments were then excised with *NotI* and inserted into the *NotI* site of pART27 to form the ledGUS and ledEIN2 vectors for plant transformation. The ledGUS construct and the existing hpGUS construct designed
5 to generate a long hpRNA with a 563 bp dsRNA stem and 1113 nt loop were separately into the GUS-expressing *N. tabacum* line PPGH24 by *Agrobacterium*-mediated transformation methods. RNA samples from independent transformants which exhibited either strong GUS silencing or little or no apparent reduction in GUS activity were used in Northern blot hybridization assays to detect the transgene-encoded
10 hpGUS or ledGUS RNA. As shown in Figure 38, much more intense hybridizing signals were detected from the ledGUS-transformed plants than from the hpGUS – transformed plants that showed strong GUS silencing (indicated by “-” in Figure 38). Indeed, most of the hybridizing signals for the hpGUS RNA samples were non-specific background signals that were also observed for RNA from the control, untransformed
15 plants (WT). Several intense hybridizing bands were observed for the ledGUS lines, presumably due to some partial processing of the full-length ledRNA.

The nucleotide sequence of the genetic construct encoding ledGUS is shown in SEQ ID NO:5. Nucleotides 1-17 correspond to a T7 RNA polymerase promoter for *in vitro* RNA synthesis, nucleotides 18-270 correspond to the 5' half of GUS antisense
20 sequence, nucleotides 271-430 correspond to loop 1 sequence, nucleotides 431-933 correspond to GUS sense sequence, nucleotides 934-1093 correspond to loop 2 sequence, and nucleotides 1094-1343 correspond to the 3' half of GUS antisense sequence.

In similar fashion, the ledEIN2 and hpEIN2 constructs were separately
25 introduced into *A. thaliana* plants of the Col-0 ecotype by *Agrobacterium*-mediated transformation. The hpEIN2 construct, encoding the hpEIN2[wt] RNA, was as described previously and contained 200 bp sense and antisense EIN2 sequences in an inverted repeat configuration, separated by the PDK intron. The nucleotide sequence of the genetic construct encoding ledEIN2 is shown in SEQ ID NO:116. Nucleotides
30 37-225 correspond to the 5' half of EIN2 antisense sequence, nucleotides 226-373 correspond to loop 1 sequence, nucleotides 374-773 correspond to EIN2 sense sequence, nucleotides 774-893 correspond to loop 2 sequence, and nucleotides 894-1085 correspond to the 3' half of EIN2 antisense sequence. Nucleotides 37-225 (antisense) are complementary to nucleotides 374-573 (sense) and nucleotides 894-
35 1085 (antisense) are complementary to nucleotides 574-773 (sense).

RNA samples from primary independent transformants were used for Northern blot hybridization analysis. As shown in Figure 39, the ledEIN2 plants showed more intense hybridizing signals than the hpEIN2 plants for larger RNA molecules (Figure 39, upper panel), indicating that ledEIN2-derived RNA accumulated at greater levels than hpEIN2-derived RNAs. For processed RNAs in the 20-25 nucleotide size range (siRNAs), siRNAs were detected in the ledEIN2 plants at greater abundance than in the hpEIN2 plants (Figure 39, lower panel), and the amount of siRNAs correlated well with the abundance of the larger RNA molecules. These results indicated that transgene-derived ledRNA was processed to some extent, but not completely, by Dicer into siRNAs. It also indicated that the ledRNA transgenes generated more siRNAs than a corresponding hpRNA transgene.

These results indicated that the ledRNA constructs, when expressed in plant cells, resulted in greater levels of accumulated transcripts, unprocessed and processed, than the corresponding hpRNA constructs. It was thought this was an indication of increased stability of the ledRNA molecules.

Example 22. Hairpin RNA is an efficient precursor of circular RNA in plants

Circular RNAs (circRNAs) are covalently linked, closed circles with no free 5' and 3' termini or polyadenylated sequences as 3' regions. They are generally non-coding in that they do not encode polypeptides and so are not translated. circRNAs are relatively resistant to digestion by RNases, in particular to exonucleases such as RNase R. circRNAs of viral or viroid origin or as satellite RNAs associated with viruses have long been observed in plants and animals. For instance, Potato Spindle Tuber Viroid, a subviral RNA pathogen in plants, has a circular RNA genome of around 360 nt in size. In plants, such satellite RNAs are often capable of being replicated in the presence of a helper virus. In contrast, viroids depend entirely on host functions including endogenous plant RNA polymerase for their replication.

Using RNA deep sequencing technologies in conjunction with specially designed bioinformatics tools, a large number of circRNAs have now been identified from plant and animal genomes. Thousands of putative circRNAs have been identified in plants including *A. thaliana*, rice and soybean which tend to show tissue-specific or biotic and abiotic stress-responsive expression patterns, but the biological function(s) of circRNAs in plants have yet to be demonstrated. The tissue-specific or stress responsive expression patterns of many putative plant circRNAs suggest that they may have potential roles in plant development and defence responses, but this has yet to be demonstrated.

A consensus view on the biogenesis of circRNAs is that they are formed by intron back-splicing, namely the splicing machinery “back-splices” pre-mRNA and covalently joins the spliced exons together. Thus, the endogenous intron splicing machinery is essential for the current model of circRNA biogenesis. This biogenesis model is based primarily on studies in mammalian systems where the majority of exonic circRNAs are shown to contain canonical intron splicing signals including the consensus GT/AG intron border dinucleotides. In animals, the intron regions flanking exonic circRNAs often contain short inverted repeats of transposable element sequences, and this has led to the suggestion that complementary intron sequences facilitate circRNA formation. Indeed, vector systems for expressing circRNAs in animals have been developed based on the naturally occurring exon-intron sequences with spliceable introns containing complementary TE repeats. However, the role of complementary flanking sequences in circRNA formation remains unclear in plants, as the proportion of identified exonic circRNAs with such flanking intron sequences is very low, ranging from 0.3% in *Arabidopsis* to 6.2% in rice.

Long hairpin RNA (hpRNA) transgenes have been widely used to induce gene silencing or RNA interference in plants (Wesley et al., 2001). An hpRNA transgene construct is typically comprised of an inverted repeat having complementary sense and antisense sequences with reference to a promoter sequence, and with a spacer sequence in between to separate and link the sense and antisense sequences. The spacer also stabilizes the inverted repeat structure in a DNA plasmid in bacterial cells during vector construction. Consequently, the RNA transcript from a typical hpRNA transgene is expected to form a stem-loop structure with a double-stranded (ds) stem of base-paired sense and antisense sequences and a “loop” corresponding to the spacer sequence. Such RNA transcripts are also referred to as self-complementary RNAs because of the ability of the sense and antisense regions to anneal by base-pairing, forming the dsRNA region or stem region of the molecule.

Loop fragments from long hpRNA accumulate in plant cells and are resistant to RNase
R

A transgene was made which encoded a long hpRNA targeting the GUS mRNA, having 563 bp sense and antisense sequences and a 1113 bp spacer (Figure 40, GUShp1100). A second transgene was also made which encoded a shorter hpRNA targeting the same GUS mRNA, having 93 bp sense and antisense sequences and a 93 bp spacer (GUShp93-1). Both constructs were introduced separately into *Nicotiana benthamiana* leaf cells for transient expression of the hairpin RNAs and were also used

to transform *A. thaliana* plants for expression as stably integrated and heritable transgenes. As previously reported, both constructs generated distinct RNA fragments of the expected size for the loop sequences when introduced and expressed in plant cells (Figure 41; Wang et al., 2008; Shen et al., 2015). In the present study, the
5 inventors wanted to determine whether the loop sequences were converted to circular RNAs.

A third construct was made having an *Arabidopsis* U6 promoter rather than the 35S promoter for expression of the shorter hpRNA (GUShp93-2). A fourth GUS hpRNA construct was also made which included a PDK intron as spacer sequence
10 (GUShpPDK in Figure 40). That construct encoded a hairpin RNA where the intron was expected to be spliced out after transcription, leaving a much shorter loop sequence. These constructs were also introduced into *N. benthamiana* leaves to examine whether the loop sequences could be detected and whether they formed circular RNA. The dsRNA stem and the loop sequences in these constructs were all
15 derived from the GUS coding sequence, and no known intron sequences were introduced. The constructs were separately introduced into *N. benthamiana* leaves using *Agrobacterium*-mediated infiltration, either in the presence or absence of a target GUS-expressing construct, together with a genetic construct encoding and expressing the cucumber mosaic virus 2b protein as a viral suppressor protein (VSP) to enhance
20 transgene expression. The accumulation and size of the loop fragments was analysed using Northern blot hybridization assays. The autoradiograph of a representative Northern blot is shown in Figure 42.

As shown in Figure 42, the long loop fragment of GUShp1100 was readily detected in *Agrobacterium*-infiltrated samples, as previously reported (Shen et al.,
25 2015). To test if this loop fragment was circular, the RNA samples were treated with RNase R and electrophoresed on polyacrylamide gels. The RNase R treatment used 10 µg of total RNA (or 50 ng of *in vitro* transcript) mixed with RNase R buffer and water in a total volume of 20µl. The mixtures were heated in boiling water for 3 min, chilled quickly on ice, then 0.5 µl RNase R was added and the tube was incubated at 37°C for
30 10 min. The enzyme was inactivated and the residual RNA recovered by precipitation with ethanol. The RNase R treatment degraded most of the RNA as indicated by the dramatic reduction in ethidium bromide stained material in the gels (Figure 42, lower panel). With all of the RNase R treatment assays, several ribosomal RNA fragments remained visible in the gels, indicating partial resistance of some RNA species to
35 RNase R. Despite the depletion of total RNA in the RNase R-treated samples, the approximately 1100 nt loop fragment remained abundant, with only about 24%

reduction in amount compared to the untreated samples. This indicated that the loop fragment was relatively resistant to RNase R digestion and was therefore circular in structure. The 24% reduction in the amount of loop RNA relative to the untreated sample was attributed to either residual amounts of endonuclease activity in the
5 commercially obtained RNase R enzyme or to reduced RNA recovery after RNase R digestion during the ethanol precipitation step.

The RNase R treatment assay was repeated with inclusion of 50 ng of *in vitro* transcribed RNA corresponding to the loop sequence as a linear RNA control. In addition, a sample of hpGUS1100-infiltrated *N. benthamiana* RNA was treated with
10 two rounds of RNase R treatment, to more stringently test RNase R resistance. It was observed that 76% of the loop fragment from GUShp1100-infiltrated *N. benthamiana* leaves remained after one RNase R treatment, whereas only about 8.5% of the linear *in-vitro* transcript remained. The two-fold RNase R treatment further reduced the loop-derived material but did not eliminate it. It was also noted that the RNA band from *N.*
15 *benthamiana* samples corresponding to the loop sequence appeared larger on the gel blot than the *in-vitro* transcript, consistent with circular RNA which has been reported to migrate more slowly in gel electrophoresis than linear RNA molecules having the same number of nucleotides. It was concluded from these experiments that the loop sequence of about 1100 nucleotides was circular.

20 Northern blot hybridization analysis of GUShp93-1 and GUShpPDK-infiltrated *N. benthamiana* RNA samples also detected RNA molecules of a size corresponding to the length of the loop sequences. For the GUShp93-1 and GUShp93-2 constructs, the U6 promoter-directed GUShp93-2 yielded more loop fragment than the 35S promoter driven GUShp93-1, indicating that the U6 promoter had stronger transcriptional
25 activity than the 35S promoter in *N. benthamiana* leaf cells or that the molecules were somehow more stable.

The GUShpPDK construct had a spacer sequence that included a spliceable PDK intron of 0.76 kb in size, and primary transcripts from this construct therefore contained an approximately 0.8 kb loop. The Northern blots were treated to remove the
30 GUS probe and re-probed with a full-length antisense probe against the PDK intron sequence. The PDK probe hybridized strongly to an unknown RNA species which was observed as an intense band across all lanes. RNase A treatment reduced but could not eliminate this non-specific band entirely. Nevertheless, a PDK intron-specific band of the expected size could be detected in the GUShpPDK-infiltrated RNA samples,
35 although the abundance of the fragment looked relatively weak, possibly because the intron sequence was spliced out from the majority of the GUShpPDK primary

transcripts. To examine if the PDK loop fragment was circular, RNA of GUShpPDK-infiltrated *N. benthamiana* leaves was treated with RNase R. The non-specific hybridizing band was almost completely removed by RNase R treatment. In contrast, the PDK intron band was readily detected after RNase R treatment, although the abundance could not be easily compared with the untreated sample due to the strong signal from the non-specific band. Taken together, these results indicated that hpRNA transcripts were an effective precursor for circular RNA formation, and suggested that the circular RNA corresponded to the whole loop sequence.

10 *RNase R-resistant loop fragment also accumulates in stably transformed Arabidopsis plants*

The hpGUS347 and the two hpGFP constructs (Figure 40) were used to transform *A. thaliana* plants of ecotype Col-0 and two plants expressing the transgene selected for each construct. The hpGUS347 construct was used in this experiment as a control for the hpGFP constructs which were designed to contain miR165/166 binding sites for testing miRNA sponge function (discussed in Example 24). Transgenic plants of the T2 generation were analysed for accumulation of RNA molecules produced from the hpGUS347 construct, in particular to detect loop sequences and whether they were circular. A band corresponding to the loop of hpGUS347 transcripts was detected in both the RNase R-treated and untreated RNA samples from two hpGUS347 lines. As for RNA samples from the *Agrobacterium*-infiltrated *N. benthamiana* tissues, there appeared to be a slight reduction in band intensity in the RNase R-treated samples compared to untreated ones, but most of the RNA signal was retained. RT-qPCR analysis, using primers designed to detect circRNA, confirmed the presence of circRNA in RNase R-treated hpGUS347 samples with a slight reduction in abundance compared to the untreated samples. These results indicated that stably integrated hpRNA transgenes which were expressed to produce hairpin RNAs also generated circRNAs from the loop sequences.

30 *The loops of hpRNA transcripts were excised at the dsRNA stem-loop junction and formed circular RNA*

To further confirm the circular nature of the RNA molecules derived from the loop sequences and to characterise their junction sequences, loop sequences were amplified by RT-PCR from GUShp1100, GUShp93 and GUShpPDK-infiltrated samples using oligonucleotide primers that would amplify putative junction sequences. The RT-PCR products were then cloned into pGEM-T Easy vector and sequenced,

confirming the nucleotide sequences at the junctions. The nucleotide positions of loop excision and joining in the circular RNAs were somewhat variable, with the 5' sites located within the 3' end of the dsRNA stem and the 3' sites near the 3' end of the loop, but the 5' sites showed a clear preference for the G nucleotide located 10 nucleotides from the 3' end of the dsRNA stem. It was noted that the excision and joining sites of the PDK intron circular RNA followed the same pattern as those from GUShp1100 and GUShp93 RNA, and were outside the canonical intron splicing sites. It was concluded that the formation of the circular RNA was determined by the stem-loop structure independently of intron splicing. It was also concluded that, at least in this example, the hairpin RNA was processed to release and circularise the loop sequence by a 5' cleavage within the 3' end of the dsRNA stem and a 3' cleavage near the 3' end of the loop sequence, with a covalent linkage formed between the 5' and 3' ends of the excised sequence.

15 **Example 23. hpRNA expressed in *Saccharomyces cerevisiae* was not processed into circular RNA**

The yeast species, *Saccharomyces cerevisiae*, is a eukaryotic organism and possesses intron splicing machinery as do all eukaryotes. As the current, consensus model for circular RNA formation is based on intron splicing, the inventors investigated whether hpRNA could form circular RNA in *S. cerevisiae* as it did in plant cells. To generate a construct to express a hpRNA, the inverted repeat region of GUShp1100 was excised from the plant expression vector and inserted into a yeast expression vector under the control of a yeast ADH1 promoter (Figure 43), and the resultant genetic construct introduced into *S. cerevisiae* cells. As shown in Figure 43, Northern blot hybridization analysis of RNA extracted from each of three independent transgenic yeast strains detected one, high molecular weight band corresponding to the GUShp1100 transcript. This indicated that the GUShp1100 transcript was not processed in *S. cerevisiae* but remained full-length. To confirm this, the *S. cerevisiae*-expressed and *N. benthamiana*-expressed GUShp1100 transcripts were compared for their response to RNase R treatment. As shown in Figure 44, the *S. cerevisiae*-expressed RNA showed a high molecular weight band which was highly sensitive to RNase R treatment and therefore not circular. That is, the yeast RNA samples did not exhibit circular molecules derived from the loop sequence as was produced in the *N. benthamiana* cells. The results indicated that the GUShp1100 transcript expressed in *S. cerevisiae* was not processed and remained full-length. The size of the *S. cerevisiae* RNA band appeared larger by gel electrophoresis than the *in vitro* GUShp1100

transcript, presumably due to the 5' and 3' UTR and poly(A) sequences that were present in the *S. cerevisiae*-expressed RNA but not in the *in vitro* transcript. Thus, the presence of intron splicing machinery in *S. cerevisiae* was not sufficient to allow processing of the hpRNA loop and formation of circular RNA as occurred in the plant cells.

In a similar fashion, the genetic construct GUShp347 was introduced into *S. cerevisiae* and expressed. Northern blot hybridisation analysis again showed that the hpRNA appeared full-length and was apparently not processed, at least not with cleavage of the loop sequence or the dsRNA region.

The inventors concluded that the yeast *S. cerevisiae* and its related budding yeasts, which do not have Dicer enzymes (Drinnenberg et al., 2003), are advantageous as an organism for the production of full length hairpin and ledRNAs, including the modified RNA molecules described herein. Such full-length RNAs are useful where the unprocessed dsRNA is desired, for example for silencing gene activity by topical application to insects.

Example 24. hpRNA loops can function as an effective “sponge” to suppress miRNA function

A few circular RNAs in animals have been found to contain multiple sequences which are complementary to specific miRNAs and thereby act as binding sites for those miRNAs, referred to as miRNA “sponges”. The inventors tested whether circular RNA produced from long hpRNA constructs could function as a miRNA sponge in plant cells. Two GFP hpRNA constructs were designed (Figure 40) which had the same GUS sequence-derived spacer except that one was modified in sequence to have two *Arabidopsis* miR165/166 binding sites. That construct, GFPhp[G:U], had an inverted repeat sequence which had the same antisense sequence as the second (control) construct GFPhp[WT] but with a modified sense sequence in which all cytosine nucleotides were replaced with thymines. The transcript from GFPhp[G:U] would therefore form a dsRNA region corresponding to the GFP sequence except that about 25% of the basepairs were G:U basepairs. The other construct, GFPhp[WT], encoded a hairpin RNA with a fully canonically basepaired dsRNA stem of the same length as the hairpin from GFPhp[G:U], and was used as a control (Figure 40). The GUS hpRNA construct GUShp347, containing a spacer without miR165/166 binding sites, was included as second control.

The constructs were used to separately transform *A. thaliana* and transgenic plants were obtained for each of the three constructs. The transformed plants were

examined visually for phenotypes related to reduction in miR165/166, which included a distinctive folding of leaves into “trumpets”. As expected, the GUShp347 transformed plants showed no phenotypes associated with miR165/166 repression. Similarly, no clear phenotype was observed in GFPhp[WT]-transformed plants. In contrast, the majority of the GFPhp[G:U] plants showed various levels of phenotypes reminiscent of miR165/166 repression including the trumpet phenotype.

Northern blot hybridization was performed on RNA extracted from GFPhp[G:U] transformed plants with a range of mild, moderate and strong to severe phenotypes to examine the accumulation of hpRNA expression. The probe used was a full-length antisense RNA corresponding to GUS mRNA. The probe had a 822 bp continuous sequence complementarity with the sense and adjacent loop of the GUShp347 transcript. The probe had less sequence complementarity to the GFPhp transcripts which had a total of 228 bp of the loop region as GUS-derived sequence, in three non-contiguous regions of 49, 109 and 70 bp in length flanking the two miRNA binding sequences. As shown in Figure 45B, highly abundant amounts of GFP hpRNA molecules were detected in the GFPhp[G:U] plants, and the amounts of RNA molecules detected in the Northern blots correlated positively with the severity of phenotypes. The GFPhp[WT] plants exhibited low levels of accumulation of hpRNA molecules which were only just detectable in the Northern blot analyses, consistent with relatively low transcription levels of conventional hpRNA transgenes compared to G:U modified hpRNA transgenes. That is, as shown in the Examples above, the hpRNA[G:U] transgenes were less subject to self-silencing compared to the corresponding hpRNA[WT] transgene.

RT-qPCR was used to quantitate the accumulation of the circular RNA molecules derived from the loop sequences. The results showed that high amounts of the circRNA were present in the GFPhp[G:U] transgenic plants that correlated with the levels of full-length hpRNA accumulation (Figure 45C). Northern blot hybridization analyses to detect small RNAs in the 20-25 nt size range confirmed the down-regulation of miR165/166 in the GFPhp[G:U] plants. The extent of reduction correlated with the amount of hpRNA and circRNA and the severity of phenotypes. Expression analysis of the miR165/166 target gene using RT-qPCR showed that target gene repression by miR165/166 was released in the plants that showed strong miR165/166 down-regulation and severe phenotypes. Taken together, these results showed that hpRNA loops could be used as a specific miRNA sponge to repress miRNA function in plants.

The inventors also conceived of the use of the circular RNAs, produced at high levels in plant cells as stable molecules, to be translated as a means to produce high levels of polypeptides. For initiation of cap-independent translation, internal ribosome entry sites (IRES) are ideally used. Numerous IRES sequences have been identified.

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Example 25. RNA constructs targeting genes involved in modulating flowering in plants

LedRNAi targeting the VRN2 gene conferring vernalisation responsiveness to wheat

The genes encoding the VRN2 protein in wheat (*Triticum aestivum*) regulate the vernalisation response and therefore the timing of flowering. The wheat VRN2A, VRN2B and VRN2D candidate genes as identified in TGACv1_scaffold_374416_5AL, TGACv1_scaffold_320642_4BL and TGACv1_scaffold_342601_4DL being homologs of the wheat *ZCCT1* gene (Genbank Accession No. AAS58481.1) were identified as targets for design of a ledRNAi construct. A 310 bp region of the VRN2B gene that was conserved in the VRN2A and VRN2D genes and corresponding to nucleotides 2-311 in SEQ ID NO:145 was used for the dsRNA region of the ledRNAi construct designated LedTaVRN2. The two antisense sequences corresponded to the complement of nucleotides 1-156 and of nucleotides 157-311 of SEQ ID NO:145. The two loops in LedTaVRN2, each of 120 nucleotides, were from a GUS sequence, so unrelated to the VRN2 sequence. Led RNAi was produced by *in vitro* transcription using T7 RNA polymerase and diluted in water. The solution was used to imbibe wheat grains for germination at 4°C for 3 days, using 150 µl of solution for six seeds with 10 µg LedTaVRN2 (SEQ ID NO:146) per seed. Seeds of the vernalisation sensitive wheat variety CSIRO W7 were used. Treated seeds were planted in soil and the resultant plants grown at 24°C under 16hr light per day. The plants were observed over time for the transition from vegetative growth to floral development. The time of flowering, as indicated by emergence of the ear from the boot, and the number of leaves on the main stem at the time of flowering were recorded.

Plants derived from seeds contacted with LedTaVRN2 flowered on average at least 17 days earlier than plants derived from seeds treated with buffer only or non-specific dsRNA controls (Figure 46). Furthermore, plants derived from seeds incubated with LedTaVRN2 had on average 2.3 fewer leaves on the main stem at the time of flowering indicating fewer nodes were dedicated to leaf production and more nodes dedicated to flowers/grain. The treated seeds were not affected in their germination rate.

In a second experiment, seeds of the winter wheat variety Longsword were treated with either a) 10µg of LedTaVRN2 in RNA buffer and water, treated as in the first experiment, b) as a control for non-specific effects of an ledRNA molecule, 10µg of ledGFP suspended in RNA buffer and water, or c) water containing an equivalent amount of RNA buffer as the LedTaVRN2 and ledGFP treatments. Seeds were incubated at 4°C for 72 hours then planted to soil in a controlled temperature room at 24°C with 16 hr light per day. The number of days to flowering, assessed as the emergence of the head from the boot, was recorded. The total number of leaves on the main stem at the time of flowering was also recorded. Longsword plants treated with ledTaVRN2 flowered on average 27.6 days earlier than non-treated seeds and contained 4.1 fewer leaves on the main stem.

In a third experiment, seeds of the vernalisation responsive wheat variety CSIRO W7 were treated with either a) 10µg of LedTaVRN2 in RNA buffer and water, by soaking as before, b) as a control for non-specific effects of an ledRNA molecule, 10µg of ledGFP suspended in RNA buffer and water, c) water containing an equivalent amount of RNA buffer as the LedTaVRN2 and ledGFP treatments or d) water only. Seeds of the early flowering (non-vernalisation responsive) parental lines Sunstate A (SSA) and Sunstate B (SSB) were incubated in water only. All seeds were incubated at 4°C for 72 hours then planted to soil in a glasshouse. The number of days to flowering, assessed as the emergence of the head from the boot, and the total number of leaves on the main stem at the time of flowering was recorded. Plants treated with LedVRN2 showed on average 10.3 days earlier flowering and 1.2 fewer leaves than non-treated seeds (Figure 47).

RNA is prepared from the wheat plants grown from the treated seeds and RT-PCR experiments are carried out to observe reduction in the level of mRNA expressed from the *VRN2* genes.

LedRNAi targeting the FLC gene controlling flowering in *A. thaliana*

A target gene encoding the flowering locus C (FLC) regulatory protein of *A. thaliana* was selected as another exemplary target gene to test whether the modified RNA molecules could modulate flowering time, this time in a dicotyledonous plant. A 520 nucleotide sequence was selected consisting of two non-contiguous regions of the FLC mRNA sequence (Accession No. AF537203, Michaels and Amasino, 1999), namely nucleotides 31-474 of AF537203 joined to nucleotides 516-591 of AF537203. These regions were selected on the basis that they were less conserved in another, homologous gene sequence in *A. thaliana* (Accession No. AT1G77080) that was not

intended to be down-regulated, thus providing greater specificity for down-regulation of FLC. A ledRNA molecule was designed and produced by *in vitro* transcription. Seeds of the late flowering, winter line MS-0 of *A. thaliana* were soaked in a buffer solution containing the ledRNA. The seeds were sown onto soil and the resultant plants
5 are grown to flowering, defined here as opening of the first flower. Flowering time of the plants produced from the ledRNA-treated seeds is reduced compared to the flowering time of control, mock-treated seeds of MS-0. RT-PCR experiments show that the level of FLC mRNA is reduced in the plants produced from treated seeds.

10 LedRNAi targeting the FLC gene controlling flowering in *Brassica napus*

An analogous experiment is carried out targeting an FLC gene of *Brassica napus*, namely the MADS-box protein encoded by the LOC106383096 gene, transcript variant X1 (Accession No. XM_013823208). A ledRNA molecule was designed and made, having a sense sequence corresponding to nucleotides 354-744 of Accession No.
15 XM_013823208 and two antisense sequences, one corresponding to the complement of nucleotides 354-546 and the other to the complement of nucleotides 547-744 of XM_013823208. Seeds of a late flowering, winter line of *B. napus* are soaked in a buffer solution containing the ledRNA. The seeds are sown onto soil and the resultant plants are grown to flowering, defined here as opening of the first flower. Flowering
20 time of the plants produced from the ledRNA-treated seeds is reduced compared to the flowering time of control, mock-treated seeds of the same genotype of *B. napus*. RT-PCR experiments show that the level of FLC mRNA is reduced in the plants produced from treated seeds.

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It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as
30 illustrative and not restrictive.

The present application claims priority from AU2020900327 filed 6 February 2020, and PCT/AU2019/050814 filed 2 August 2019, the entire contents of both of which are incorporated herein by reference.

All publications discussed and/or referenced herein are incorporated herein in
35 their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the
5 field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. An RNA molecule comprising a first RNA component, a second RNA component which is covalently linked to the first RNA component and, optionally, one or more or all of (i) a linking ribonucleotide sequence which covalently links the first and second RNA components, (ii) a 5' leader sequence and (iii) a 3' trailer sequence, wherein the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair with each other in the first RNA component, wherein the first RNA sequence comprises a first sense ribonucleotide sequence of at least 20 contiguous ribonucleotides, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence of at least 20 contiguous ribonucleotides, wherein the first antisense ribonucleotide sequence hybridises with the first sense ribonucleotide sequence in the RNA molecule, wherein the first antisense ribonucleotide sequence is capable of hybridising to a first region of a target RNA molecule which modulates the timing of plant flowering, wherein the second RNA component is covalently linked, via the linking ribonucleotide sequence if present or directly if the linking ribonucleotide sequence is not present, to the first 5' ribonucleotide or the first 3' ribonucleotide, wherein the second RNA component consists of, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide, wherein the second 5' and 3' ribonucleotides basepair to each other in the RNA molecule, wherein the second RNA sequence comprises a second sense ribonucleotide sequence, a second loop sequence of at least 4 ribonucleotides and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence hybridises with the second antisense ribonucleotide sequence in the RNA molecule, wherein the 5' leader sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide, and wherein the 3' trailer sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the second 3' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the first 3' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide.

2. An RNA molecule comprising a first RNA component, a second RNA component which is covalently linked to the first RNA component and, optionally, one or more or all of (i) a linking ribonucleotide sequence which covalently links the first and second RNA components, (ii) a 5' leader sequence and (iii) a 3' trailer sequence,
- 5 wherein the first RNA component consists of, in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide, wherein the first 5' and 3' ribonucleotides basepair, wherein the first RNA sequence comprises a first sense ribonucleotide sequence, a first loop sequence of at least 4 ribonucleotides and a first antisense ribonucleotide sequence, wherein the first sense ribonucleotide sequence and
- 10 first antisense ribonucleotide sequence each of at least 20 contiguous ribonucleotides whereby the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence fully basepair with the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence, wherein the at least 20 contiguous ribonucleotides of the first sense ribonucleotide sequence are identical in sequence to a first region of a
- 15 target RNA molecule which modulates the timing of plant flowering, wherein the second RNA component is covalently linked, via the linking ribonucleotide sequence if present, to the first 5' ribonucleotide or the first 3' ribonucleotide, wherein the second RNA component consists of, in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide, wherein the
- 20 second 5' and 3' ribonucleotides basepair, wherein the second RNA sequence comprises a second sense ribonucleotide sequence, a second loop sequence of at least 4 ribonucleotides and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence basepairs with the second antisense ribonucleotide sequence,
- 25 wherein the 5' leader sequence, if present, consists of a sequence of ribonucleotides which is covalently linked to the first 5' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the second 5' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide, and wherein the 3' trailer sequence, if present, consists of a sequence of ribonucleotides
- 30 which is covalently linked to the second 3' ribonucleotide if the second RNA component is linked to the first 3' ribonucleotide or to the first 3' ribonucleotide if the second RNA component is linked to the first 5' ribonucleotide.
3. The RNA molecule of claim 1 or claim 2, wherein the at least 20 contiguous
- 35 ribonucleotides of the first antisense ribonucleotide sequence are all capable of basepairing to nucleotides of the first region of the target RNA molecule.

4. The RNA molecule according to any one of claims 1 to 3, wherein the first sense ribonucleotide sequence is linked covalently to the first 5' ribonucleotide without any intervening nucleotides, or the first antisense ribonucleotide sequence is linked
5 covalently to the first 3' ribonucleotide without any intervening nucleotides, or both.
5. The RNA molecule according to any one of claims 1 to 4 which comprises the linking ribonucleotide sequence, wherein the linking ribonucleotide sequence is less than 20 ribonucleotides.
- 10 6. The RNA molecule of claim 5, wherein the linking ribonucleotide sequence hybridizes to the target RNA molecule.
7. The RNA molecule of claim 5 or claim 6, wherein the linking ribonucleotide
15 sequence is identical to a portion of the complement of the target RNA molecule.
8. The RNA molecule according to any one of claims 5 to 7, wherein the linking ribonucleotide sequence is between 1 and 50 ribonucleotides in length.
- 20 9. The RNA molecule according to any one of claims 5 to 7, wherein the linking ribonucleotide sequence is between 1 and 10 ribonucleotides in length.
10. The RNA molecule according to any one of claims 1 to 9 which comprises two or more sense ribonucleotide sequences, and antisense ribonucleotide sequences fully
25 based paired thereto, which are identical in sequence to a region of a target RNA molecule.
11. The RNA molecule of claim 10, wherein the two or more sense ribonucleotide sequences are identical in sequence to different regions of the same target RNA
30 molecule.
12. The RNA molecule of claim 10, wherein the two or more sense ribonucleotide sequences are identical in sequence to a region of different target RNA molecules.
- 35 13. The RNA molecule according to any one of claims 1 to 12 which comprises two or more antisense ribonucleotide sequences, and sense ribonucleotide sequences fully

based paired thereto, which are each complementary to a region of a target RNA molecule.

14. The RNA molecule of claim 13, wherein the two or more antisense
5 ribonucleotide sequences are complementary to different regions of the same target RNA molecule.

15. The RNA molecule of claim 13 or claim 14, wherein the second of the two or
10 more antisense ribonucleotide sequences are complementary to region of a different target RNA molecule than the first of the two or more antisense ribonucleotide sequences.

16. The RNA molecule according to any one of claims 1 to 15, wherein the two or
15 more sense ribonucleotide sequences have no intervening loop sequences.

17. The RNA molecule according to any one of claims 2 to 16 which is a single
strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence
which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is
fully base paired with each sense ribonucleotide sequence over at least 21 contiguous
20 nucleotides, at least two loop sequences and a 3' end.

18. The RNA molecule according to any one of claims 1, or 3 to 16 which is a
single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide
sequence which is at least 21 nucleotides in length, an antisense ribonucleotide
25 sequence which is fully base paired with each sense ribonucleotide sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end.

19. The RNA molecule according to any one of claims 1 to 18 which is a single
strand of ribonucleotides comprising a 5' end, the first RNA component comprising a
30 first sense ribonucleotide sequence which is at least 21 nucleotides in length, at least one loop sequence, a first antisense ribonucleotide sequence which hybridises with the first sense ribonucleotide sequence over a length of at least 21 contiguous nucleotides, and the second RNA component comprising a second sense ribonucleotide sequence which is at least 21 nucleotides in length, a loop sequence, a second antisense
35 ribonucleotide sequence which hybridises with the second sense ribonucleotide

sequence over a length of at least 21 contiguous nucleotides, and a 3' end, wherein the RNA molecule has only one 5' end and only one 3' end.

20. The RNA molecule of claim 19, wherein the ribonucleotide at the 5' end and the
5 ribonucleotide at the 3' end are adjacent, each base paired and are not directly covalently bonded.

21. The RNA molecule according to any one of claims 1 to 20 which comprises a
10 first antisense ribonucleotide sequence which hybridizes to a first region of a target RNA, a second antisense ribonucleotide sequence which hybridizes to a second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one sense ribonucleotide sequence which hybridizes to the target RNA, wherein the two antisense sequences are not contiguous in the RNA molecule.

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22. The RNA molecule according to any one of claims 1 to 20 which comprises a
first sense ribonucleotide sequence which is at least 60% identical to a first region of a target RNA, a second sense ribonucleotide sequence which is at least 60% identical to a
20 second region of a target RNA, the second region of the target RNA being different to the first region of the target RNA, and the RNA molecule comprising only one antisense ribonucleotide sequence which hybridizes to the target RNA, wherein the two sense sequences are not contiguous in the RNA molecule.

23. The RNA molecule according to any one of claims 1 to 22 which has the 5'
25 leader sequence.

24. The RNA molecule according to any one of claims 1 to 23 which has the 3'
trailer sequence.

30 25. The RNA molecule according to any one of claims 1 to 24, wherein each ribonucleotide is covalently linked to two other nucleotides.

26. The RNA molecule of claim 25, wherein at least one or all of the loop sequences are longer than 20 nucleotides.

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27. The RNA molecule according to any one of claims 1 to 15, wherein the RNA molecules has none, or one, or two or more bulges, or a double-stranded region of the RNA molecule comprises one, or two, or more nucleotides which are not basepaired in the double-stranded region.
- 5
28. The RNA molecule according to any one of claims 1 to 27 which has three, four or more loops.
29. The RNA molecule according to any one of claims 1 to 27 which only has two
10 loops.
30. The RNA molecule according to any one of claims 1 to 29, wherein the target RNA is in a plant cell.
- 15 31. The RNA molecule of claim 30, wherein the plant cell is from *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye.
32. The RNA molecule of claim 30 or claim 31, which is present in a plant cell.
- 20
33. The RNA molecule of claim 32 which is expressed in the cell.
34. The RNA molecule according to any one of claims 1 to 33, wherein at least one of the loops is between 4 and 1,000 ribonucleotides, or between 4 and 200
25 ribonucleotides, in length.
35. The RNA molecule of claim 34, wherein all of the loops are between 4 and 1,000 ribonucleotides, or between 4 and 200 ribonucleotides, in length.
- 30 36. The RNA molecule of claim 35, wherein all of the loops are between 4 and 50 ribonucleotides in length.
37. The RNA molecule according to any one of claims 1 to 36, wherein each loop is
35 between 20 and 30 ribonucleotides in length.

38. The RNA molecule according to any one of claims 1 to 37, wherein the target RNA encodes a protein.
39. The RNA molecule according to any one of claims 1 to 38 which comprises an
5 nucleotide sequence set forth in SEQ ID NO:146 or SEQ ID NO:147.
40. A chimeric ribonucleic acid (RNA) molecule, comprising a double-stranded RNA (dsRNA) region which comprises a first sense ribonucleotide sequence of at least
20 contiguous nucleotides in length and a first antisense ribonucleotide sequence of at
10 least 20 contiguous nucleotides in length, whereby the first sense ribonucleotide sequence and the first antisense ribonucleotide sequences are capable of hybridising to each other to form the dsRNA region, wherein
- i) the first sense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a first 5' ribonucleotide, a first RNA sequence and a first 3' ribonucleotide,
 - 15 ii) the first antisense ribonucleotide sequence consists of, covalently linked in 5' to 3' order, a second 5' ribonucleotide, a second RNA sequence and a second 3' ribonucleotide,
 - iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide to form a terminal basepair of the dsRNA region,
 - 20 iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide to form a terminal basepair of the dsRNA region,
 - v) between about 5% and about 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired,
 - 25 vi) the dsRNA region does not comprise 20 contiguous canonical basepairs,
 - vii) the RNA molecule is capable of being processed in a plant cell or *in vitro* whereby the first antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length,
 - viii) the RNA molecule or at least some of the asRNA molecules, or both, are
30 capable of reducing the expression or activity of a target RNA molecule which modulates the timing of plant flowering, and
 - ix) the RNA molecule is capable of being made enzymatically by transcription *in vitro* or in a cell, or both.
- 35 41. The chimeric RNA molecule of claim 40, wherein the first sense ribonucleotide sequence is covalently linked to the first antisense ribonucleotide sequence by a first

linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides, or between 4 and 1,000 ribonucleotides, or between 4 and 200 ribonucleotides, or between 4 and 50 ribonucleotides, or at least 10 nucleotides, or between 10 and 1,000 ribonucleotides, or between 10 and 200 ribonucleotides, or
5 between 10 and 50 ribonucleotides, in length, whereby the first linking ribonucleotide sequence is covalently linked to either the second 3' ribonucleotide and the first 5' ribonucleotide or, preferably, to the first 3' ribonucleotide and the second 5' ribonucleotide, so that the sequences are comprised in a single, contiguous strand of RNA.

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42. The chimeric RNA molecule of claim 41, wherein the loop sequence in the RNA molecule comprises one or more binding sequences which are complementary to an RNA molecule which is endogenous to the plant cell, and/or the loop sequence in the RNA molecule comprises an open reading frame which encodes a polypeptide or a
15 functional polynucleotide.

43. The chimeric RNA molecule according to any one of claims 40 to 42, wherein between about 5% and about 40% of the ribonucleotides of the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence of the dsRNA,
20 in total, are basepaired in non-canonical basepairs, preferably G:U basepairs.

44. The chimeric RNA molecule according to any one of claims 40 to 43, the first antisense ribonucleotide sequence is fully complementary to a region of the target RNA and the first sense ribonucleotide sequence is different in sequence to the region of the
25 target RNA by the substitution of C nucleotides in the region of the target RNA with U nucleotides.

45. The chimeric RNA molecule according to any one of claims 40 to 44 which comprises a second sense ribonucleotide sequence and the first sense ribonucleotide
30 sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the first 3' ribonucleotide and the second 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence
35 of at least 4 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and the second sense ribonucleotide sequence.

46. The chimeric RNA molecule according to any one of claims 40 to 45 which comprises a second antisense ribonucleotide sequence and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking
5 ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the second 3' ribonucleotide and the first 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and
10 the second antisense ribonucleotide sequence.

47. The chimeric RNA molecule according to any one of claims 40 to 45 which comprises a second sense ribonucleotide sequence and a second antisense ribonucleotide sequence, wherein the second sense ribonucleotide sequence and the
15 second antisense ribonucleotide sequences are capable of hybridising to each other to form a second dsRNA region, and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the first 3' ribonucleotide and the
20 second 5' ribonucleotide, and the RNA molecule optionally comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the second 3' ribonucleotide and the second sense ribonucleotide sequence or which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence.

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48. The chimeric RNA molecule according to any one of claims 40 to 45 which comprises a second sense ribonucleotide sequence and a second antisense ribonucleotide sequence and the first sense ribonucleotide sequence and the first antisense ribonucleotide sequence are linked by a first linking ribonucleotide sequence
30 comprising a loop sequence of at least 4 nucleotides in length, whereby the first linking ribonucleotide sequence is covalently linked to the second 3' ribonucleotide and the first 5' ribonucleotide, and the RNA molecule further comprises a second linking ribonucleotide sequence which comprises a loop sequence of at least 4 nucleotides in length and which is covalently linked to the first 3' ribonucleotide and the second
35 antisense ribonucleotide sequence, or which covalently links the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence.

49. The chimeric RNA molecule according to any one of claims 45 to 48, wherein the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence each comprise at least 20 contiguous nucleotides in length.
- 5
50. The chimeric RNA molecule according to any one of claims 45 to 49, wherein the first and second sense ribonucleotide sequences are covalently linked by an intervening ribonucleotide sequence which is unrelated in sequence to the target RNA molecule, or which is related in sequence to the target RNA molecule, or the first and
- 10 second sense ribonucleotide sequences are covalently linked without an intervening ribonucleotide sequence.
51. The chimeric RNA molecule according to any one of claims 45 to 50, wherein the first and second antisense ribonucleotide sequences are covalently linked by an
- 15 intervening ribonucleotide sequence which is unrelated in sequence to the complement of a target RNA molecule, or which is related in sequence to the complement of a target RNA molecule, or the first and second antisense ribonucleotide sequences are covalently linked without an intervening ribonucleotide sequence.
- 20 52. The chimeric RNA molecule according to any one of claims 45 to 51, wherein between 5% and 40% of the ribonucleotides of the second sense ribonucleotide sequence and the second antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, preferably basepaired in G:U basepairs, wherein the second dsRNA region does not comprise 20 contiguous
- 25 canonical basepairs, and wherein the RNA molecule is capable of being processed in a eukaryotic cell or *in vitro* whereby the second antisense ribonucleotide sequence is cleaved to produce short antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length.
- 30 53. The chimeric RNA molecule according to any one of claims 45 to 52, wherein each linking ribonucleotide sequence is independently between 4 and about 2000 nucleotides in length, preferably between 4 and about 1200 nucleotides in length, more preferably between 4 and about 200 nucleotides in length and most preferably between 4 and about 50 nucleotides in length.

54. The chimeric RNA molecule according to any one of claims 40 to 53 which further comprises a 5' leader sequence or a 3' trailer sequence, or both.

55. A chimeric RNA molecule comprising a first RNA component and a second
5 RNA component which is covalently linked to the first RNA component,
wherein the first RNA component comprises a first double-stranded RNA (dsRNA)
region, which comprises a first sense ribonucleotide sequence and a first antisense
ribonucleotide sequence which are capable of hybridising to each other to form the first
dsRNA region, and a first intervening ribonucleotide sequence of at least 4 nucleotides
10 which covalently links the first sense ribonucleotide sequence and the first antisense
ribonucleotide sequence,

wherein the second RNA component comprises a second sense ribonucleotide
sequence, a second antisense ribonucleotide sequence and a second intervening
ribonucleotide sequence of at least 4 ribonucleotides which covalently links the second
15 sense ribonucleotide sequence and the second antisense ribonucleotide sequence,
wherein the second sense ribonucleotide sequence hybridises with the second antisense
ribonucleotide sequence in the RNA molecule,
wherein in the first RNA component,

i) the first sense ribonucleotide sequence consists of at least 20 contiguous
20 ribonucleotides covalently linked, in 5' to 3' order, a first 5' ribonucleotide, a first
RNA sequence and a first 3' ribonucleotide,

ii) the first antisense ribonucleotide sequence consists of at least 20 contiguous
ribonucleotides covalently linked, in 5' to 3' order, a second 5' ribonucleotide, a
second RNA sequence and a second 3' ribonucleotide,

25 iii) the first 5' ribonucleotide basepairs with the second 3' ribonucleotide,

iv) the second 5' ribonucleotide basepairs with the first 3' ribonucleotide,

v) between 5% and 40% of the ribonucleotides of the first sense ribonucleotide
sequence and the first antisense ribonucleotide sequence, in total, are either basepaired
in a non-canonical basepair or are not basepaired, and

30 vi) the first dsRNA region does not comprise 20 contiguous canonical basepairs,
wherein the chimeric RNA molecule is capable of being processed in a plant cell or *in*
vitro whereby the first antisense ribonucleotide sequence is cleaved to produce short
antisense RNA (asRNA) molecules of 20-24 ribonucleotides in length, and wherein

(d) the chimeric RNA molecule or at least some of the asRNA molecules, or both,
35 are capable of reducing the expression or activity of a target RNA molecule
which modulates plant flowering, or

- (e) the first antisense ribonucleotide sequence comprises a sequence of at least 20 contiguous ribonucleotides which is at least 50% identical in sequence, preferably at least 90% or 100% identical in sequence, to a region of the complement of the target RNA molecule, or
- 5 (f) both (a) and (b).

56. The chimeric RNA molecule according to any one of claims 40 to 55, wherein the at least 20 contiguous ribonucleotides of the first antisense ribonucleotide sequence are all capable of basepairing to nucleotides of a first region of the target RNA
10 molecule.

57. The chimeric RNA molecule according to any one of claims 40 to 56, wherein the RNA molecule comprises two or more antisense ribonucleotide sequences, and sense ribonucleotide sequences based paired thereto, which antisense sequences are
15 each complementary, preferably fully complementary, to a region of a target RNA molecule.

58. The chimeric RNA molecule of claim 57, wherein the two or more antisense ribonucleotide sequences are complementary to different regions of the same target
20 RNA molecule.

59. The chimeric RNA molecule of claim 57, wherein the two or more antisense ribonucleotide sequences are complementary to regions of different target RNA
25 molecules.

60. The chimeric RNA molecule according to any one of claims 40 to 59 which comprises a hairpin RNA (hpRNA) structure having a 5' end, a sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense ribonucleotide sequence which is fully base paired with the sense ribonucleotide sequence over at least
30 21 contiguous nucleotides, an intervening loop sequence and a 3' end.

61. The chimeric RNA molecule according to any one of claims 40 to 59 which comprises a single strand of ribonucleotides having a 5' end, at least one sense ribonucleotide sequence which is at least 21 nucleotides in length, an antisense
35 ribonucleotide sequence which is fully base paired with each sense ribonucleotide

sequence over at least 21 contiguous nucleotides, at least two loop sequences and a 3' end.

62. The chimeric RNA molecule according to any one of claims 40 to 61, wherein
5 between about 15% and about 30%, or between about 16% and about 25%, of the ribonucleotides of the sense ribonucleotide sequence and the antisense ribonucleotide sequence, in total, are either basepaired in a non-canonical basepair or are not basepaired, preferably basepaired in non-canonical basepairs, more preferably basepaired in G:U basepairs.

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63. The chimeric RNA molecule according to any one of claims 40 to 62, wherein at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, or 100% of the non-canonical basepairs are G:U basepairs.

15 64. The chimeric RNA molecule according to any one of claims 40 to 63, wherein less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 1% or none, of the ribonucleotides in the dsRNA region are not basepaired.

20 65. The chimeric RNA molecule according to any one of claims 40 to 64, wherein every one in four to every one in six ribonucleotides in the dsRNA region form a non-canonical basepair or are not basepaired, preferably form a G:U basepair.

66. The chimeric RNA molecule according to any one of claims 40 to 65, wherein the dsRNA region does not comprise 8 contiguous canonical basepairs.

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67. The chimeric RNA molecule according to any one of claims 40 to 66, wherein the dsRNA region comprises at least 8 contiguous canonical basepairs, preferably at least 8 but not more than 12 contiguous canonical basepairs.

30 68. The chimeric RNA molecule according to any one of claims 40 to 67, wherein all of the ribonucleotides in the dsRNA region, or in each dsRNA region, are basepaired with a canonical basepair or a non-canonical basepair.

35 69. The chimeric RNA molecule according to any one of claims 40 to 67, wherein one or more ribonucleotides of the sense ribonucleotide sequence or one or more ribonucleotides of the antisense ribonucleotide sequence, or both, are not basepaired.

70. The chimeric RNA molecule according to any one of claims 40 to 69, wherein the antisense RNA sequence is less than 100% identical, or between about 80% and 99.9% identical, or between about 90% and 98% identical, or between about 95% and 5 98% identical, in sequence to the complement of a region of the target RNA molecule.

71. The chimeric RNA molecule according to any one of claims 40 to 69, wherein the antisense RNA sequence is 100% identical in sequence to a region of the target RNA molecule.

10

72. The chimeric RNA molecule according to any one of claims 40 to 71, wherein the sense and/or antisense ribonucleotide sequence, preferably both, is at least 50, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least 15 about 1,000, or about 100 to about 1,000, or 20 to about 1000 nucleotides, or 20 to about 500 nucleotides, in length.

73. The chimeric RNA molecule according to any one of claims 40 to 72, wherein the number of ribonucleotides in the sense ribonucleotide sequence is between about 20 90% and about 110% of the number of ribonucleotides in the antisense ribonucleotide sequence.

74. The chimeric RNA molecule according to any one of claims 40 to 73, wherein the number of ribonucleotides in the sense ribonucleotide sequence is the same as the 25 number of ribonucleotides in the antisense ribonucleotide sequence.

75. The chimeric RNA molecule according to any one of claims 40 to 74, wherein the chimeric RNA molecule further comprises a 5' extension sequence which is covalently linked to the first 5' ribonucleotide or a 3' extension sequence which is 30 covalently linked to the second 3' ribonucleotide, or both.

76. The chimeric RNA molecule according to any one of claims 40 to 75, wherein the chimeric RNA molecule further comprises a 5' extension sequence which is covalently linked to the second 5' ribonucleotide or a 3' extension sequence which is 35 covalently linked to the first 3' ribonucleotide, or both.

77. The chimeric RNA molecule according to any one of claims 40 to 76, which comprises two or more dsRNA regions which are the same or different.
78. The chimeric RNA molecule according to any one of claims 40 to 77, wherein
5 when expressed in a plant cell more asRNA molecules are formed that are 22 and/or 20 ribonucleotides in length when compared to processing of an analogous RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs.
- 10 79. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein the target RNA
- i) encodes VERNALIZATION1 (VRN1), VERNALIZATION2 (VRN2), EARLYINSHORTDAYS4, FLOWERING LOCUS T1 (FT1), FLOWERING LOCUS T2 (FT2), Flowering Locus C (FLC), FRIGIDA (FRI) or CONSTANS in the plant
15 species of interest, and/or
 - ii) comprises a region of a nucleotide sequence set forth in any one or more of SEQ ID NO's 146, 147, or 151 to 228 (where the T's are replaced with U's), or a complement (antisense) of the region of the sequence, or both the region and the complement, or a nucleotide sequence 95%, preferably, 99%, identical thereto (where
20 the T's are replaced with U's).
80. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein the target RNA is a gene transcript of the following from wheat, VRN1/VRN-A1 (SEQ ID NO:151); VRN2
25 (SEQ ID NO:145); FT (SEQ ID NO:152) or homologous genes in other species, preferably cereal species.
81. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein the target RNA is a gene
30 transcript of the following from canola, BnFLC1 (SEQ ID NO:179); BnFLC2 (SEQ ID NO:180); BnFLC3 (SEQ ID NO:181); BnFLC4 (SEQ ID NO:182); BnFLC5 (SEQ ID NO:183); BnFRI (SEQ ID NO:184); BnFT (SEQ ID NO:185) or homologous genes in other species, preferably a *Brassica* sp.
- 35 82. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein the target RNA is a gene

transcript of the following from *Arabidopsis*, FRI; FLC; VRN1; VRN2; VIN3; FT; SOC1; CO (constans); LFY; AP1, or homologous genes in other species.

83. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA
5 molecule according to any one of claims 40 to 78, wherein the target RNA is a gene transcript of the following from rice, OsPhyB (SEQ ID NO:156); OsCol4 (SEQ ID NO:157); RFT1 (SEQ ID NO:158); OsSNB (SEQ ID NO:159); OsIDS1 (SEQ ID NO:160); OsGI (SEQ ID NO:161), OsMADS50 (SEQ ID NO:162), OsMADS55 (SEQ ID NO:163) or OsLFY (SEQ ID NO:164), or homologous genes in other species.

10

84. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein

i) the target RNA is a gene transcript of the following from *Medicago truncatula*, MtFTa1 (SEQ ID NO:186); MtFTb1 (SEQ ID NO:187), MtYFL (SEQ ID
15 NO:210), MtSOC1a, MtSOC1b, MtSOC1c, or homologous genes in other species,

ii) the target RNA is a gene transcript of the one of the following from maize (*Zea mays*): ZmMADS1/ZmM5 (SEQ ID NO:165), PHYA1 (SEQ ID NO:166), PHYA2 (SEQ ID NO:167), PHYB1 (SEQ ID NO:168), PHYB2 (SEQ ID NO:169), PHYC1 (SEQ ID NO:170), PHYC2 (SEQ ID NO:171), ZmLD (SEQ ID NO:172),
20 ZmFL1 (SEQ ID NO:173), ZmFL2 (SEQ ID NO:174), DWARF8 (SEQ ID NO:175), ZmAN1 (SEQ ID NO:176), ZmID1 (SEQ ID NO:177), ZCN8 (SEQ ID NO:178), or homologous genes in other species, preferably cereal species,

iii) the target RNA is a gene transcript of one of the following from alfalfa (*Medicago sativa*), MsFRI-L (SEQ ID NO:188), MsSOC1a (SEQ ID NO:189), or
25 MsFT (SEQ ID NO:190), or homologous genes in other species,

iv) the target RNA is a gene transcript of one of the following from soybean (*Glycine max*): encoded by the gene GLYMA_05G148700 with any one or more of the following transcript variants GmFLC-X1 (SEQ ID NO:191), GmFLC-X2 (SEQ ID NO:192), GmFLC-X3 (SEQ ID NO:193), GmFLC-X4 (SEQ ID NO:194), GmFLC-X5
30 (SEQ ID NO:195), GmFLC-X6 (SEQ ID NO:196), GmFLC-X7 (SEQ ID NO:197), GmFLC-X8 (SEQ ID NO:198), GmFLC-X9 (SEQ ID NO:199), SUPPRESSOR OF FRI (SEQ ID NO:200), GmFRI (SEQ ID NO:201), GmFT2A (SEQ ID NO:202), GmPHYA3 (SEQ ID NO:203), or GIGANTEA (SEQ ID NO:204), or homologous genes in other species,

v) the target RNA is a gene transcript of the following from sugarbeet (*Beta vulgaris*), BvBTC1 (SEQ ID NO:205), preferably BvFT1 (SEQ ID NO:206) and/or BvFT2 (SEQ ID NO:207), or homologous genes in other species,

vi) the target RNA is a gene transcript of one of the following genes from
5 *Brassica rapa*, which may be turnip, cabbage, bok choy, turnip rape or related crucifers: BrFLC2 (SEQ ID NO:208), BrFT or BrFRI (SEQ ID NO:209), or homologous genes in other species,

vii) the target RNA is a gene transcript of one of the following from cotton (*Gossypium hirsutum*): GhCO, GhFLC, GhFRI, GhFT, GhLFY, GhPHYA, GhPHYB,
10 GhSOC1, GhVRN1, GhVRN2, GhVRN5, or homologous genes in other species,

viii) the target RNA is a gene transcript of one of the following from onion (*Allium cepa*): AcGI (SEQ ID NO:211), AcFKF (SEQ ID NO:212), AcZTL (SEQ ID NO:213), AcCOL (SEQ ID NO:214), AcFTL (SEQ ID NO:215), AcFT1 (SEQ ID NO:216), AcFT2 (SEQ ID NO:217), AcFT6 (SEQ ID NO:218), AcPHYA (SEQ ID
15 NO:219), AcCOP1 (SEQ ID NO:220), or homologous genes in other species,

ix) the target RNA is a gene transcript of one of the following from asparagus (*Asparagus officinalis*): FPA, TWIN SISTER of FT-like, MOTHER of FT, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1, FLOWERING LOCUS T-like, Flowering locus K, Flowering time control protein FY, flowering time control
20 protein FCA-like, or homologous genes in other species,

x) the target RNA is a gene transcript of one of the following from lettuce (*Lactuca sativa*): LsFT (SEQ ID NO:221), TFL1-like (SEQ ID NO:222), TFL1 homolog 1-like (SEQ ID NO:223), LsFLC (SEQ ID NO:224), LsSOC1-like (SEQ ID NO:225, SEQ ID NO:226 or SEQ ID NO:227), TsLFY (SEQ ID NO:228), or
25 homologous genes in other species, or

xi) the target RNA is a gene transcript of the one of the following from barley: HvVRN1 (SEQ ID NO:153), HvVRN2 (SEQ ID NO:154) or HvFT (SEQ ID NO:155), or homologous genes in other species, preferably cereal species.

30 85. The RNA molecule according to any one of claims 1 to 39, or the chimeric RNA molecule according to any one of claims 40 to 78, wherein the target RNA is a miRNA.

86. The RNA molecule or the chimeric RNA molecule according to claim 86, wherein the miRNA is miR-156 or miR-172.

87. The RNA molecule according to any one of claims 1 to 39 or 79 to 86, or the chimeric RNA molecule according to any one of claims 40 to 86 which reduces the time to flowering compared to an isogenic plant lacking the RNA molecule or chimeric RNA molecule.
- 5
88. The RNA molecule according to any one of claims 1 to 39 or 79 to 86, or the chimeric RNA molecule according to any one of claims 40 to 86 which delays the time to flowering compared to an isogenic plant lacking the RNA molecule or chimeric RNA molecule.
- 10
89. The RNA molecule according to any one of claims 1 to 39 or 79 to 88, or the chimeric RNA molecule according to any one of claims 40 to 88, wherein the plant is *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye.
- 15
90. The RNA molecule according to any one of claims 1 to 39 or 79 to 89, or the chimeric RNA molecule according to any one of claims 40 to 89, wherein the plant is genetically unmodified.
- 20
91. An isolated and/or exogenous polynucleotide encoding an RNA molecule according to any one of claims 1 to 39 or 79 to 90, or a chimeric RNA molecule according to any one of claims 40 to 90.
92. The polynucleotide of claim 91 which is a DNA construct.
- 25
93. The polynucleotide of claim 91 or claim 92 which is operably linked to a promoter capable of directly expression of the RNA molecule in a plant cell.
94. The polynucleotide of claim 93, wherein the promoter is an RNA polymerase promoter such as an RNA polymerase III promoter, an RNA polymerase II promoter, or a promoter which functions *in vitro*.
- 30
95. The polynucleotide according to any one of claims 91 to 94 which encodes an RNA precursor molecule comprising an intron in at least one loop sequence which is
- 35
- capable of being spliced out during transcription of the polynucleotide in a plant cell or *in vitro*.

96. The polynucleotide according to any one of claims 91 to 94 which comprises a nucleotide sequence set forth in SEQ ID NO:150.
- 5 97. A vector comprising a polynucleotide according to any one of claims 91 to 96.
98. The vector of claim 97 which is a viral vector.
99. A host cell comprising one or more or all of an RNA molecule according to any
10 one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of
claims 40 to 90, small RNA molecules (20-24nt in length) produced by processing of
the RNA molecule or chimeric RNA molecule, a polynucleotide according to any one
of claims 91 to 96, or a vector of claim 97 or claim 98.
- 15 100. The host cell of claim 99 which is a plant cell.
101. The polynucleotide claim 93 or claim 94 or the host cell of claim 99 or claim
100 which encodes and/or comprises the chimeric RNA molecule according to any one
of claims 40 to 90, wherein the promoter region of the polynucleotide has a lower level
20 of methylation, such as less than about 50%, less than about 40%, less than about 30%
or less than about 20%, when compared to the promoter of a corresponding
polynucleotide encoding an RNA molecule which has a corresponding dsRNA region
which is fully basepaired with canonical basepairs.
- 25 102. A host cell according to any one of claims 99 to 101 which is a plant cell
comprising the chimeric RNA molecule or small RNA molecules produced by
processing of the chimeric RNA molecule, or both, wherein the chimeric RNA
molecule comprises, in 5' to 3' order, the first sense ribonucleotide sequence, the first
linking ribonucleotide sequence which comprises a loop sequence, and the first
30 antisense ribonucleotide sequence.
103. The host cell according to any one of claims 99 to 102 which comprises at least
two copies of the polynucleotide encoding a chimeric RNA molecule according to any
one of claims 40 to 90, and wherein

i) the level of reduction in the expression or activity of the target RNA molecule in a plant cell is at least the same when compared to if the cell had a single copy of the polynucleotide, and/or

ii) the level of reduction in the expression or activity of the target RNA molecule in a plant cell is lower when compared to a corresponding cell comprising an RNA molecule which has a corresponding dsRNA region which is fully basepaired with canonical basepairs.

104. The host cell according to any one of claims 99 to 103, wherein the cell encodes and/or comprises the chimeric RNA molecule according to any one of claims 40 to 90 and the level of sense ribonucleotide sequence in the cell is less than 50 to 99% the level of the antisense ribonucleotide.

105. A plant comprising one or more or all of an RNA molecule according to any one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of claims 40 to 90, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, a polynucleotide according to any one of claims 91 to 96, a vector of claim 97 or claim 98, or a host cell according to any one of claims 99 to 104 which is a plant cell.

20

106. The plant of claim 105 which comprises the polynucleotide according to any one of claims 91 to 96.

107. The plant of claim 106, wherein the polynucleotide is stably integrated into the genome of the plant.

25

108. A method of producing an RNA molecule according to any one of claims 1 to 39 or 79 to 90, or a chimeric RNA molecule according to any one of claims 40 to 90, or small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, the method comprising expressing the polynucleotide according to any one of claims 91 to 96 or 101 in a host cell or cell-free expression system.

109. The method of claim 108 which further comprises at least partially purifying the RNA molecule.

35

110. A method of producing the plant according to any one of claims 105 to 107, the method comprising introducing the polynucleotide according to any one of claims 91 to 96 or 101 into a plant cell so that it is stably integrated into the genome of the cell, and generating the plant from the cell.

5

111. An extract of a host cell according to any one of claims 99 to 104, wherein the extract comprises the RNA molecule according to any one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of claims 40 to 90, or small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or
10 chimeric RNA molecule, and/or the polynucleotide according to any one of claims 91 to 96 or 101.

112. A composition comprising one or more of an RNA molecule according to any one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of
15 claims 40 to 90, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or chimeric RNA molecule, a polynucleotide according to any one of claims 91 to 96 or 101, a vector of claim 97 or claim 98, a host cell according to any one of claims 99 to 104, or an extract of claim 111, and one or more suitable carriers.

20 113. The composition of claim 112 suitable for application to a field.

114. The composition of claim 113, wherein the field comprises plants.

115. The composition according to any one of claims 112 to 114 which further
25 comprises at least one compound which enhances the stability of the RNA molecule, chimeric RNA molecule or polynucleotide and/or which assists in the RNA molecule, chimeric RNA molecule or polynucleotide being taken up by a cell of a plant.

116. The composition of claim 115, wherein the compound is a transfection
30 promoting agent.

117. A method for down-regulating the level and/or activity of a target RNA molecule which modulates plant flowering in a plant, the method comprising delivering to the plant one or more of an RNA molecule according to any one of claims 1 to 39 or
35 79 to 90, a chimeric RNA molecule according to any one of claims 40 to 90, small RNA molecules (20-24nt in length) produced by processing of the RNA molecule or

chimeric RNA molecule, a polynucleotide according to any one of claims 91 to 96 or 101, a vector of claim 97 or claim 98, a host cell according to any one of claims 99 to 104, an extract of claim 111, or a composition according to any one of claims 112 to 116.

5

118. The method of claim 117, wherein the target RNA molecule encodes a protein.

119. The method of claim 117 or claim 118, wherein the chimeric RNA molecule, or small RNA molecules produced by processing of the chimeric RNA molecule, or both,
10 are contacted with the cell or plant by topical application to the cell or plant.

120. A method of modulating flowering of a plant, the method comprising delivering to the plant one or more of an RNA molecule according to any one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of claims 40 to 90, a
15 polynucleotide according to any one of claims 91 to 96 or 101, a vector of claim 97 or claim 98, a host cell according to any one of claims 99 to 104, an extract of claim 111, or a composition according to any one of claims 112 to 116.

121. The method of claim 120 which results in early flowering of the plant.

20

122. The method of claim 120 or claim 121, wherein the plant is from *Arabidopsis*, corn, canola, cotton, soybean, alfalfa, lettuce, wheat, barley, rice, legume, *Medicago truncatula*, sugarbeet or rye.

25 123. The method of claim 120 which results in late flowering of the plant.

124. The method of claim 123, wherein the plant is a grass.

125. A method of modulating the flowering time of a plant, or a plant produced from
30 a seed, the method comprising contacting the plant or seed with a composition comprising an RNA molecule which comprises at least one double stranded RNA region, and/or a polynucleotide(s) encoding the RNA molecule, wherein the at least one double stranded RNA region comprises an antisense ribonucleotide sequence which is capable of hybridising to a region of a target RNA molecule which modulates the
35 timing of plant flowering.

126. The method of claim 125, wherein the composition is an aqueous composition.
127. The method of claim 125 or claim 126, wherein the composition comprises a transfection promoting agent.
- 5 128. The method according to any one of claims 125 to 127, wherein the method comprises soaking the seed in the composition.
129. The method according to any one of claims 125 to 127, wherein the plant is a
10 seedling, and the method comprises soaking at least a part of the seedling in the composition.
130. The method according to any one of claims 125 to 127, wherein the plant is in a field and the method comprises spraying the composition on at least a part of the plant.
- 15 131. The method according to any one of claims 125 to 130, wherein the polynucleotide is a hairpin RNA, a microRNA, a siRNA or an ledRNA.
132. The method according to any one of claims 125 to 131, wherein the plant has an
20 early flowering time when compared to a control plant that has not been applied with the composition.
133. The method according to any one of claims 125 to 131, wherein the plant has a late flowering time when compared to a control plant that has not been applied with the
25 composition.
134. A kit comprising one or more of an RNA molecule according to any one of claims 1 to 39 or 79 to 90, a chimeric RNA molecule according to any one of claims 40 to 90, small RNA molecules (20-24nt in length) produced by processing of the RNA
30 molecule or chimeric RNA molecule, a polynucleotide according to any one of claims 91 to 96 or 101, a vector of claim 97 or claim 98, a host cell according to any one of claims 99 to 104, an extract of claim 111, or a composition according to any one of claims 112 to 116.

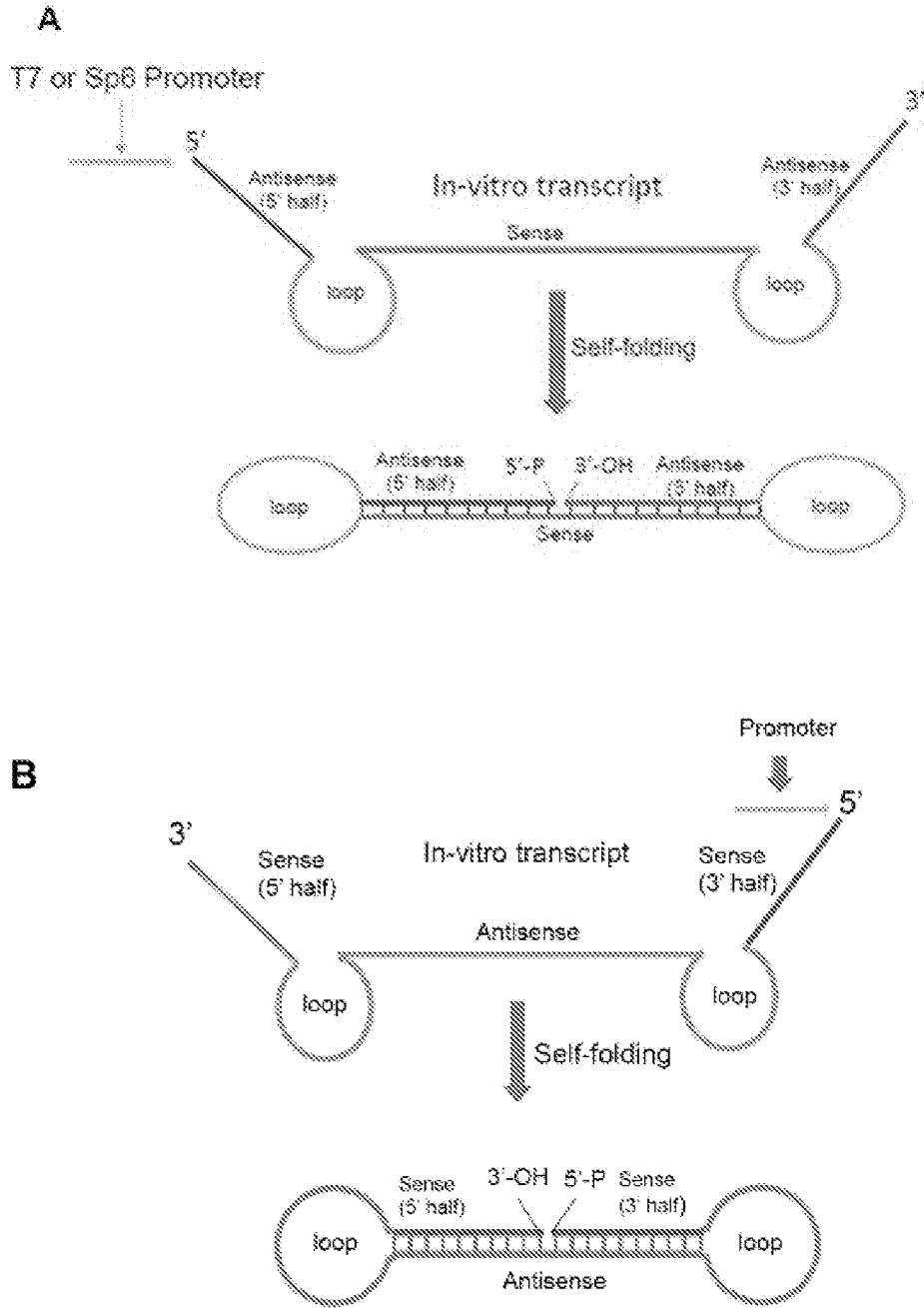


FIGURE 1

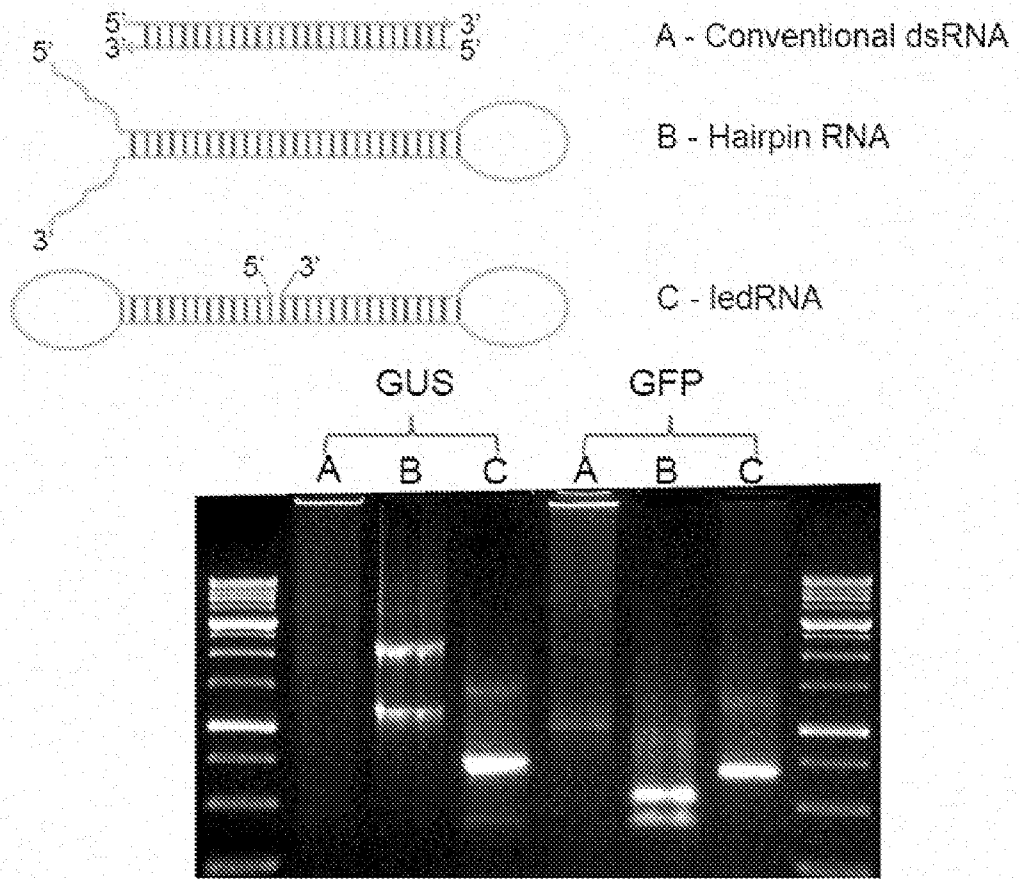


FIGURE 2

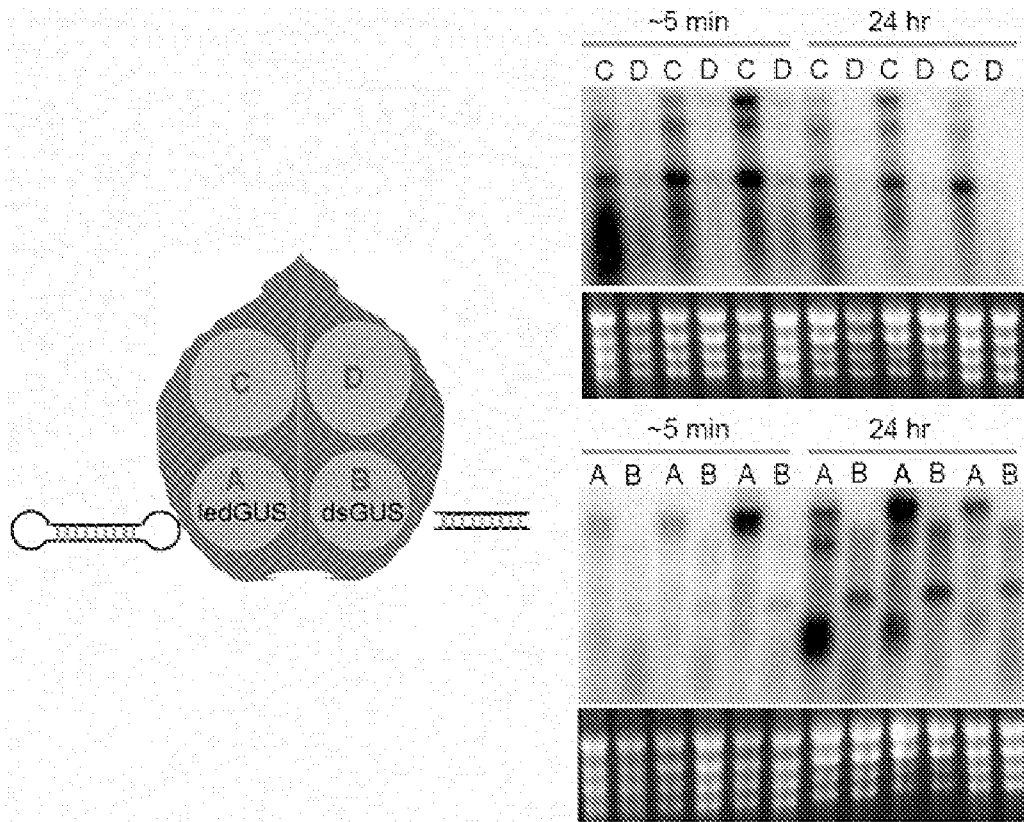


FIGURE 3

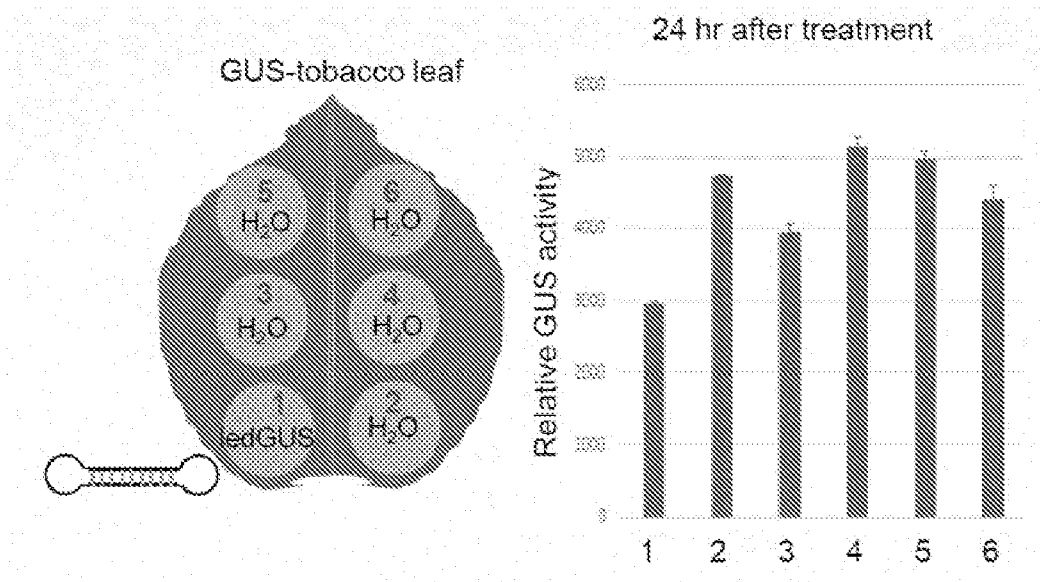


FIGURE 4

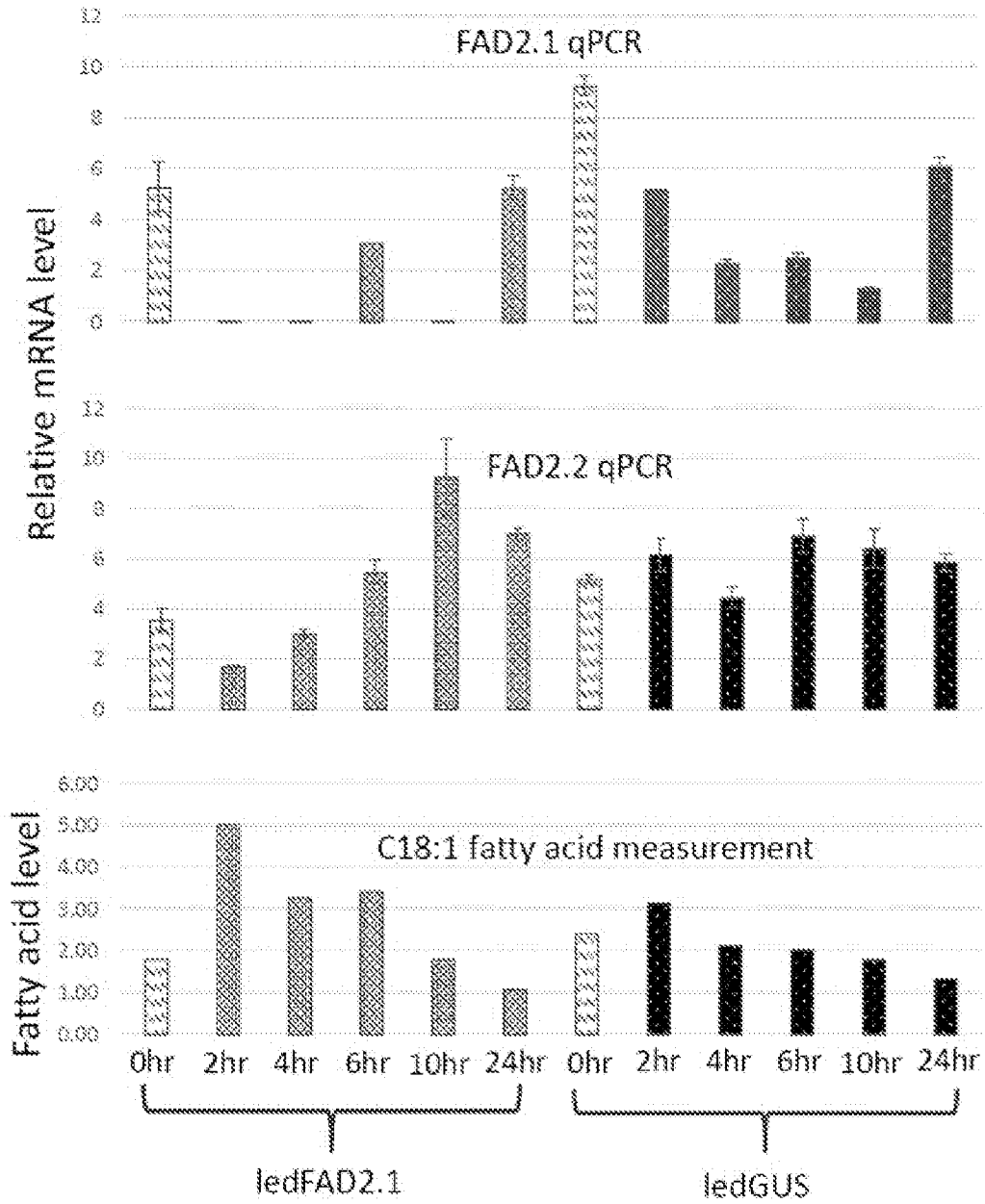


FIGURE 5

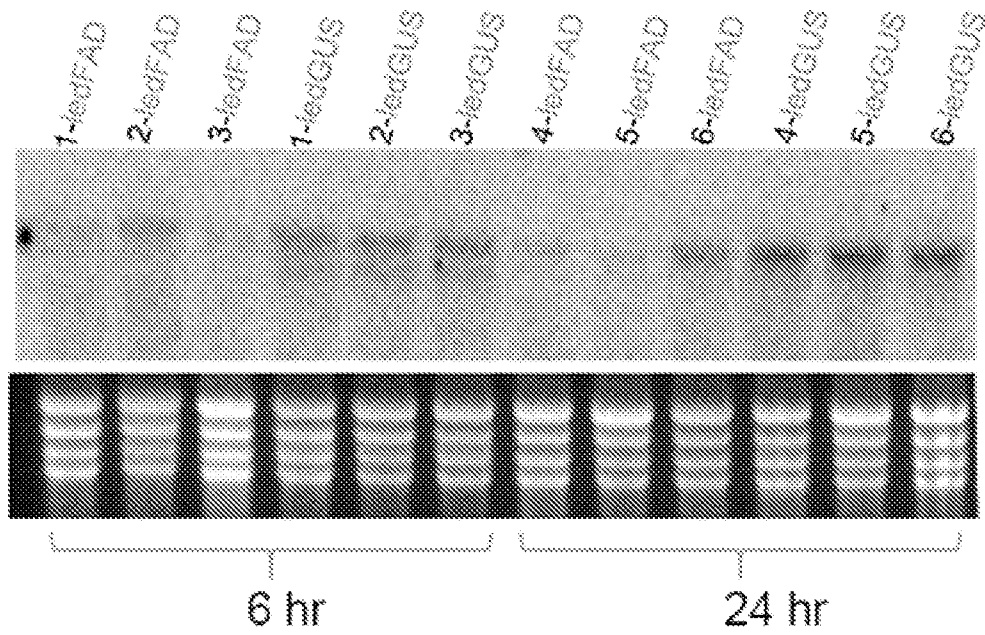


FIGURE 6

Alignment with WT GUS ORF

```

GUS          GAGTGTGATATCTACCCGCTTCGGCTGGGCATCCGGTCAGTGGCAGTGAAGGGCGAACAG 840
GUS-GU      -----TTGTGTGGTATTGGTTAGTGGTASTGAAGGTTGAATAG 40
              * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GUS          TTCCTGATTAACCAACAACCGTTCTACTTTACTGGCTTTCGGTCGTCATGAAGATGCGGAC 900
GUS-GU      TTTTTCATTAATTATTAATTTGTTTATTTTATTTGGTTTTCGGTTGTTATGAAGATGCGGAT 100
              ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GUS          TTGCTGGCAAGGATTCGATAACGTCCTGATGGTGCACGACCACCGCATTAATGGACTGG 960
GUS-GU      TTGTGCGTTAAAGGATTTGATAATGTCGTTGATGGTGTATGATTATGTATTAAATGGACTGG 160
              *** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GUS          ATFGGGGCCAACTCCTACCGTACCTCGCATTACCCCTTACGGTGAAGAGATGCTCGACTGG 1020
GUS-GU      ATFGGGGTTAATTTTATGTAATTTGTATTATTTTATG----- 200
              * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
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FIGURE 7

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GUS          GAGTGTGATATCTACCCGCTTCGCGTCGGCATCCGGTCCAGTGGCAGTGAAGGGCGAACAG 840
GUS-4M      -----TCGGGTCCGCAACCGCTCAGTGGGAGTCAAGCGCGTACAC 40
              *** ***,*** ***,*** ***,*** ***,*** ***,***

GUS          TTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCCGTCATGAAGATGCGGAC 900
GUS-4M      TTCCTGAATAAGCACTAACCGTTGTACATTAGTGGGTTTCGTCCCTCAAGAACATGGGGAG 100
              *** ***,*** ***,*** ***,*** ***,*** ***,*** ***,***

GUS          TTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCCACGCATTAATGGACTGG 960
GUS-4M      TTGGGTGCCAATGGAATCGTTAAGGTGGTGAAGGTCCACCACCTCGCTTTTATGGTCTGC 160
              *** ***,*** ***,*** ***,*** ***,*** ***,*** ***,***

GUS          ATTGGGGCCAACTCCTACCGTACCTCGCATTACCTTACGCTGAAGAGATGCTCGACTGG 1020
GUS-4M      ATTCGGGGCAAGTCCAACCCCTACGTGGGATTTCCCATACC----- 200
              *** ***,*** ***,*** ***,*** ***,*** ***,***

```

FIGURE 8

Alignment with WT GUS ORF

```

GUS          GAGTGTGATATCTACCGCTTCGCGTCCGGCATCCGGTCAGTGGCAGTGAAGGGCGAACAG 840
GUS-10M     -----TCGCGTCCGGCATCCGGTCTCTGGCAGTGTGGGGCGAACTC 40
                *****  *****; *****;*****;

GUS          TTCCTGAFATAACCACAAACCGTTCTACTTTACTGGGCTTTGGTCCGCATGAAGATGCCGSAC 900
GUS-10M     TTCCTGATATAACCACAAAGGGTCTACTAAACTGGCTTACGTCCGCATCTAGATGCCGGTG 100
                *****;***** *****;*****; ***** ;*****;

GUS          TTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGCCACGCATTAATGGACTGG 960
GUS-10M     TTGCGTGGGTAAGGATTCCTTAACGTGCACATGGTGCAGCACCACGCAAAAATGGACTCC 160
                ***** ;***** ;*****; ***** *****;*****

GUS          ATTGGGGCCAACTCCTACCGTACCTCGCATTACCCCTTACGCTGAAGAGATGCTCGACTGG 1020
GUS-10M     ATTGGGGCGTACTCCTACGCTACCTCGCTATACCCCTAGC----- 200
                ***** ;***** *****;*****

```

FIGURE 9

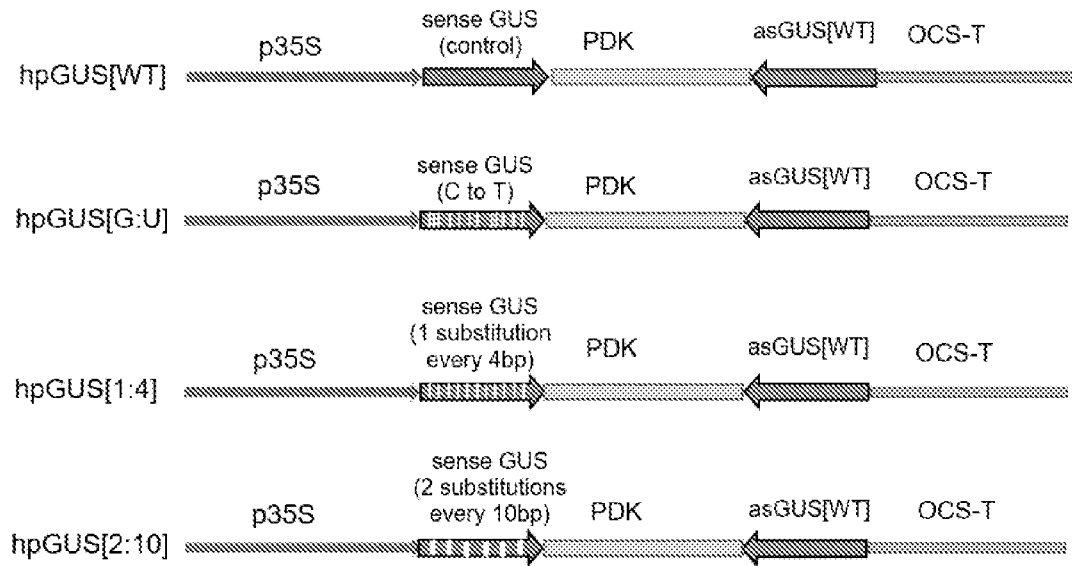


FIGURE 10

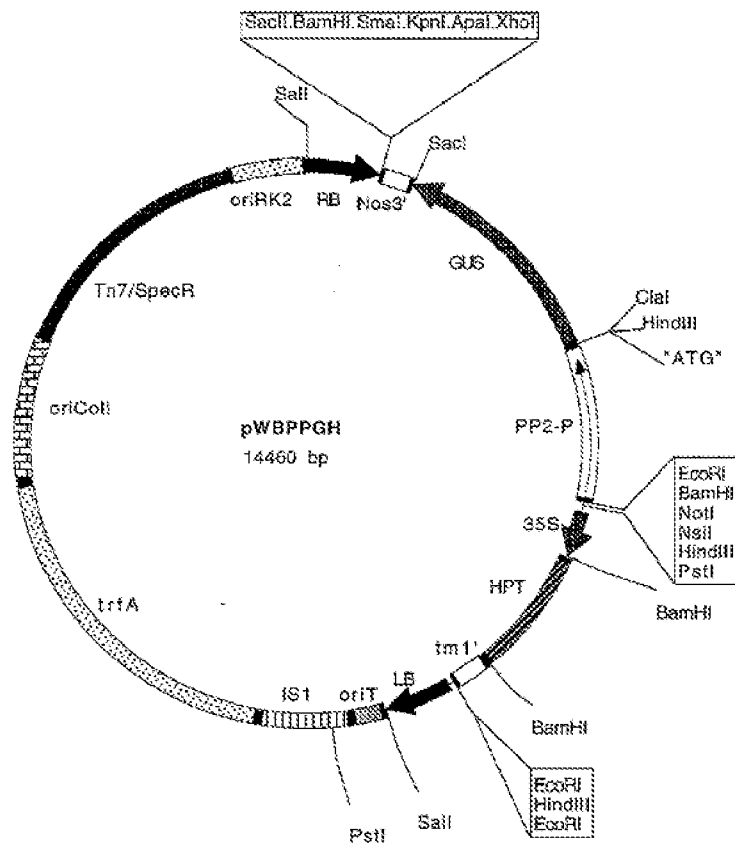


FIGURE 11

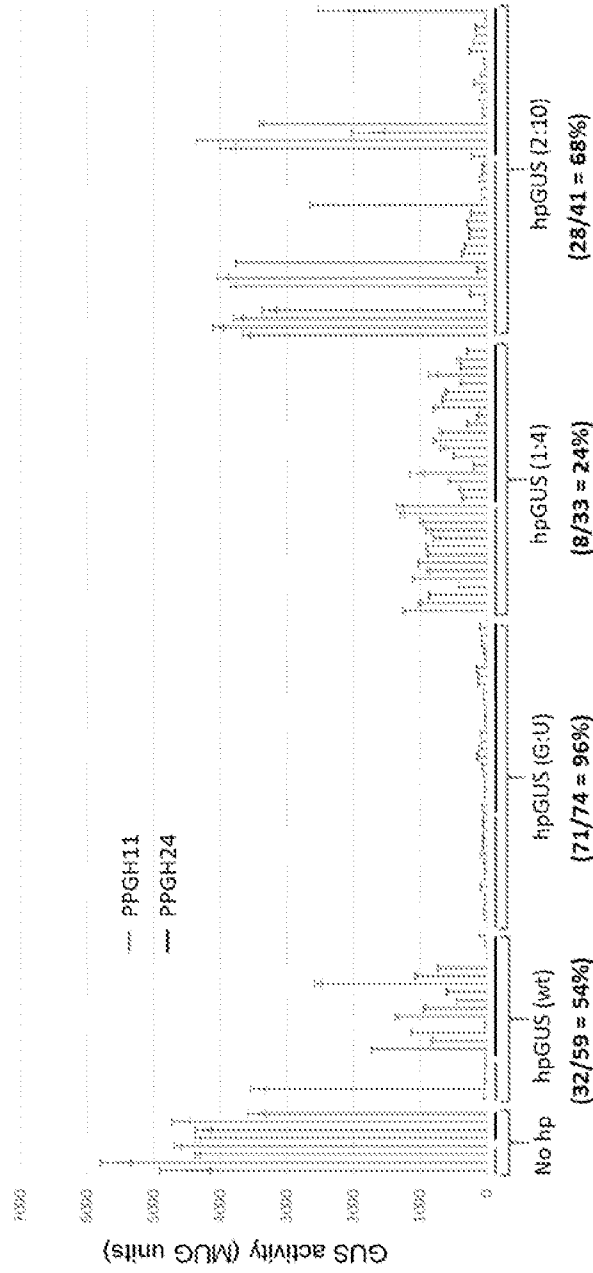


FIGURE 12

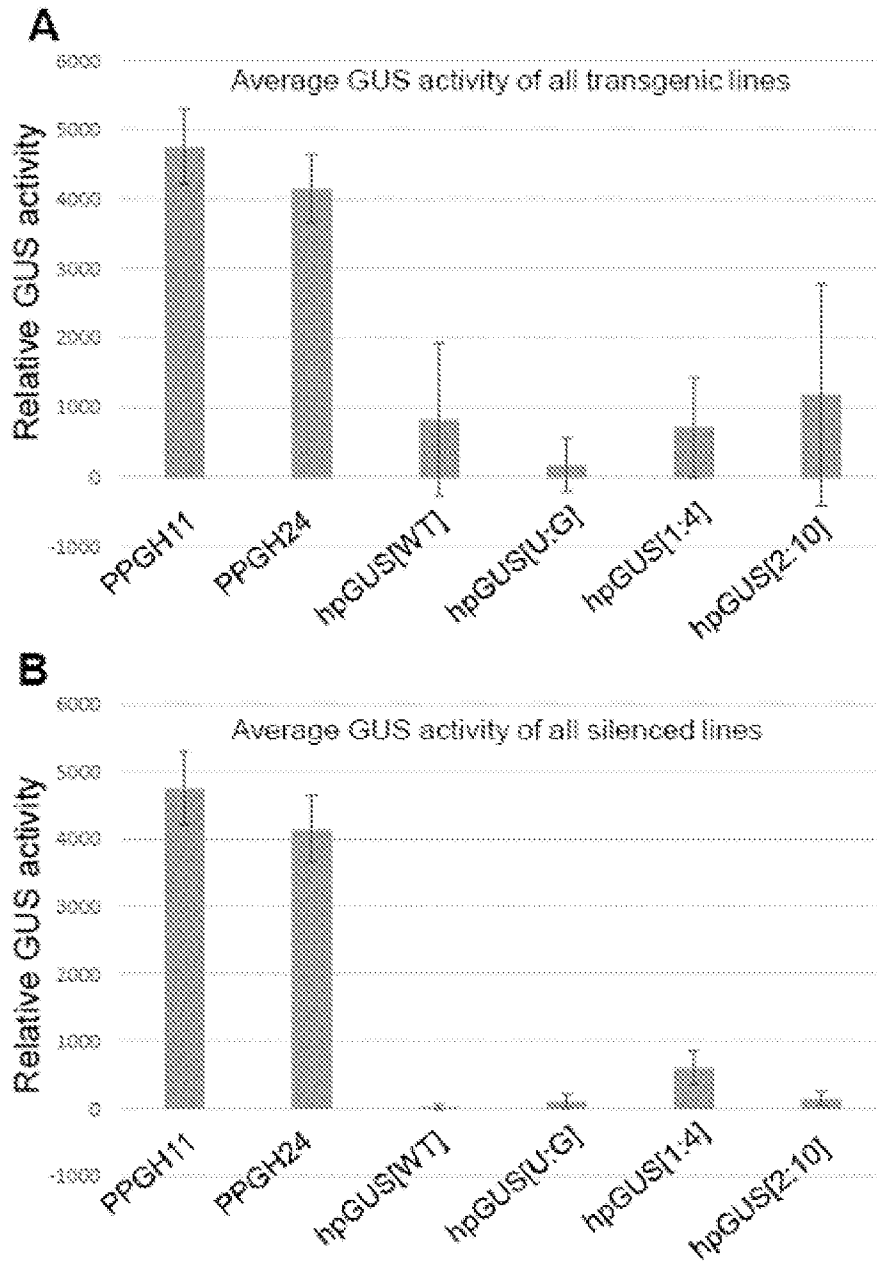


FIGURE 13

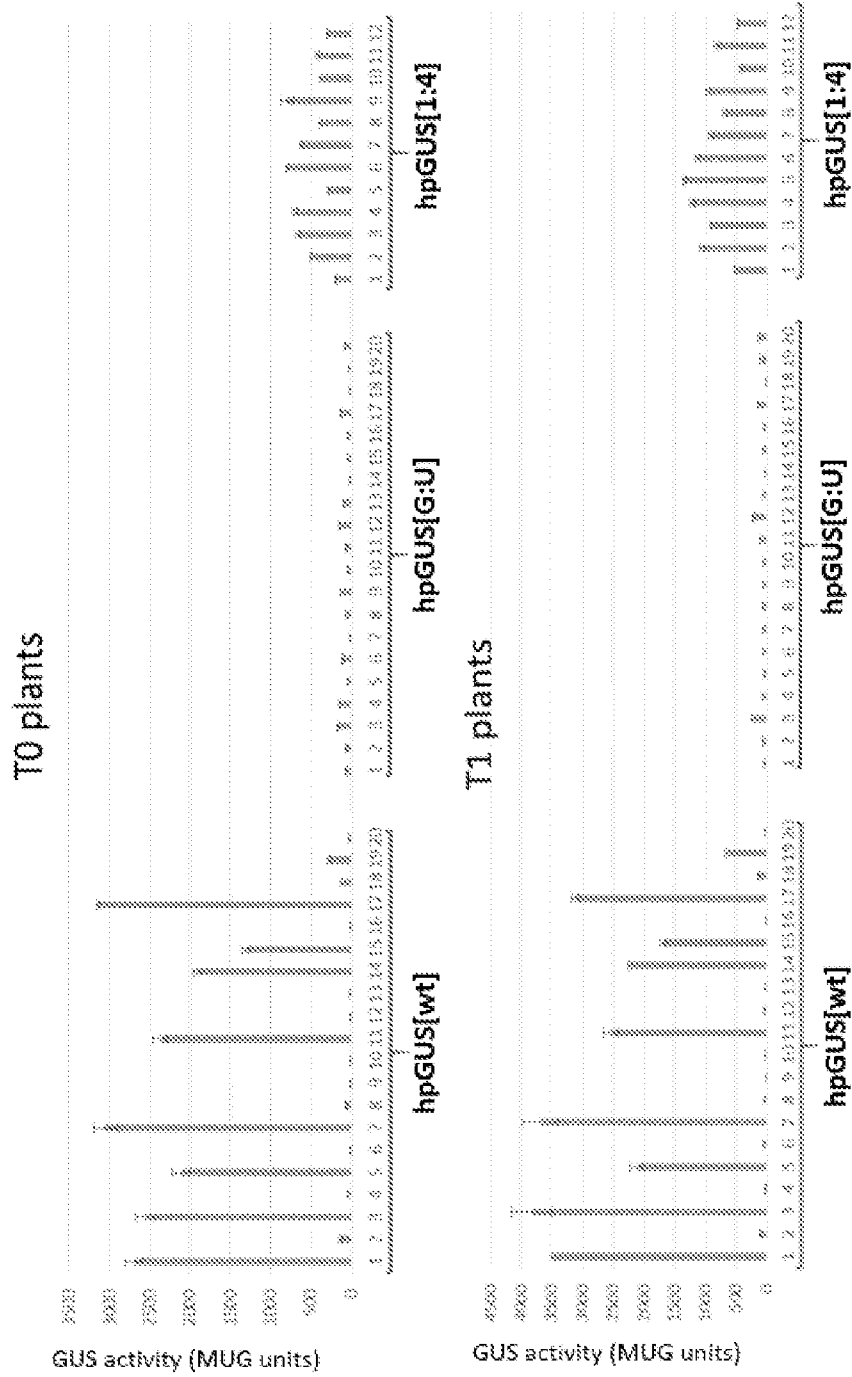


FIGURE 14

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

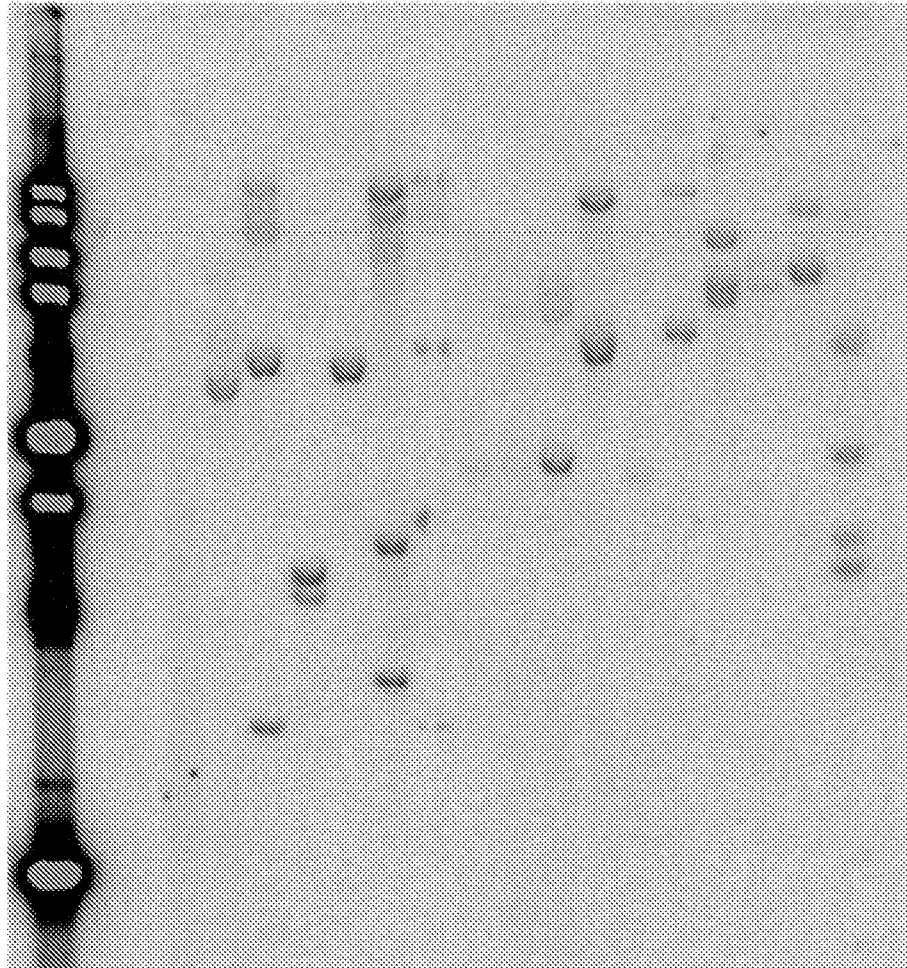


FIGURE 15

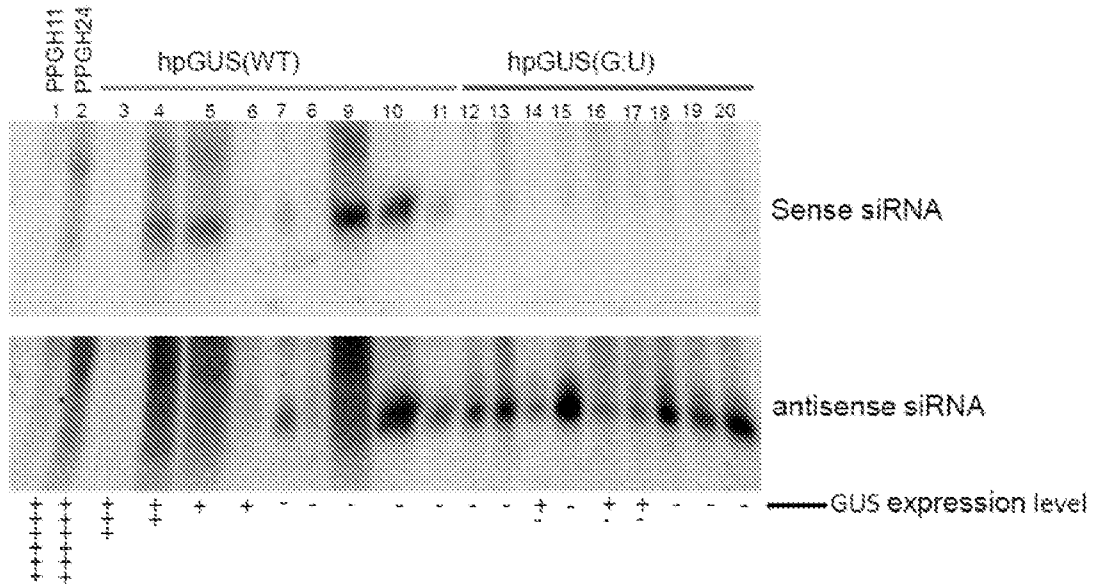


FIGURE 16

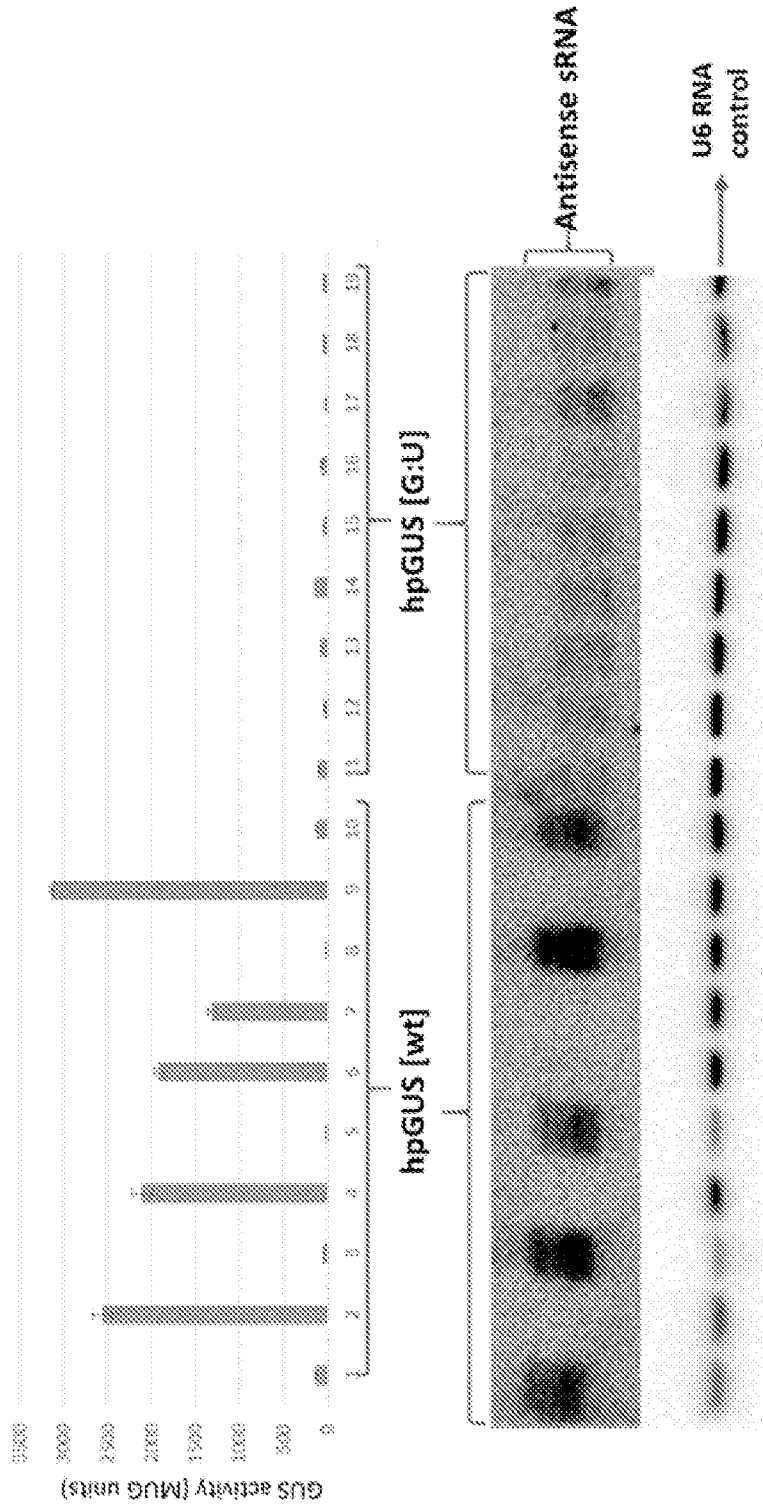


FIGURE 17

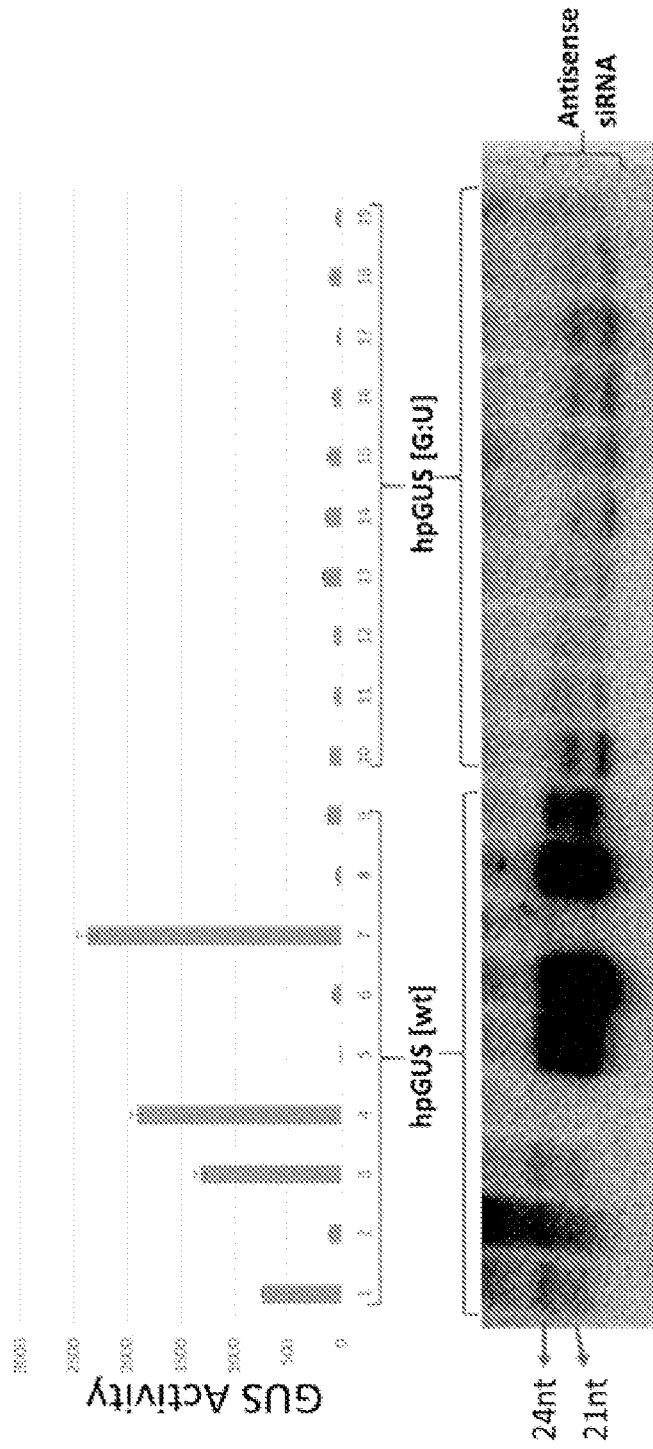


FIGURE 18

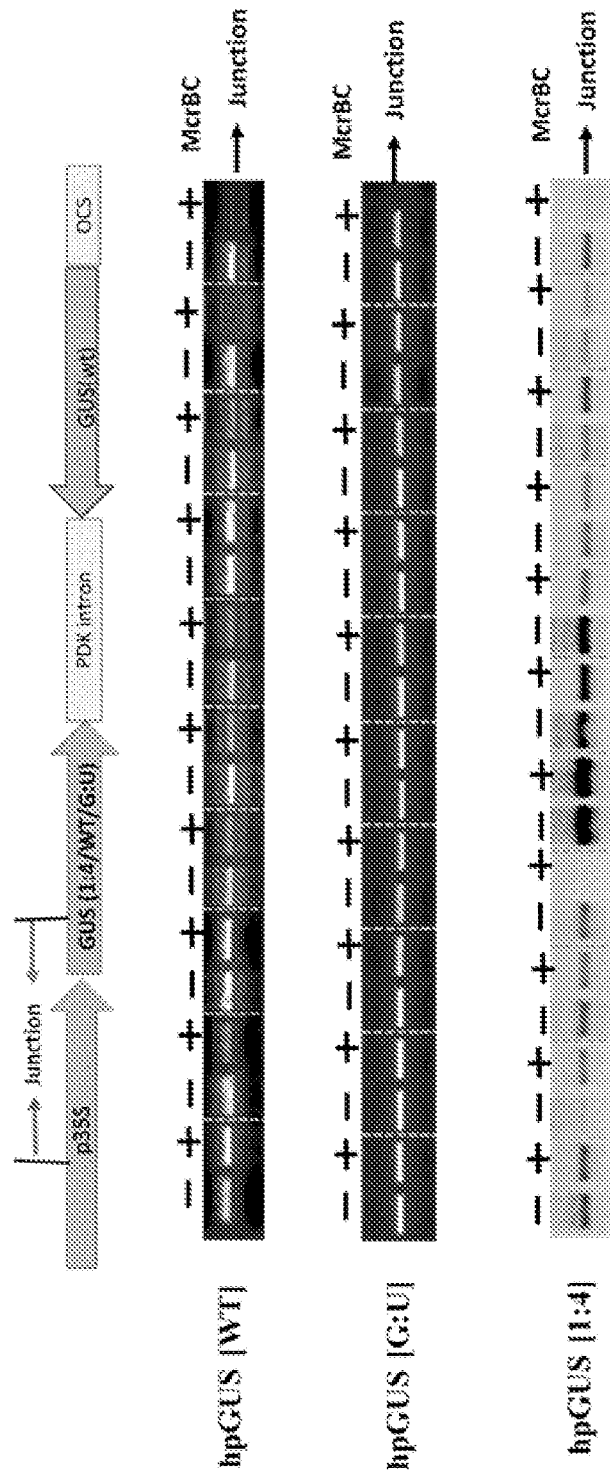


FIGURE 19

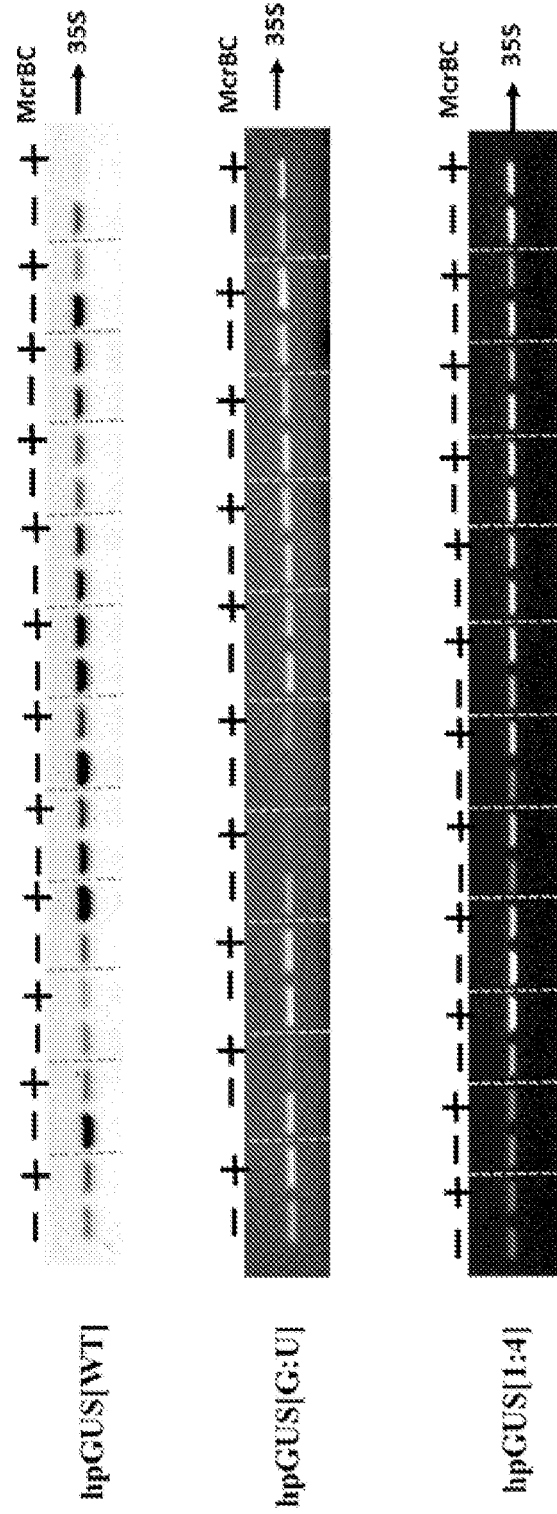
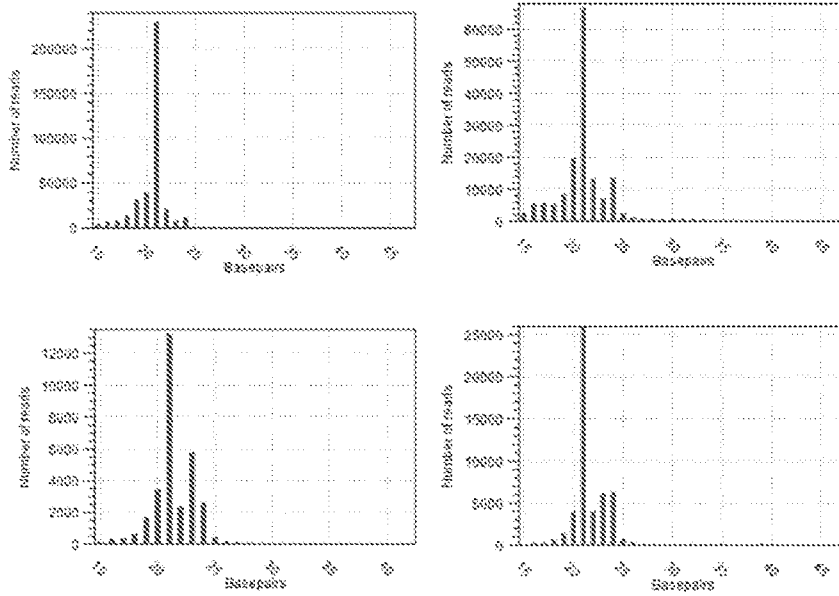


FIGURE 20

A



B

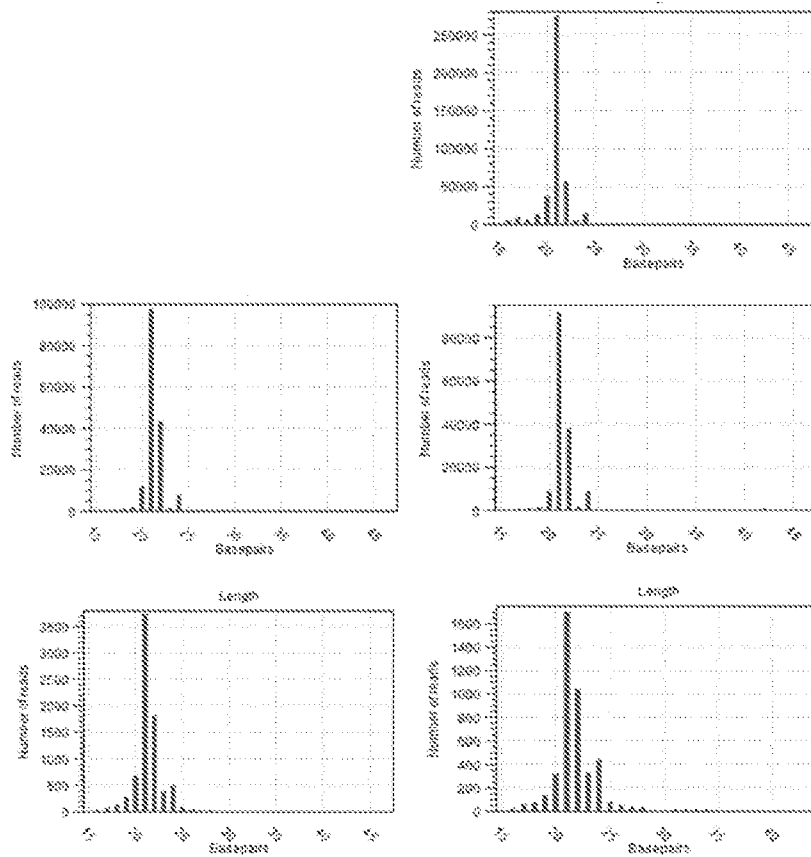


FIGURE 21

```

EIN2-GU      ~~~~~GATTTTA
EIN2-cDNA    AGTAATTATATTATCAGATAGATTTAGGATGGAAGCTGAAATTTGGAATGTGAGACCTCA
                                     ** **

EIN2-GU      GFTAGGTTTATTTGAGAAATGCTTTTCTGTTTATTTTTSTTTTTTGGTTTTGTTG
EIN2-cDNA    GCTAGGTTTATCCAGAAATGTTTCTGCTCTACTTCTGCTCCTTETGTTTCTGTGG
* ***** ** * ** * ** * ***** **

EIN2-GU      ATATATGATTTTGGGAAATGGTTTAAATATGAAGGAGGTGTTTGGTTTGGTATGA
EIN2-cDNA    ATATATGATTCCTGGGAAATGGTTTCAAAATATGGAAGGAGGTGCTTCTGTTTGGGTATGA
***** ***** ***** ***** * ****

EIN2-GU      TTTGGTGGTAAATATTTTGTTTTAAATTTGTTGTTATTTATSTTAAATAGCTGTAGT
EIN2-cDNA    CTGGTGGCAATTAATCTGCTTTTCAATTTGCGCCATCTTATGCCAATATGTTGAGC
***** ***** * ** **** ***** * ** ***** *****

EIN2-GU      TCGTATAAGTGT-----
EIN2-cDNA    TCGCTAAGCGTTGTGACTGGTAAACACTTGGCTCAGATCTGCBATGAGAAATGTGCA
* * **** *

```

FIGURE 22

```

CHS~GU -----GTTGTTTTGTT
CHS~cDNA TCCGTATCGCTAAGGATCTCCGCCGAGAACAAATCGTGGASACAGGTGTCCTCGTTGCTGCT
          ***** ** *

CHS~GU TTCAGATTATAGTTTGTATTTTTTGTGGTTTTTTTTGATATTTAATTTGATTTTTTTTGTTC
CHS~cDNA CTGASATCACAGCCGTTACCTTCCCTGGTCCCTCTGACACCCACCTTCACTCCCTCGTCC
          ***** * ** **** ** ***** * *** * * **** * ** *

CHS~GU GTTAGGTTTTTTTTTAAAGTATGCTGTTGTTGTATTTAATTTGGGGGTTGGATTTTGAATAT
CHS~cDNA STCASSCTCTTTTTCAATGATGGCSCCCGCCACTCATTGTGSSSTCSBACCCCTGACACAT
          ** *** * **** ***** * * * * * ***** *** ** * **

CHS~GU TTGTTGGAGAGAAATTTATTTTTGAGATGGTGTGTTTGTGTTTATATTATTTTTTAGATT
CHS~cDNA CTGTCCGAGAGAAACCCATCTTTGAGATGGTGTCTCCCGCTCAGACCCATCCTTCCAGACT
          *** ***** ** ***** ** * * ** ** ** **

CHS~GU TTGATGGTGT-----
CHS~cDNA CTGATGGTGGCCATAGACGGACATTTGAGGGGAAGTTGGTCTCAGCTTCCATCTCCTCAAGS
          *****

```

FIGURE 23

Antisense EIN2-GU align with antisense EIN2 cDNA

```

As-EIN2-GU      -----AAAGCTTTAGGTGAGTTGTAAAT
asEIN2-cDNA    TCCTTCATTGCAGATCTGAGCCAAAGTGTTTACCAGTCCACACGCTTATGCCGAGCTGCACAC
                ** * ***** ** * * * *
As-EIN2-GU      TATTGGTATAAGATGGTTGGTAAAATTGAAAAGTAGAGTAATTTTATTAAAGTTATATTTG
asEIN2-cDNA    TATTGGCATAAGATGGCCGGCAAAATTCGAAAAGTCAGAGTAATTCGCCACCAAGTCTATACCCG
***** ***** ** ***** ***** * **** ** *
As-EIN2-GU      AAATGAGTATTTTTTTTGAATATTTGTAATTTATTTTTTGGGATTAATATATTTGATAGAA
asEIN2-cDNA    AAACGAGTACCTTCCTTCGATATTTGCAACCCATTTCCCGGATCAGATATACCGACAGAA
*** ** * * ** ***** ** **** ***** ***** ** ****
As-EIN2-GU      ATTAAGAAGGTGGAAGTAGAGTAGAATTTATTTTTTGGATTAATTTTAGTTGAGGTT--
asEIN2-cDNA    ACCAAAGGACAGGAGTAGAGCAGGAAACCATTTCTCTGGATAAACCTAGCTGAGGTTTC
* ***** ***** ***** ** * ***** ** * *****

```

FIGURE 24

Antisense CH5-GU align with antisense CH5 cDNA

```

As-CH5-GU      -----GTAATATTAAGACTTGGAA
asCH5-cDNA    GATGGAGGGTGGACCAACTTCCCTCAAAATGTCCTATGCGACCCATCGAATCTTGGAA
                * * ** ***** *****

As-CH5-GU      GGATGGTTTCGATGCTEGAFATTTATTTAAAGATGGGTTTTTTTTGGATAGATCTGTAG
asCH5-cDNA    GGATGGTTTCGATGCTEGACCTCCATCCCTCTCAAAAGATGGGTTTTCTCTCCGACAGTGTGTCA
***** ** ** * ** * ***** * * ** ***** **

As-CH5-GU      GGTTCGATTTTAAATGATGATGTTGGTGTATTATTGAAAGAGGTTTGGATGATGGG
asCH5-cDNA    GGTTCGACCCGCAATGATGATGCTGGGCGGCGCTCACTGAAAGAGGCTGACCCGACGGGG
** ** * ***** ** * * ** * ***** ** ** ** *****

As-CH5-GU      AGTAAAGGTGGGTGTTAGAGGATTAAGGAGGATATGGTTGATTTTAACTAGATA
asCH5-cDNA    AGTCAAGGTGGGTGTTAGAGGATCCAGGAGGATTAAGGATGCTGATCTCAAGCCAGACA
** ***** ***** * ***** ** ***** * ** ** ** **

```

FIGURE 25

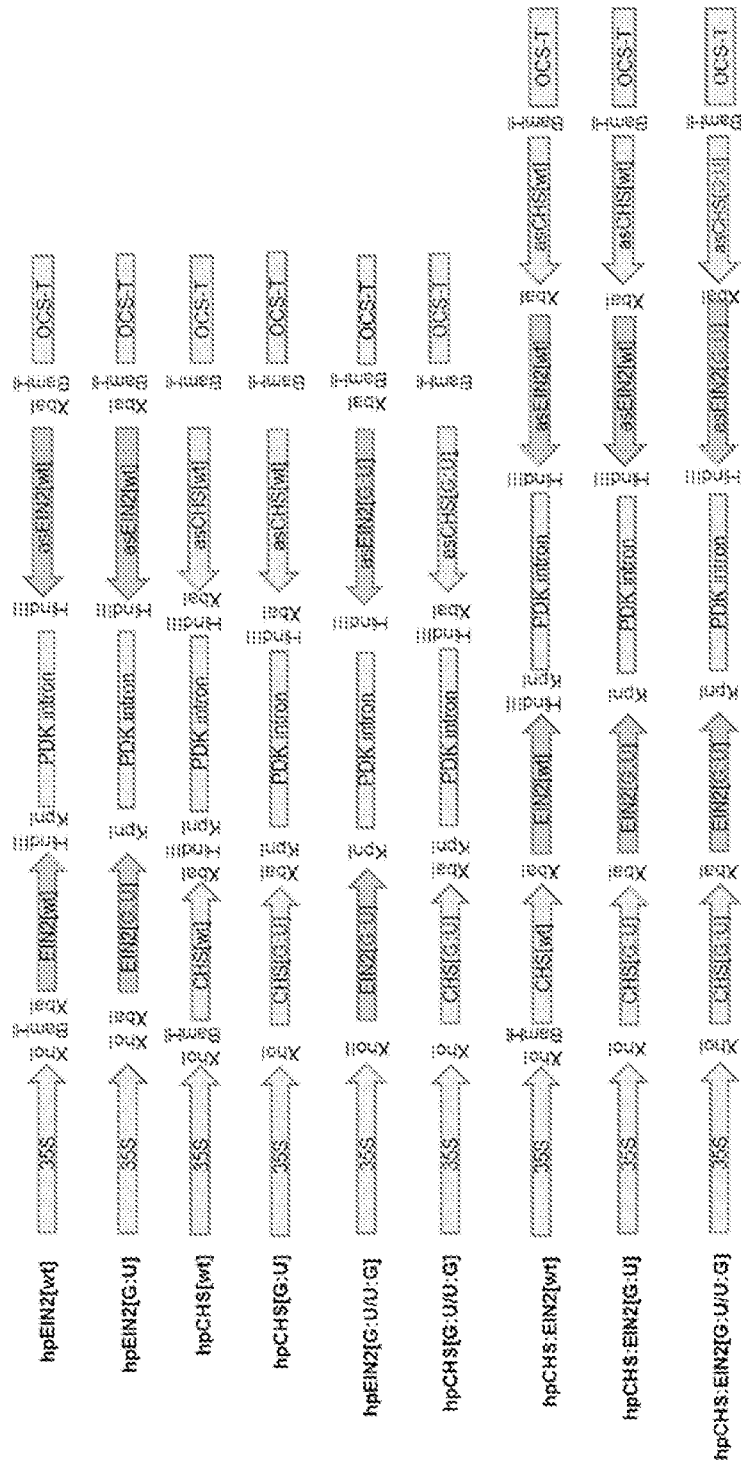


FIGURE 26

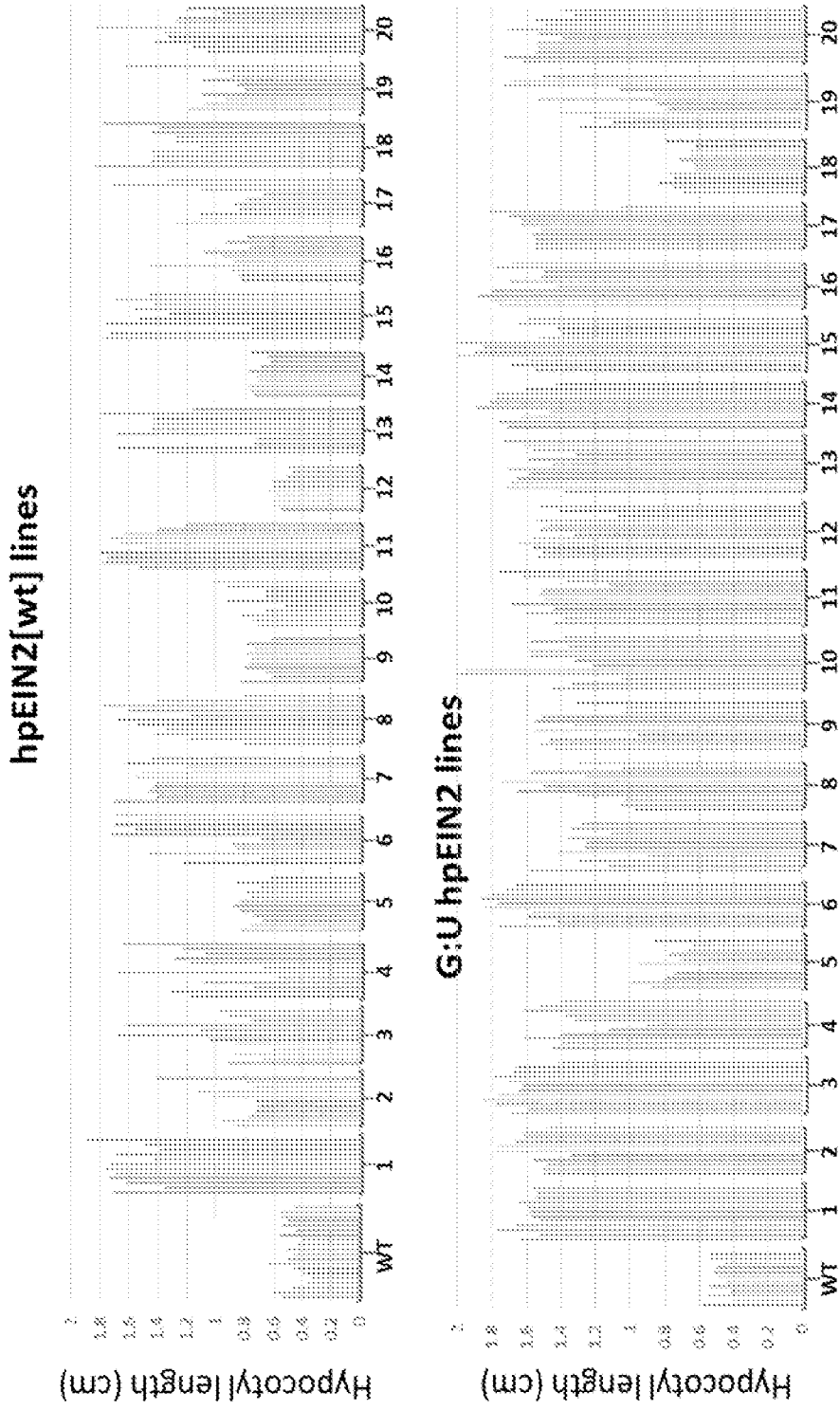


FIGURE 27

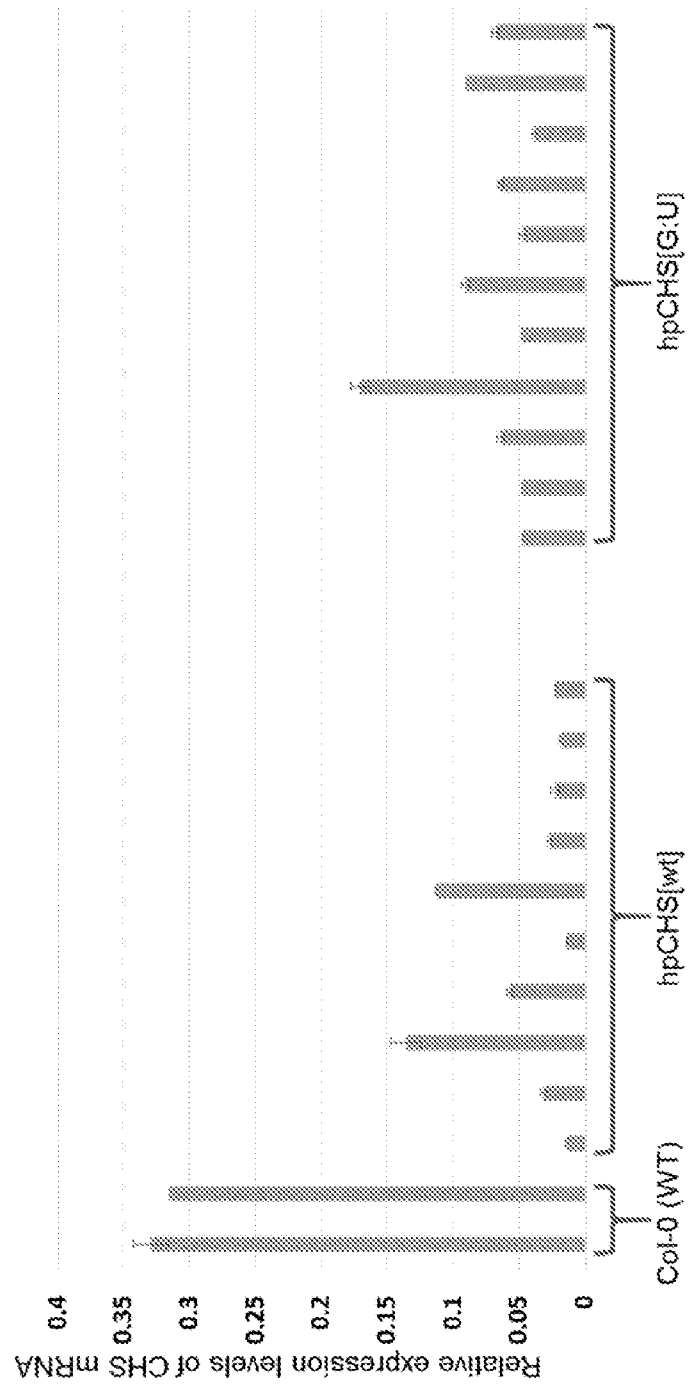


FIGURE 28

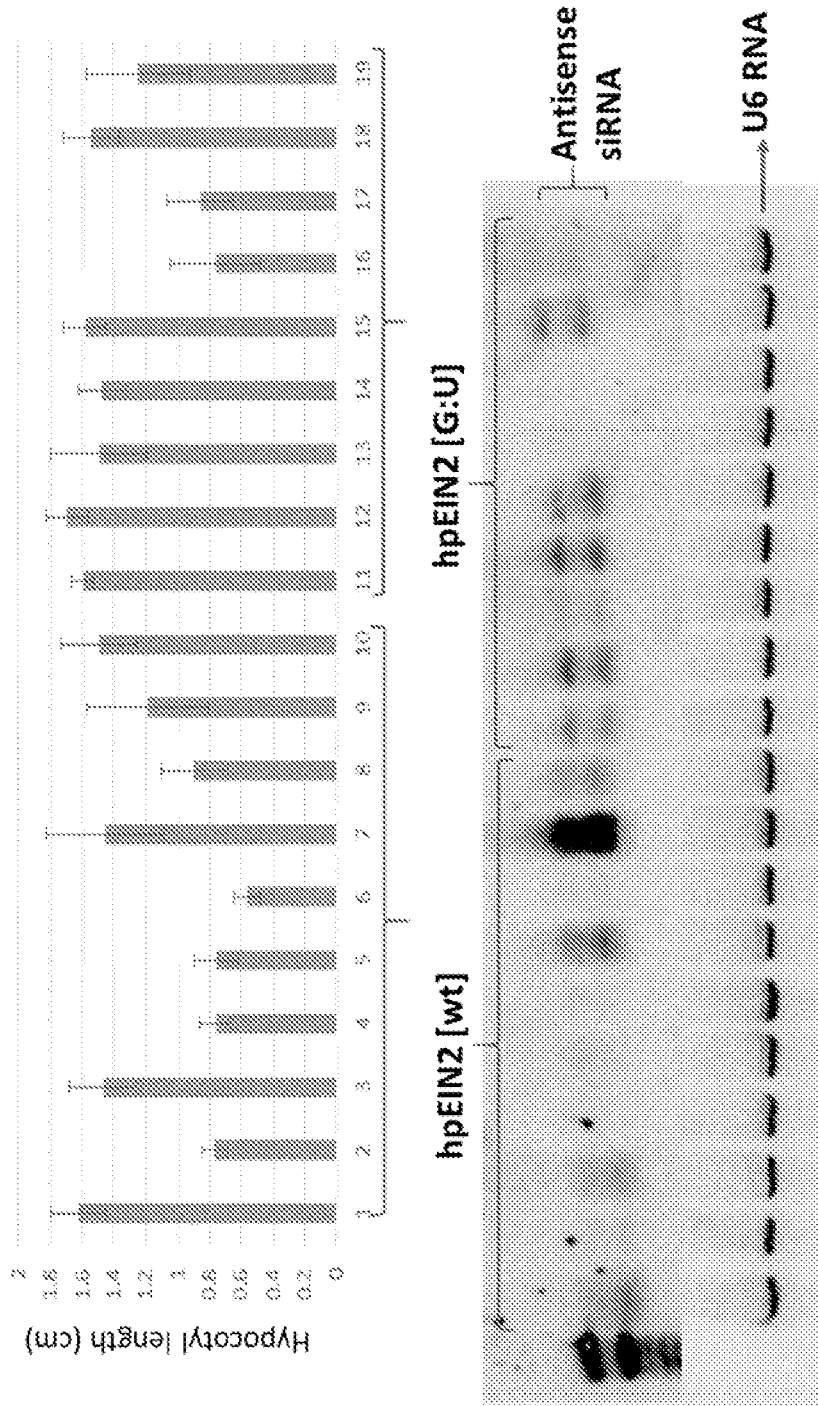


FIGURE 29

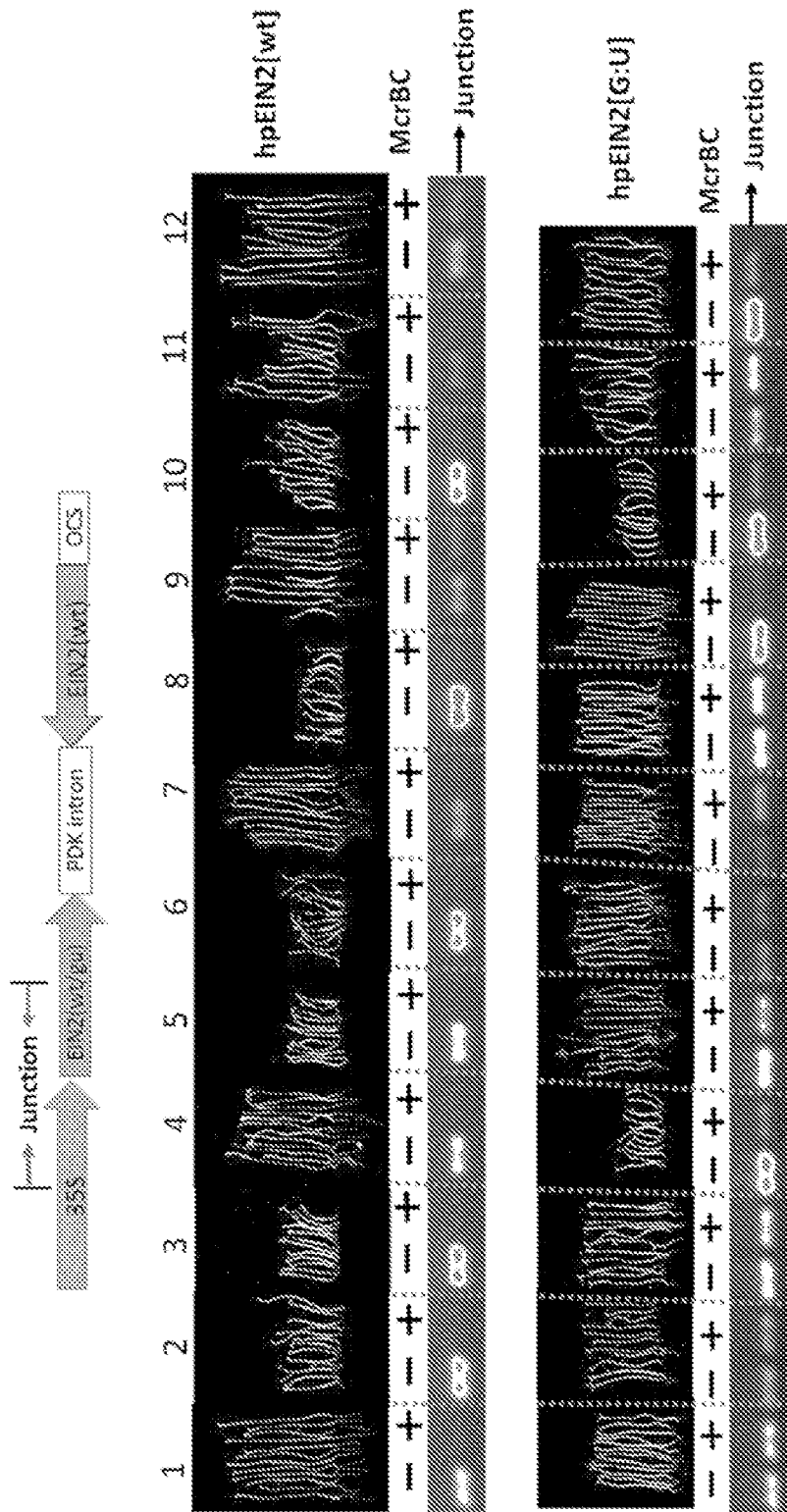


FIGURE 30

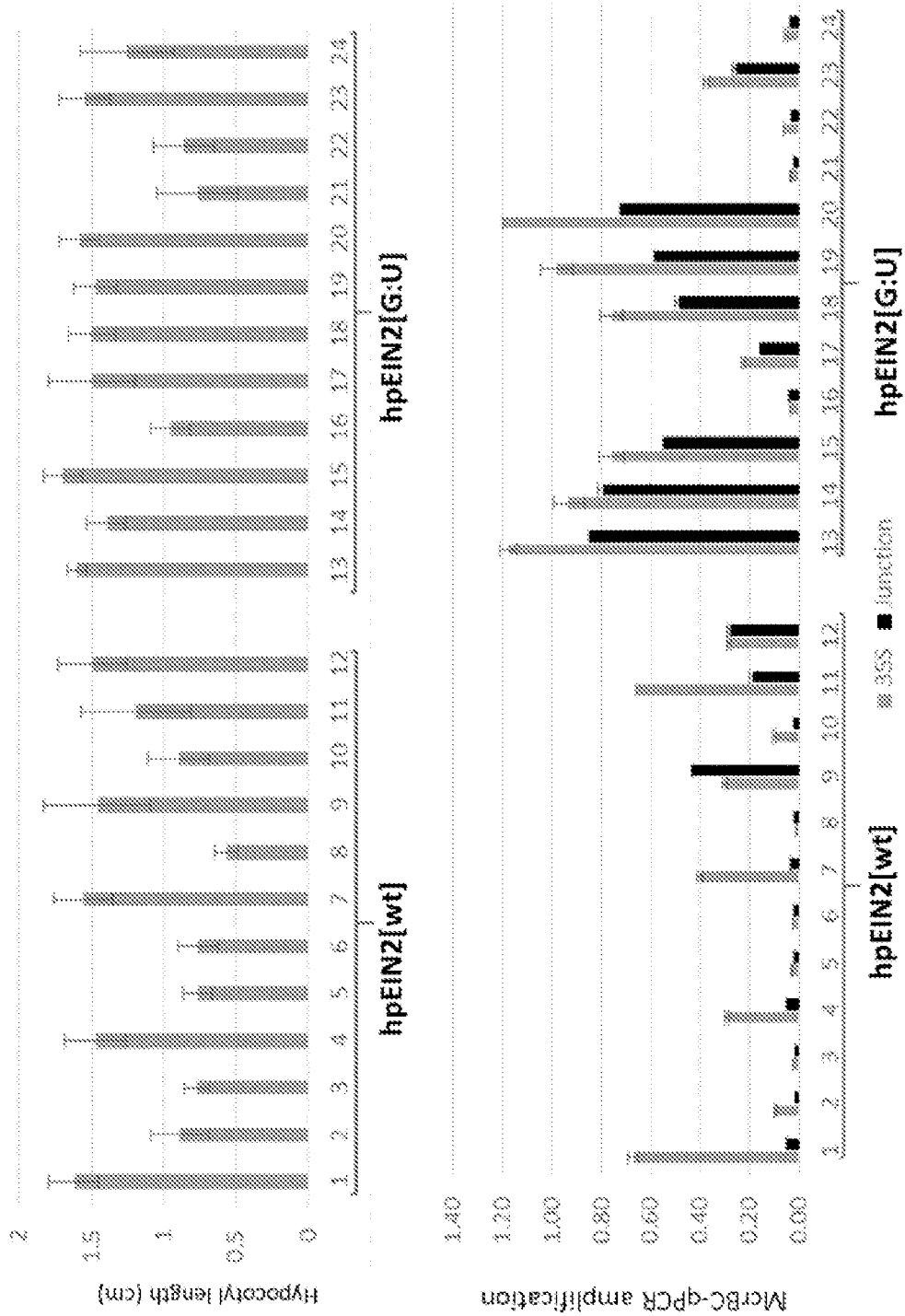


FIGURE 31

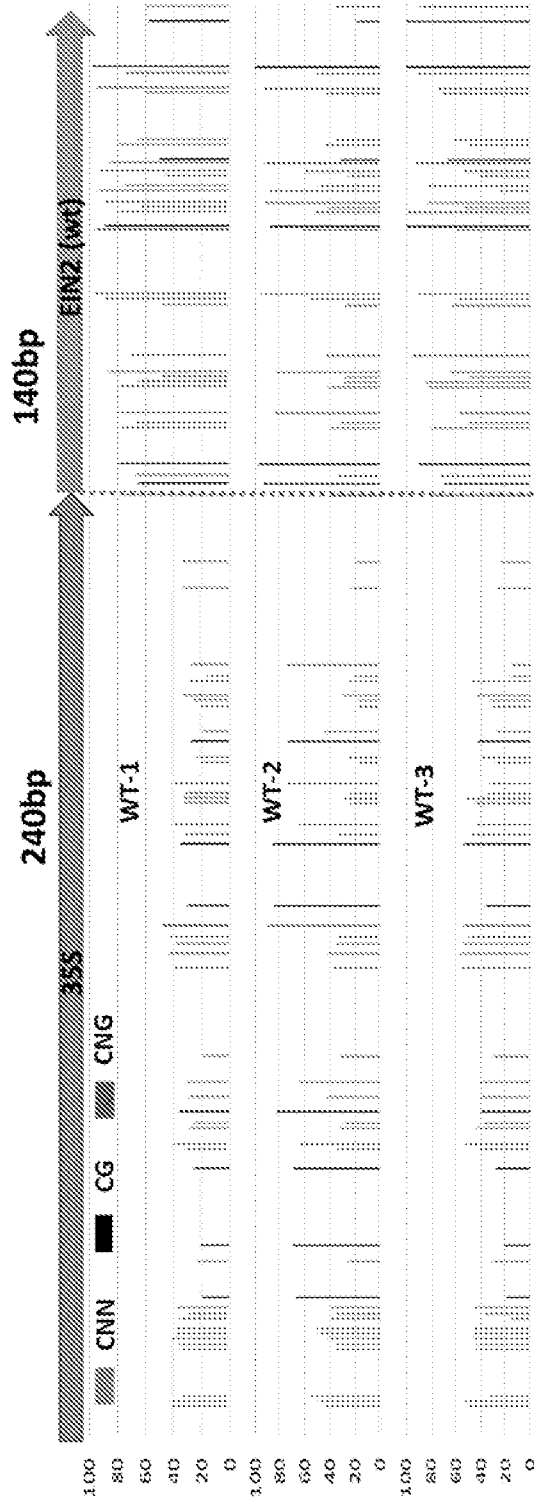


FIGURE 32

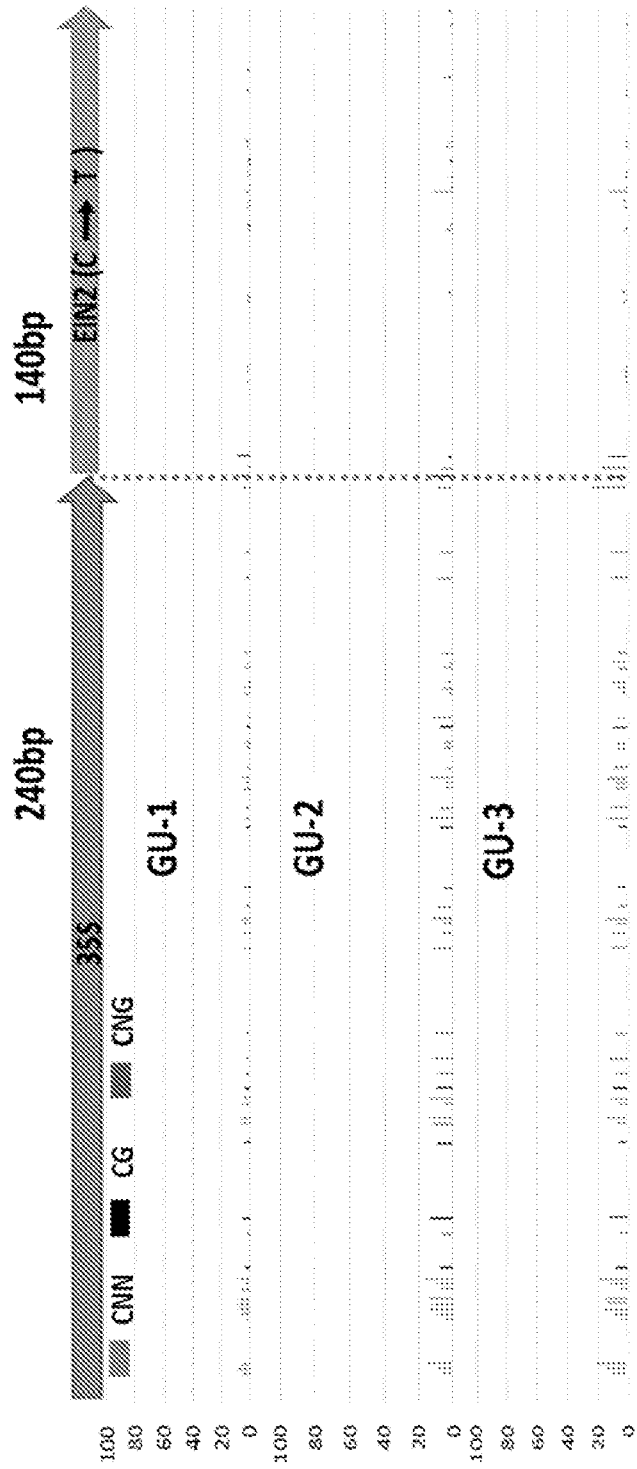


FIGURE 33

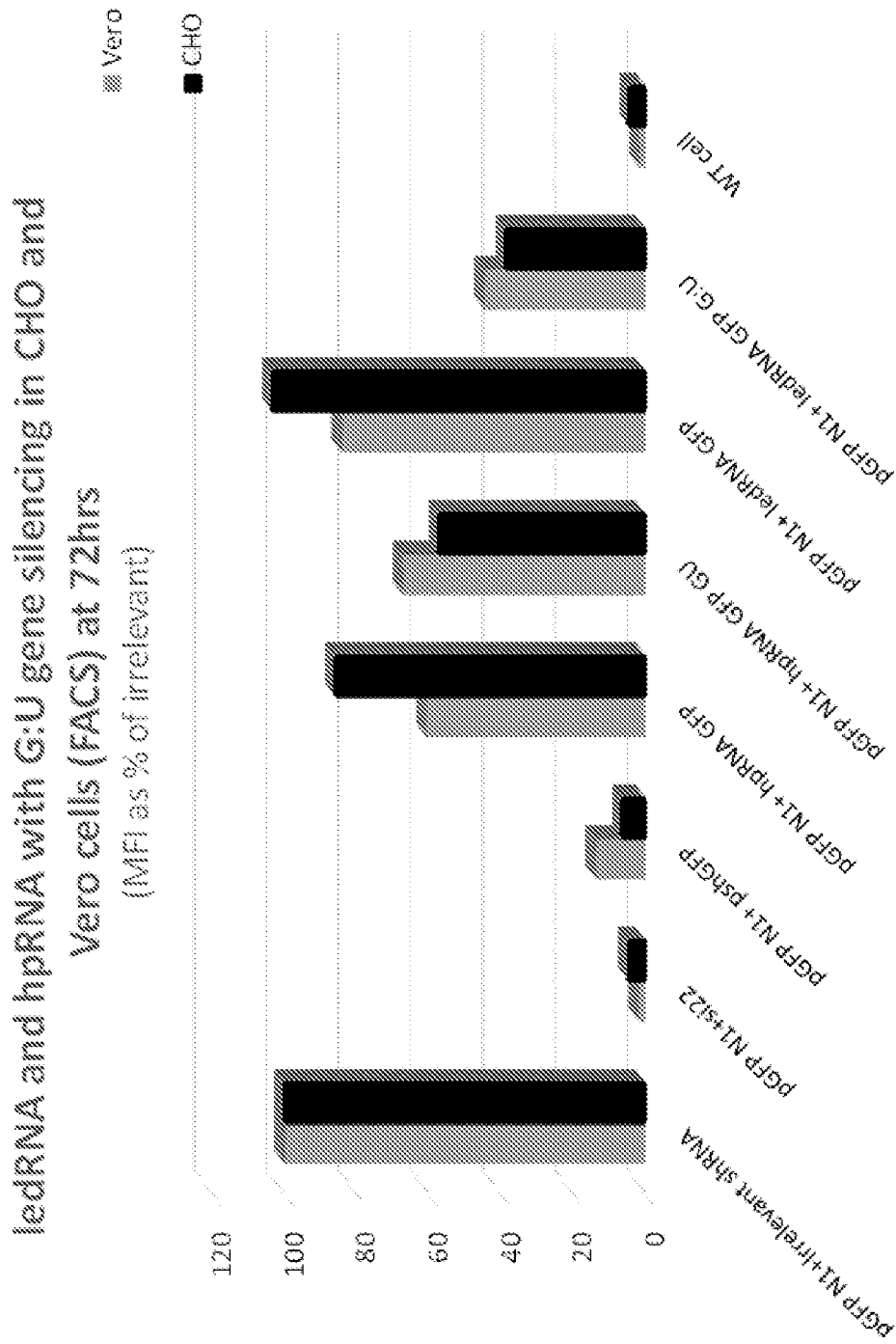
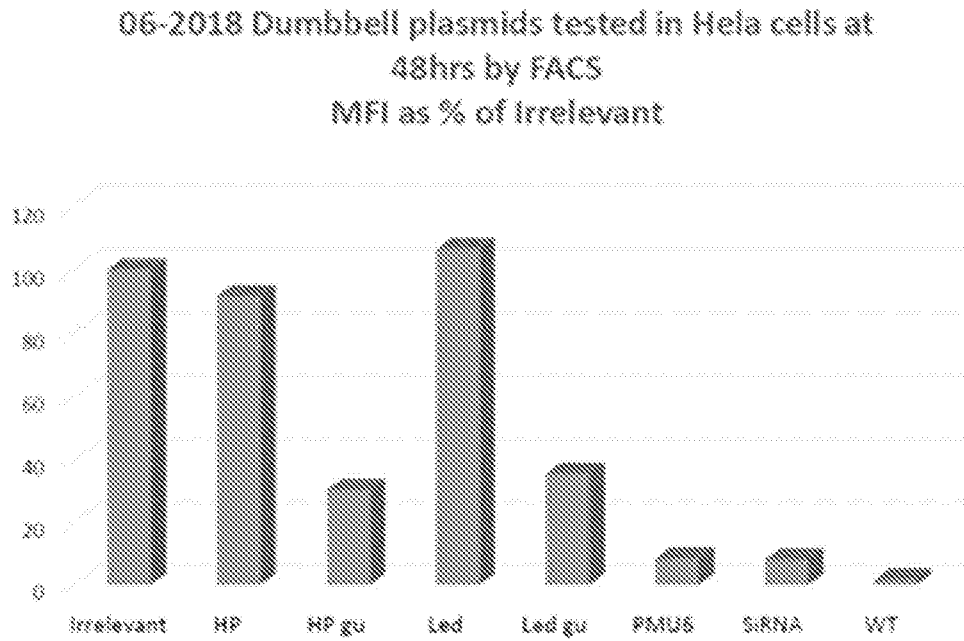


FIGURE 34

**FIGURE 35**

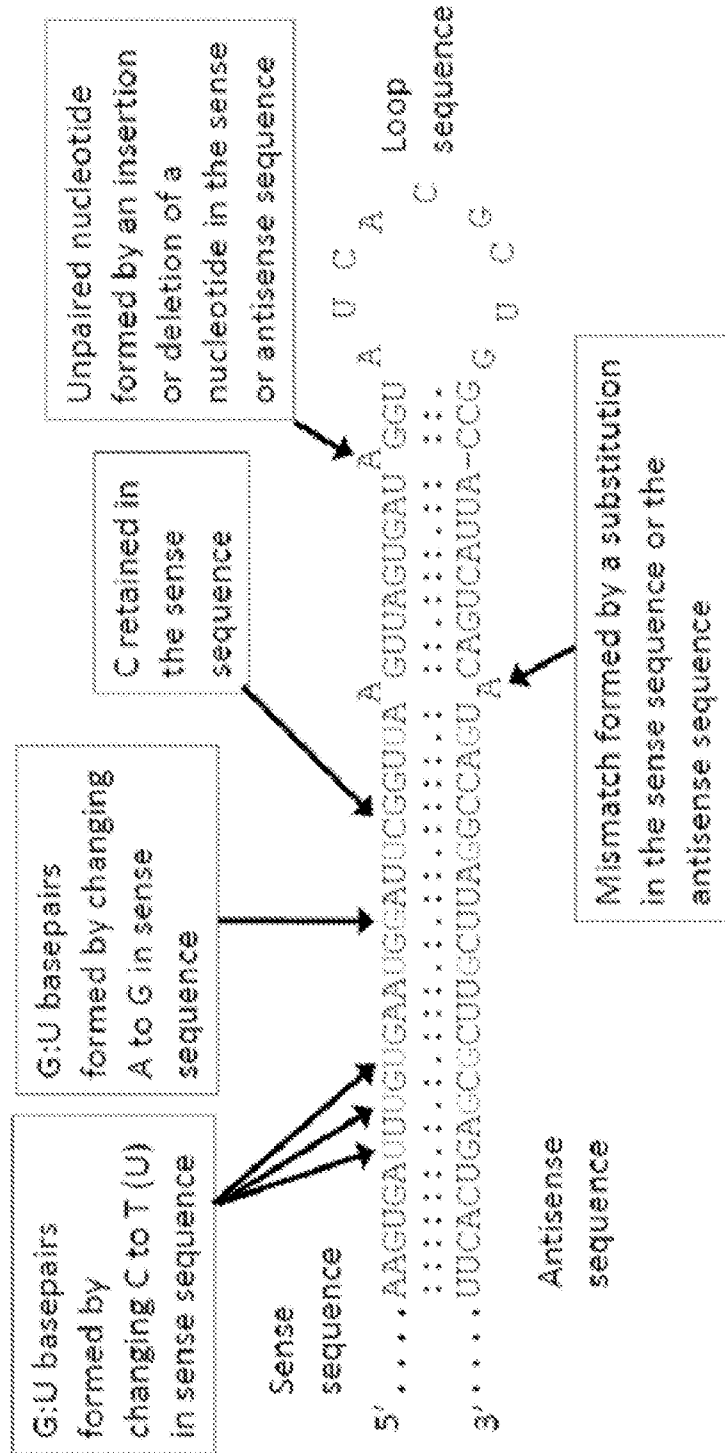
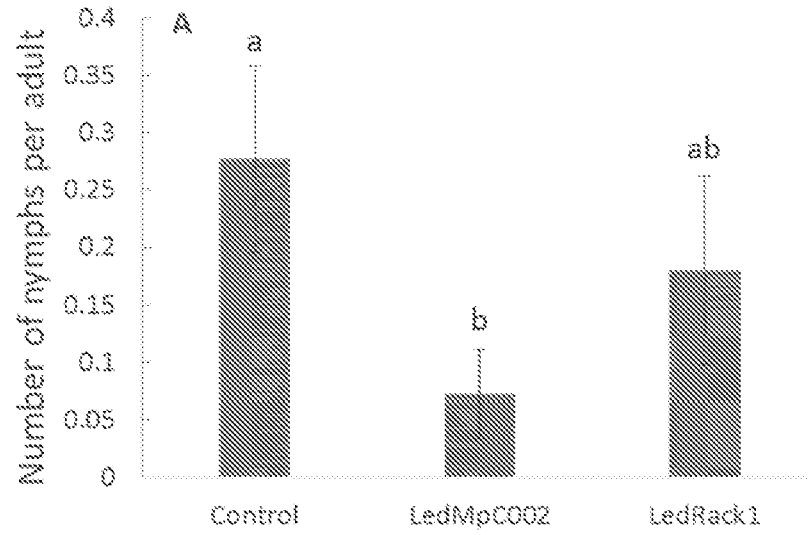


FIGURE 36

A



B

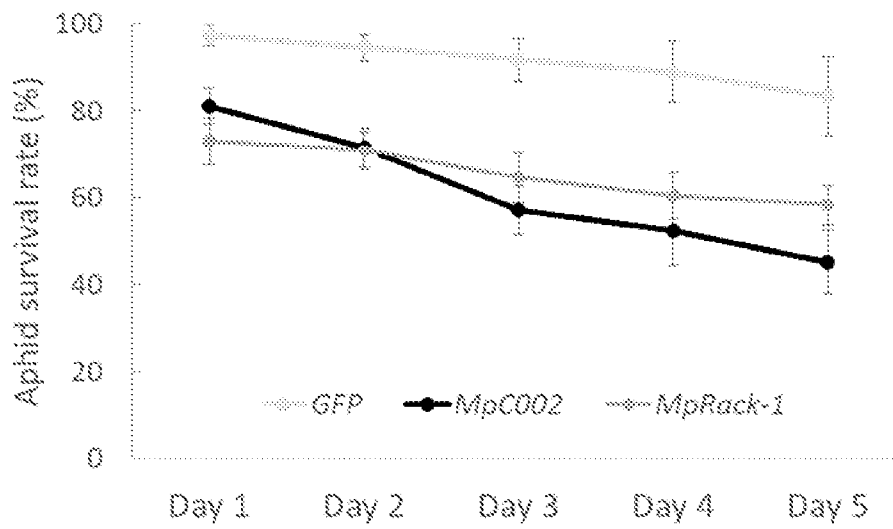


FIGURE 37

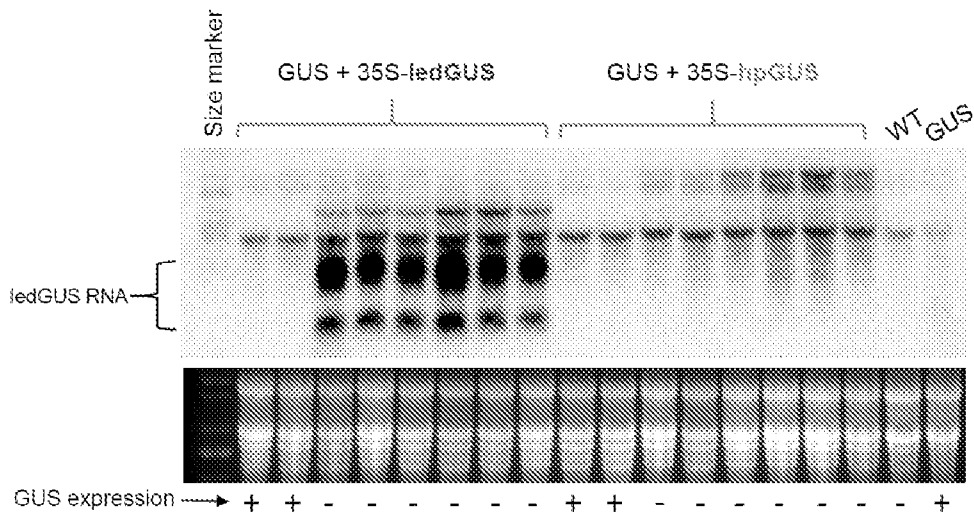


FIGURE 38

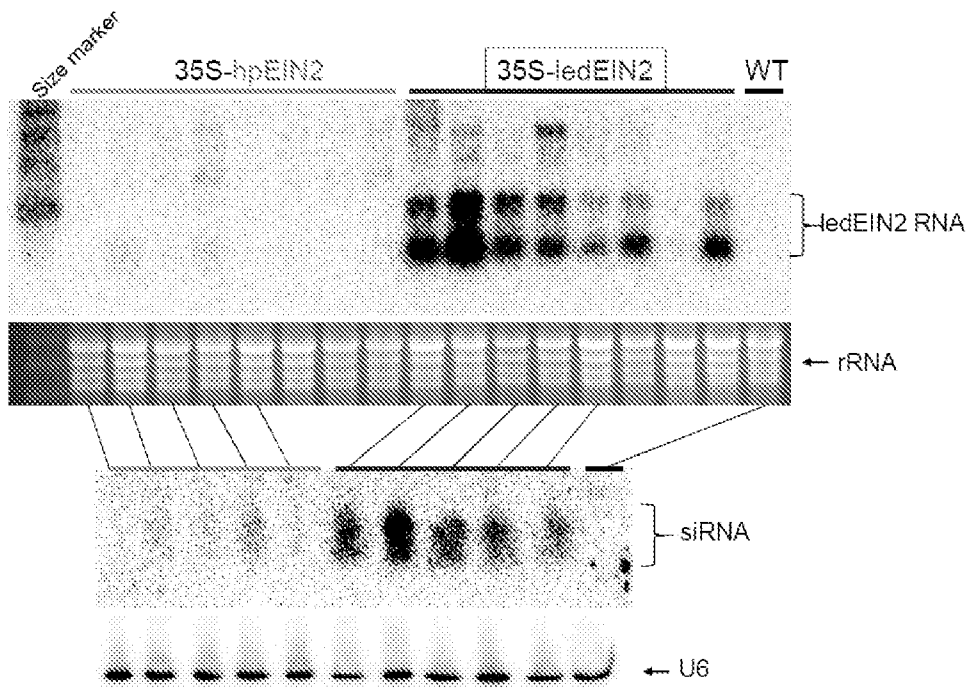


FIGURE 39

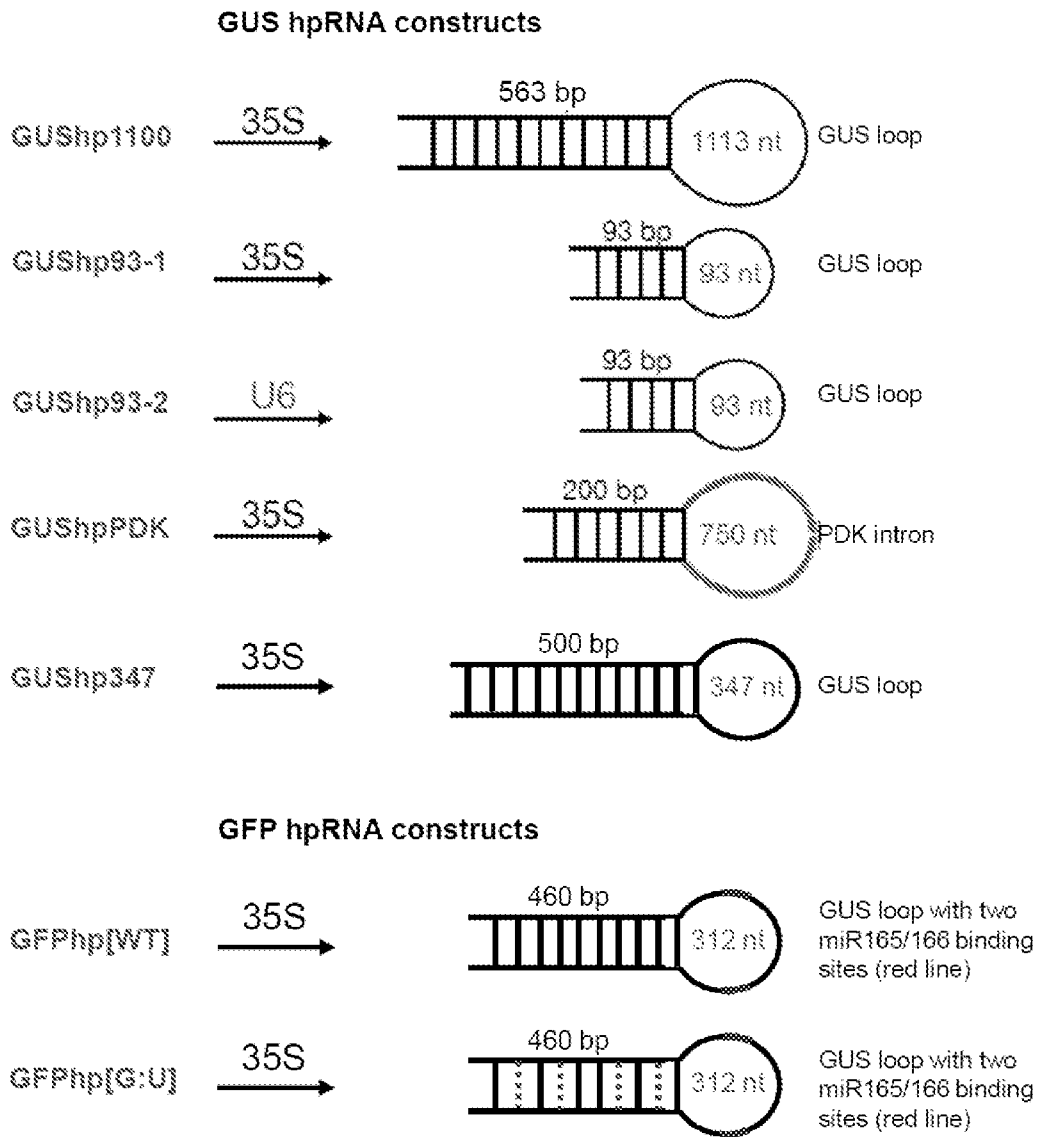


FIGURE 40

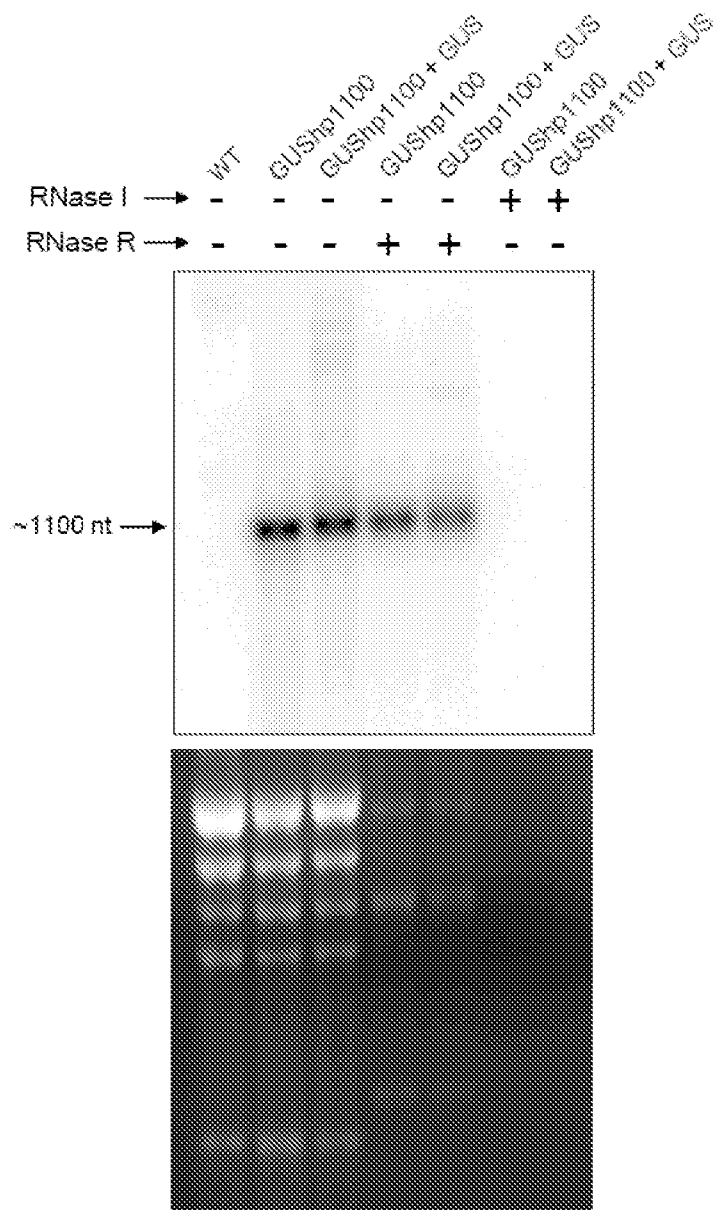


FIGURE 42

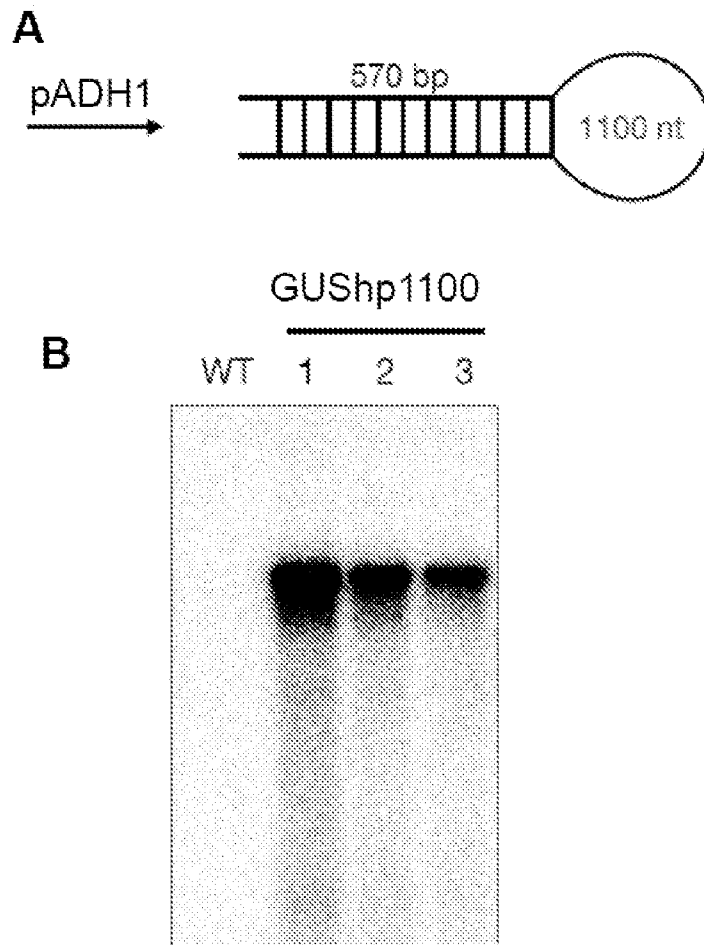


FIGURE 43

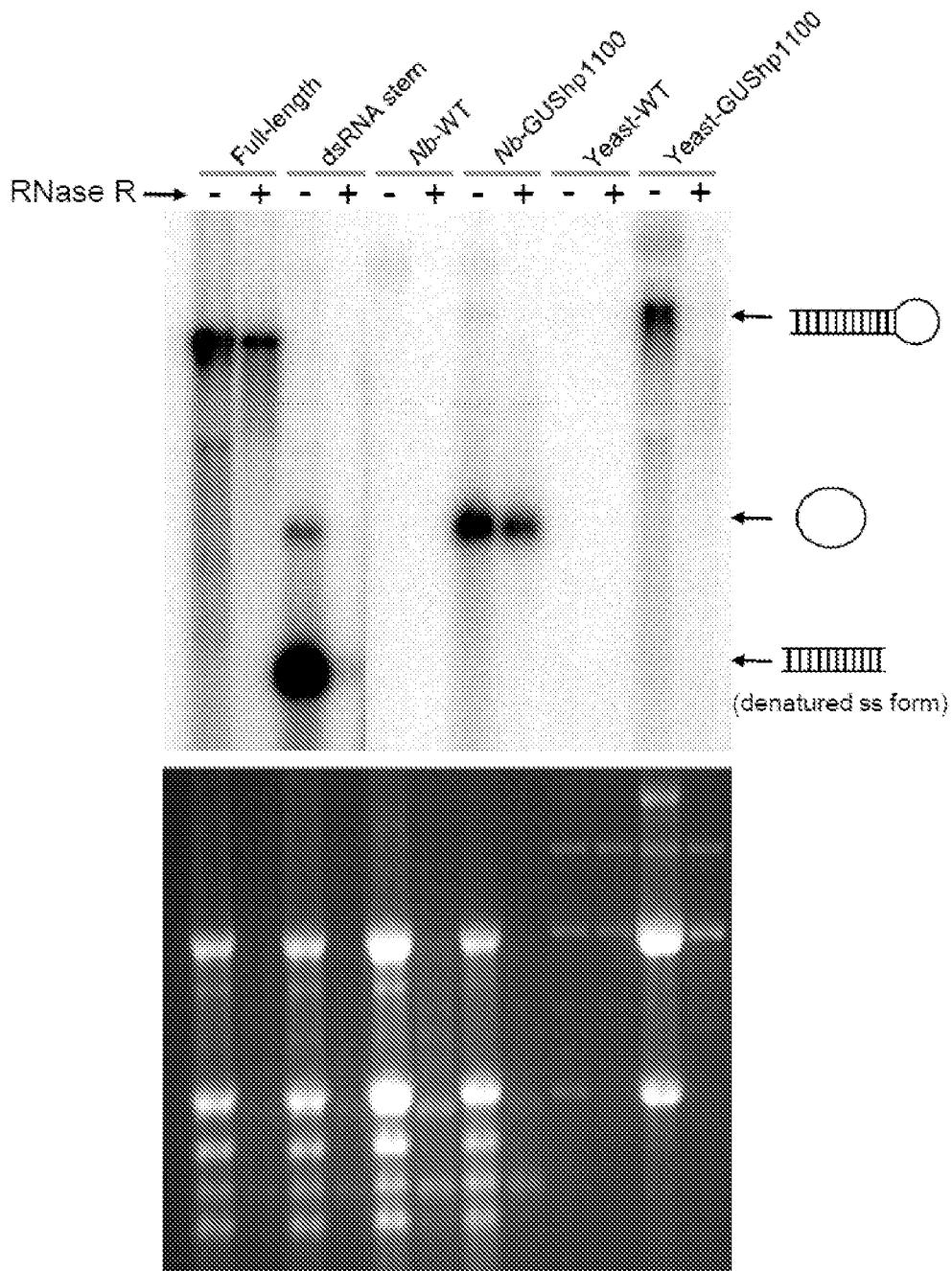


FIGURE 44

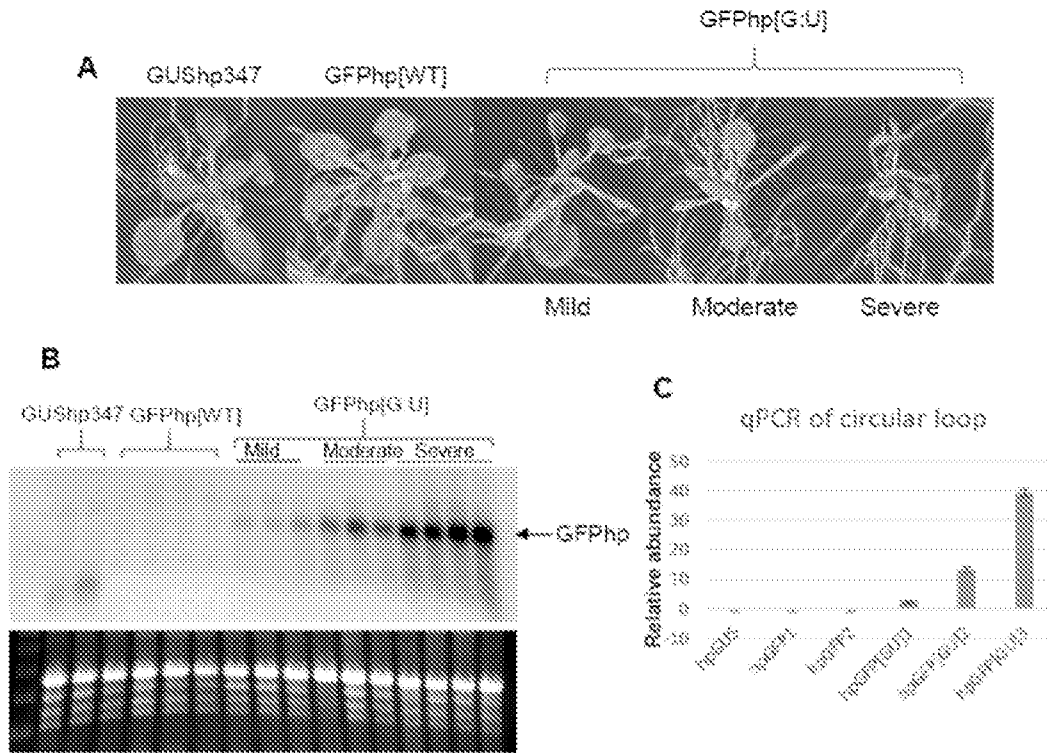


FIGURE 45

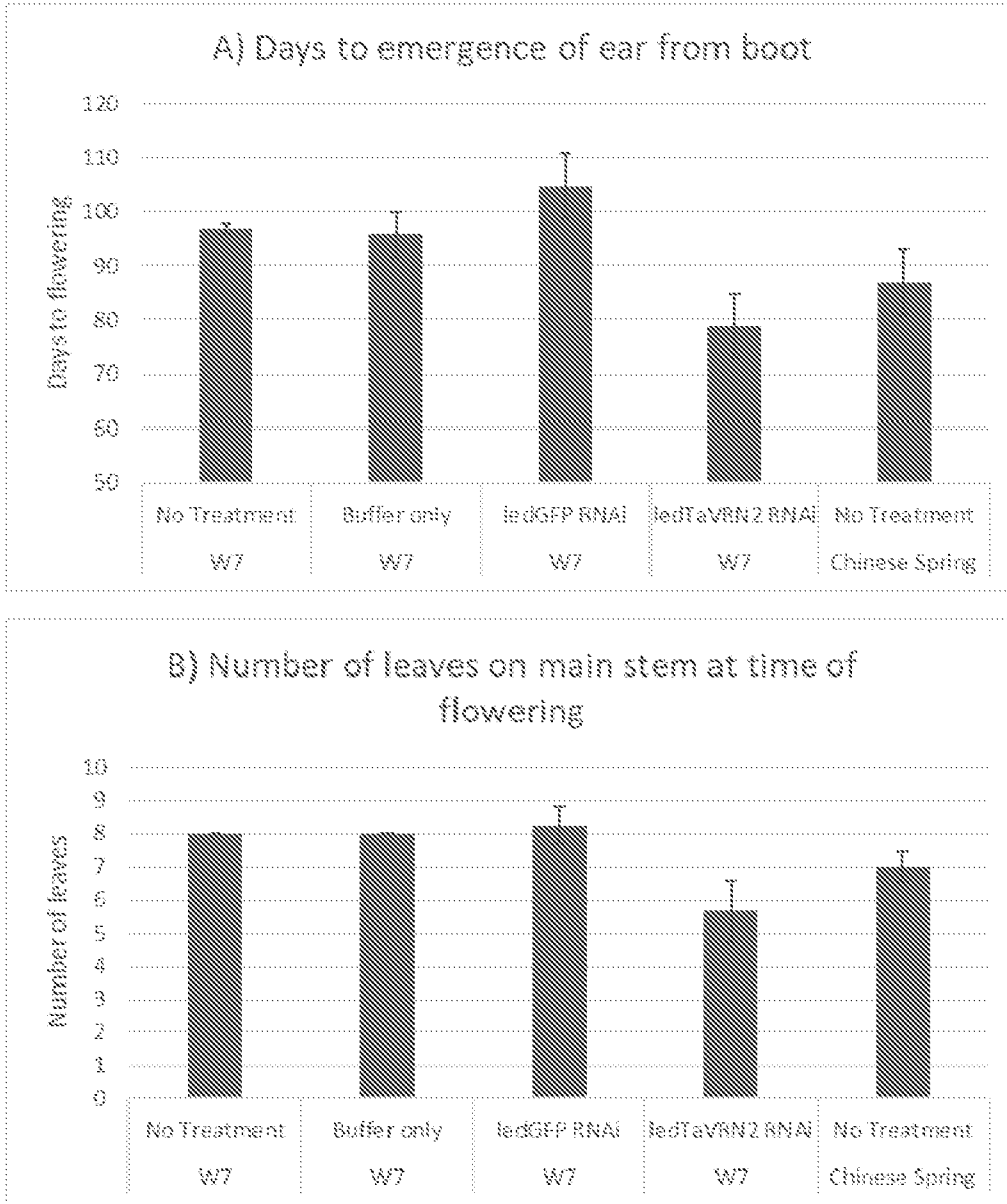


FIGURE 46

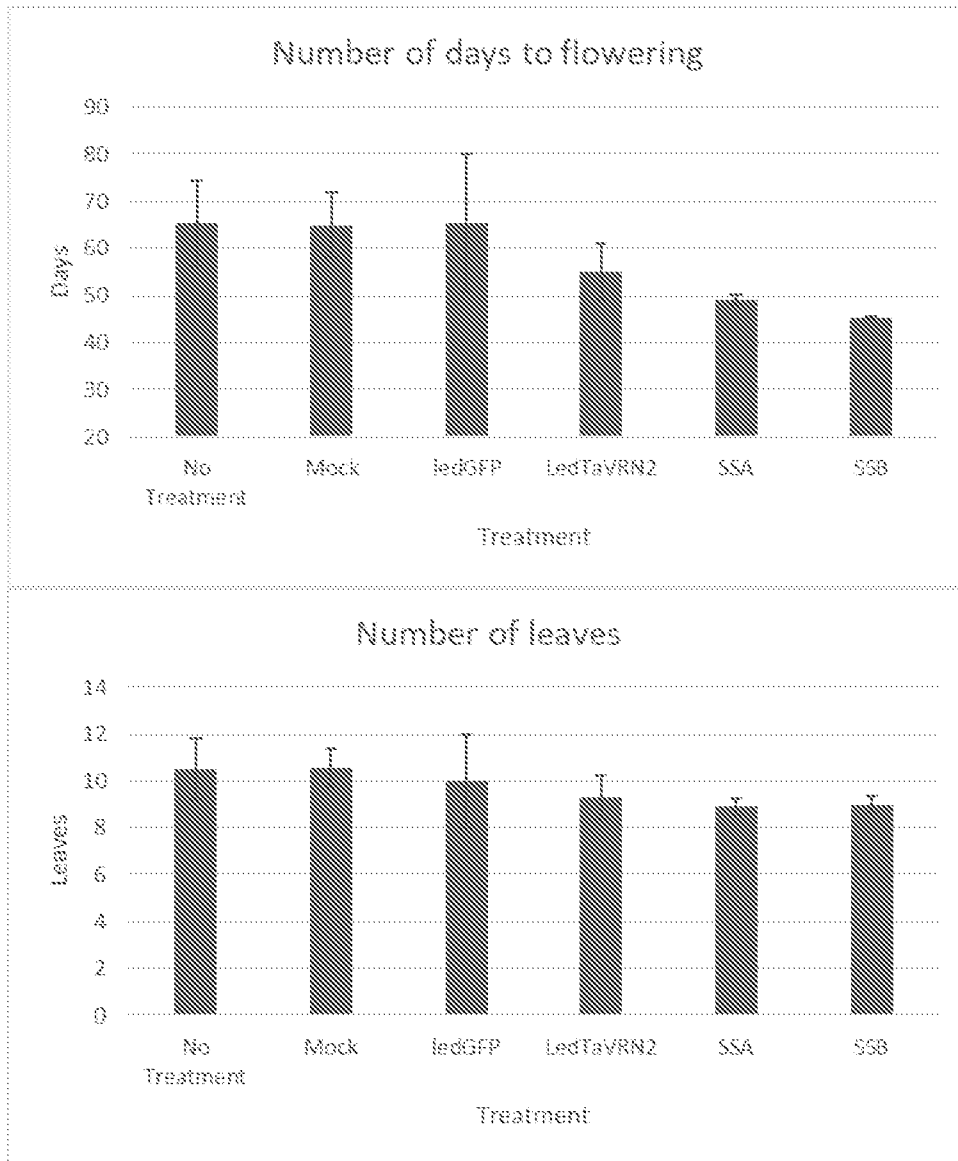


FIGURE 47