miRNA sequences of the duplex within pre-miRNAs [Tiwari et al. (2014) supra]. These amiRNAs are introduced as a transgene within an artificial expression cassette (including a promoter, terminator etc.) [Carbonell et al., *Plant Physiology* (2014) pp.113.234989], are processed via small RNA biogenesis and silencing machinery and downregulate target expression. According to Schwab et al. [Schwab et al. *The Plant Cell* (2006) Vol. 18, 1121–1133], amiRNAs are active when expressed under tissue-specific or inducible promoters and can be used for specific gene silencing in plants, especially when several related, but not identical, target genes need to be downregulated.

Senis et al. [Senis et al., *Nucleic Acids Research* (2017) Vol. 45(1): e3] disclose engineering of a promoterless anti-viral RNAi hairpin into an endogenous miRNA locus. Specifically, Senis et al. insert an amiRNA precursor transgene (hairpin pri-amiRNA) adjacent to a naturally occurring miRNA gene (e.g. miR122) by homology-directed DNA recombination that is induced by sequence-specific nuclease such as Cas9 or TALEN nucleases. This approach uses promoter- and terminator-free amiRNAs by utilizing transcriptionally active DNA that expresses a natural miRNA (miR122), that is, the endogenous promoter and terminator drove and regulated the transcription of the inserted amiRNA transgene.

Various DNA-free methods of introducing RNA and/or proteins into cells have been previously described. For example, RNA transfection using electroporation and lipofection has been described in U.S. Patent Application No. 20160289675. Direct delivery of Cas9/sgRNA ribonucleoprotein (RNPs) complexes to cells by microinjection of the Cas9 protein and sgRNA complexes was described by Cho [Cho et al., "Heritable gene knockout in Caenorhabditis elegans by direct injection of Cas9-sgRNA ribonucleoproteins," *Genetics* (2013) 195:1177-1180]. Delivery of Cas9 protein/sgRNA complexes via electroporation was described by Kim [Kim et al., "Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins" *Genome Res.* (2014) 24:1012-1019]. Delivery of Cas9 protein-associated sgRNA complexes via liposomes was reported by Zuris [Zuris et al., "Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo" *Nat Biotechnol.* (2014) doi: 10.1038/nbt.3081].

SUMMARY

In one aspect, the present disclosure refers to a method of generating a micro RNA (miRNA) molecule having a silencing activity in a cell, the method comprising:

(a) identifying a nucleic acid sequence that encodes an RNA molecule, wherein:

i. said nucleic acid sequence exhibits 60-99.9% sequence identity with respect to a nucleic acid sequence encoding an RNA molecule engaged with RNA-induced silencing complex (RISC);

ii. said nucleic acid sequence is determined to be transcribable; and

iii. said RNA molecule is determined to be aberrantly processed; and

(b) modifying said nucleic acid sequence so as to impart a secondary structure of said aberrantly processed RNA molecule, which results in the processing of said RNA molecule into small RNAs that are engaged with RISC and are complementary to a target RNA,

wherein the modifying in step (b) comprises introducing into the cell a DNA editing agent and a donor oligonucleotide,

wherein said DNA editing agent comprises an endonuclease and at least one guide RNA (gRNA),

thereby generating the miRNA molecule having the silencing activity in the cell.

In another aspect, the present disclosure refers to a genetically modified cell comprising a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type cell of the same origin devoid of said nucleic acid sequence alteration.

In another aspect, the present disclosure refers to a plant cell generated according to the method as disclosed herein, wherein the plant cell comprises a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type cell of the same origin devoid of said nucleic acid sequence alteration.

In another aspect, the present disclosure refers to a plant comprising the plant cell as disclosed herein, optionally wherein said plant is non-transgenic.

In another aspect, the present disclosure refers to a method of producing a plant with reduced expression of a target gene, the method comprising:

(a) breeding the plant as disclosed herein; and

(b) selecting for progeny plants that have reduced expression of said target RNA of interest, or progeny that comprise a silencing specificity in said miRNA molecule towards said target RNA of interest, and which do not comprise said DNA editing agent,

thereby producing said plant with reduced expression of a target gene.

In yet another aspect, the present disclosure refers to a method of producing a plant comprising a miRNA molecule having a silencing activity towards a target RNA of interest, the method comprising:

(a) breeding the plant as disclosed herein; and

(b) selecting for progeny plants that comprise said miRNA molecule having said silencing activity towards said target RNA of interest, or progeny that comprise a silencing specificity in said miRNA molecule towards said target RNA of interest, and which do not comprise said DNA editing agent,

thereby producing the plant comprising the miRNA molecule having the silencing activity towards the target RNA of interest.

In yet another aspect, the present disclosure refers to a method of producing a plant or plant cell as disclosed herein comprising growing the plant or plant cell under conditions which allow propagation.

In yet another aspect, the present disclosure refers to a seed of the plant as disclosed herein, or of the plant produced by the method as disclosed herein, wherein the seed comprises a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type seed of the same origin devoid of said nucleic acid sequence alteration.

In yet another aspect, the present disclosure refers to a method of introducing silencing activity to a first RNA molecule in a cell to generate a miRNA molecule having a silencing activity, the method comprising:

(a) selecting a first nucleic acid sequence within said cell, wherein:

i. said first nucleic acid sequence is transcribed into said first RNA molecule within the cell;

ii. the sequence of said first RNA molecule has 60-99.9% sequence identity to the sequence of a second RNA molecule; wherein said second RNA molecule is processable to a third RNA molecule having a silencing activity; and wherein said

second RNA molecule is encoded by a second nucleic acid sequence in said cell; and

iii. said first RNA molecule is not processable, or is processable differently than the second RNA molecule, such that the first RNA molecule is not processed to an RNA molecule having a silencing activity of the same nature as the third RNA molecule; and

(b) modifying the first nucleic acid sequence so as to impart a secondary structure such that it encodes a modified first RNA molecule, said modified first RNA molecule being processable to a fourth RNA in the same way that said second RNA molecule is processable to the third RNA molecule, such that the fourth RNA molecule has a silencing activity of the same nature as the third RNA molecule, wherein the modifying in comprises introducing into the cell a DNA editing agent and a donor oligonucleotide, wherein said DNA editing agent comprises an endonuclease and at least one guide RNA (gRNA),

thereby introducing a silencing activity to the first RNA molecule to generate a miRNA molecule having a silencing activity.

According to some examples of the present disclosure there is provided a method of generating an RNA molecule having a silencing activity in a cell, the method comprising: (a) identifying nucleic acid sequences encoding RNA molecules exhibiting a predetermined sequence homology range, not including complete identity, with respect to nucleic acid sequences encoding RNA molecules engaged with RNA-induced silencing complex (RISC);

WHAT IS CLAIMED IS:

1. A method of generating a micro RNA (miRNA) molecule having a silencing activity in a cell, the method comprising:

(a) identifying a nucleic acid sequence that encodes an RNA molecule, wherein:

i. said nucleic acid sequence exhibits 60-99.9% sequence identity with respect to a nucleic acid sequence encoding an RNA molecule engaged with RNA-induced silencing complex (RISC);

ii. said nucleic acid sequence is determined to be transcribable; and

iii. said RNA molecule is determined to be aberrantly processed; and

(b) modifying said nucleic acid sequence so as to impart a secondary structure of said aberrantly processed RNA molecule, which results in the processing of said RNA molecule into small RNAs that are engaged with RISC and are complementary to a target RNA,

wherein the modifying in step (b) comprises introducing into the cell a DNA editing agent and a donor oligonucleotide,

wherein said DNA editing agent comprises an endonuclease and at least one guide RNA (gRNA),

thereby generating the miRNA molecule having the silencing activity in the cell.

2. The method of claim 1, wherein:

(i) said RNA molecule of step (a) encoded by the identified nucleic acid sequence exhibits 60-99.9% sequence identity, with respect to an RNA molecule that is engaged with and/or that is processed into a molecule engaged with RISC; and/or

(ii) imparting processability in step (b) comprises imparting canonical processing relative to an RNA molecule encoded by a nucleic acid sequence of said nucleic acid sequence encoding RNA molecule engaged with RNA-induced silencing complex (RISC); and/or

(iii) wherein the method further comprises determining the genomic location of said nucleic acid sequence encoding said RNA molecule exhibiting the sequence identity range of step (a), optionally wherein:

(I) said genomic location is in a non-coding gene, optionally within an intron of a non-coding gene; or

(II) said genomic location is in a coding gene, optionally within an exon of coding gene, optionally within an exon encoding an untranslated region (UTR) of a coding gene, or optionally within an intron of a coding gene.

3. The method of claim 1 or 2, wherein:

(i) step (a) is affected by alignment of small RNA expression data to a genome of said cell and determining the amount of reads that map to each genomic location, optionally wherein said alignment of said small RNAs is alignment to a predetermined location in said genome of said cell with no mismatches; and/or

(ii) said modifying said nucleic acid sequence of said transcribable nucleic acid sequence encoding said aberrantly processed RNA molecule exhibiting the sequence identity range is effected at nucleic acids other than those corresponding to the binding site to said target RNA; and/or

(iii) said processability is effected by cellular nucleases selected from the group consisting of Dicer, Argonaute, tRNA cleavage enzymes, and Piwi-interacting RNA (piRNA) related proteins.

4. The method of any one of claims 1-3, further comprising modifying the specificity of said miRNA molecule having the silencing activity in the cell, wherein said DNA editing agent redirects a silencing specificity of said miRNA molecule towards a target RNA of interest, said target RNA of interest being distinct from said target RNA, thereby modifying said specificity of said miRNA molecule having said silencing activity in said cell.

5. The method of any one of claims 1-4, wherein the identified nucleic acid sequence encoding the RNA molecule of step (a) is homologous to a gene encoding a miRNA molecule whose silencing activity and/or processing into small silencing RNA is dependent on its secondary structure.

6. A genetically modified cell comprising a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type cell of the same origin devoid of said nucleic acid sequence alteration.

7. The genetically modified cell of claim 6, wherein processing is canonical processing.

8. The method of any one of claims 1-5, wherein:

said endonuclease is a CRISPR-endonuclease, a dCRISPR-endonuclease, or comprises Cas9;

and/or wherein said DNA editing agent is applied to the cell as DNA, RNA or RNP.

9. The method of any one of claims 1-5 or 8, wherein said target RNA of interest is endogenous or exogenous to said cell and/or wherein said specificity of said RNA molecule is determined phenotypically by determination of at least one phenotype selected from the group consisting of a cell size, a growth rate/inhibition, a cell shape, a cell membrane integrity, a tumor size, a tumor shape, a pigmentation of an organism, a size of an organism, a crop yield, metabolic profile, a fruit trait, a biotic stress resistance, an abiotic stress resistance, an infection parameter, and an inflammation parameter.

10. The method of any one of claims 1-5, 8 or 9, or genetically modified cell of claim 6 or claim 7 wherein said cell is a eukaryotic cell, optionally wherein said eukaryotic cell is obtained from a eukaryotic organism selected from the group consisting of a plant, a mammal, an invertebrate, an insect, a nematode, a bird, a reptile, a fish, a crustacean, a fungi and an algae, optionally wherein said eukaryotic cell is a plant cell, and optionally wherein said plant cell is a protoplast.

11. A plant cell generated according to the method of any one of claims 1-5 or 8-10, wherein the plant cell comprises a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type cell of the same origin devoid of said nucleic acid sequence alteration.

12. A plant comprising the plant cell of claim 11, optionally wherein said plant is non-transgenic.

13. A method of producing a plant with reduced expression of a target gene, the method comprising:

(a) breeding the plant of claim 12; and

(b) selecting for progeny plants that have reduced expression of said target RNA of interest, or progeny that comprise a silencing specificity in said miRNA molecule towards said target RNA of interest, and which do not comprise said DNA editing agent,

thereby producing said plant with reduced expression of a target gene.

14. A method of producing a plant comprising a miRNA molecule having a silencing activity towards a target RNA of interest, the method comprising:

(a) breeding the plant of claim 12; and

(b) selecting for progeny plants that comprise said miRNA molecule having said silencing activity towards said target RNA of interest, or progeny that comprise a silencing specificity in said miRNA molecule towards said target RNA of interest, and which do not comprise said DNA editing agent,

thereby producing the plant comprising the miRNA molecule having the silencing activity towards the target RNA of interest.

15. A method of producing a plant or plant cell of claim 12 comprising growing the plant or plant cell under conditions which allow propagation.

16. The method of claim 13 or 14, wherein said breeding comprises crossing or selfing.

17. A seed of the plant of claim 12, or of the plant produced by the method of any one of claims 13-16, wherein the seed comprises a genome comprising a polynucleotide sequence encoding a miRNA molecule having a nucleic acid sequence alteration introduced by a DNA editing agent and a donor oligonucleotide, wherein the DNA editing agent comprises an endonuclease and at least one gRNA, and wherein the alteration imparts a secondary structure which results in processing of said miRNA molecule into small RNAs that are engaged with RISC, said processing of said miRNA molecule being absent from a wild type seed of the same origin devoid of said nucleic acid sequence alteration.

18. The method or genetically modified cell of claim 10, wherein said eukaryotic cell is a human cell, optionally wherein said nucleic acid sequences encoding RNA molecules are selected from the group consisting of the nucleic acid sequences as set forth in any of SEQ ID NOs. 352 to 392 and/or wherein said eukaryotic cell is a totipotent stem cell.

19. A method of introducing silencing activity to a first RNA molecule in a cell to generate a miRNA molecule having a silencing activity, the method comprising:

(a) selecting a first nucleic acid sequence within said cell, wherein:

i. said first nucleic acid sequence is transcribed into said first RNA molecule within the cell;

ii. the sequence of said first RNA molecule has 60-99.9% sequence identity to the sequence of a second RNA molecule; wherein said second RNA molecule is processable to a third RNA molecule having a silencing activity; and wherein said second RNA molecule is encoded by a second nucleic acid sequence in said cell; and

iii. said first RNA molecule is not processable, or is processable differently than the second RNA molecule, such that the first RNA molecule is not processed to an RNA molecule having a silencing activity of the same nature as the third RNA molecule; and

(b) modifying the first nucleic acid sequence so as to impart a secondary structure such that it encodes a modified first RNA molecule, said modified first RNA molecule being processable to a fourth RNA in the same way that said second RNA molecule is processable to the third RNA molecule, such that the fourth RNA molecule has a silencing activity of the same nature as the third RNA molecule, wherein the modifying in comprises introducing into the cell a DNA editing agent and a donor oligonucleotide, wherein said DNA editing agent comprises an endonuclease and at least one guide RNA (gRNA),

thereby introducing a silencing activity to the first RNA molecule to generate a miRNA molecule having a silencing activity.

20. The method of claim 19, wherein said second RNA molecule is an RNA molecule which has a secondary structure that enables it to be processed into an RNA having a silencing activity, optionally wherein said silencing activity is mediated through engaging RISC, optionally wherein:

said modifying the first nucleic acid sequence comprises modifying the sequence such that the modified first RNA molecule has essentially the same secondary structure as that of the second RNA molecule, optionally a secondary structure which is at least 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% or 100% identical to the secondary structure of the second RNA molecule,

or wherein said first nucleic acid molecule is a gene from *H. sapiens*, wherein the gene is selected from the group consisting of the genes having the sequences set forth in any of SEQ ID NOs. 352 to 392.