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(54) Title: ENHANCED SELECTIVE EXPRESSION OF TRANSGENES IN FIBER PRODUCING PLANTS

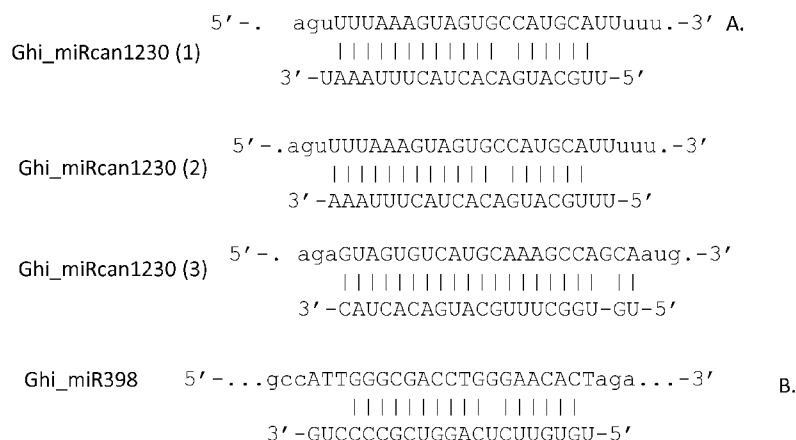


Figure 5

(57) Abstract: Methods and means are provided to enhance the selective expression of transgenes under control of a fiber-selective promoter, in fiber cells, particularly cotton fiber cells by including target sites for naturally occurring microRNAs with a specific expression profile, particularly with a differential expression profile between cells leading to fibers and other cells of the fiber producing plant, into the transcribed region of genes of interest.





— with sequence listing part of description (Rule 5.2(a))

Enhanced selective expression of transgenes in fiber producing plants

Field of the invention

[01] This invention relates to the field of agricultural biotechnology, more specifically to the use of molecular biology techniques to alter the expression pattern of transgenes in fiber-producing plants such as cotton. Naturally occurring microRNAs with a specific expression profile, particularly with a differential expression profile between cells leading to fibers and other cells of the fiber producing plant, are provided. By engineering and including target sites for such microRNAs in genes, particularly in transgenes, the expression profile of the gene comprising such microRNA target site will reflect (mirror) the expression profile of the microRNA in plants. Such microRNAs and the corresponding target sites can be used to enhance the selective expression of transgenes under control of a fiber-selective promoter, in fibers, particularly cotton fibers.

Background of the invention

[02] Cotton (*Gossypium* spp.) is the world's most important natural textile fiber and is also a significant oilseed crop. Cotton production provides income for approximately 100 million families, and approximately 150 countries are involved in cotton import and export. Its economic impact is estimated to be approximately \$500 billion/year worldwide. World consumption of cotton fiber is approximately 115 million bales or approximately 27 million metric tons per year (National Cotton Council, <http://www.cotton.org/>, 2006). The genus *Gossypium* is relatively complex and includes approximately 45 diploid ($2n = 2x = 26$) and five tetraploid ($2n = 4x = 52$) species, all exhibiting disomic patterns of inheritance. Diploid species ($2n = 26$) fall into eight genomic groups (A–G, and K). The African clade, comprising the A, B, E, and F genomes, occurs naturally in Africa and Asia, while the D genome clade is indigenous to the Americas. A third diploid clade, including C, G, and K, is found in Australia. All 52 chromosome species, including *Gossypium hirsutum* and *Gossypium barbadense*, are classic natural allotetraploids that arose in the New World from interspecific hybridization between an A genome-like ancestral African species and a D genome-like

American species. The closest extant relatives of the original tetraploid progenitors are the A genome species *Gossypium herbaceum* (A1) and *Gossypium arboreum* (A2) and the D genome species *Gossypium raimondii* (D5) 'Ulbrich'. Polyploidization is estimated to have occurred 1 to 2 million years ago, giving rise to five extant allotetraploid species. Interestingly, the A genome species produce spinnable fiber and are cultivated on a limited scale, whereas the D genome species do not. More than 95% of the annual cotton crop worldwide is *G. hirsutum*, Upland or American cotton, and the extra-long staple or Pima cotton (*G. barbadense*) accounts for less than 2% (National Cotton Council, <http://www.cotton.org>, 2006).

[03] Each cotton fiber is a differentiated single epidermal cell of the ovule. Approximately half a million fibers are produced per cotton boll, some forming fuzz and some forming lint. Initiation of an epidermal cell into fiber requires a change in cell fate, which is a fundamental biological process involving genetic, physiological and developmental "switches". Genetic mutations, polyploidy, pollination/fertilization, and hormonal regulation can affect the number of cells developing into fibers or alter fiber cell properties (fuzz vs. lint). However, it is unclear how these factors control gene expression changes that orchestrate the pattern and tempo in early stages of fiber development.

[04] In contrast, the morphological development of cotton fibers is well documented in the art. Cotton fibers undergo four overlapping developmental stages: fiber cell initiation, elongation, secondary wall biosynthesis, and maturation. Fiber initiation is a rapid process. The white fluffy fibers begin to develop immediately after anthesis and continue up to 3 days post-anthesis (DPA), which is followed by fiber cell elongation (until 20 DPA). Secondary wall biosynthesis initiates around 15 dpa and continues to 45 DPA, followed by a maturation process at 45-60 DPA. Cotton fibers are derived from ovular epidermal cells (maternal tissues). However, only ~25-30% of the epidermal cells differentiate into the commercially important lint fibers. The majority of cells does not differentiate into fibers or develop into short fibers or fuzz. For the cells committed to fiber development, cell initiation and elongation are nearly synchronous on each ovule,

indicating that changes in gene expression are orchestrated during fiber differentiation and development through intercellular signaling and/or timing mechanisms.

[05] In many instances, it may be advantageous to preferentially, selectively or specifically express genes in fiber developing cells or fibers. Such expression can influence the fiber development and result in longer or stronger fibers. WO 98/00549 describes the expression of cellulose synthase gene in fiber-developing cells. WO 08/012058 and WO02/45485 describe expression of sucrose synthase genes in cotton fiber developing cells. WO05/017157 describes reduction of the expression of β -1,3-glucanase in fiber-developing cells.

[06] Cotton fiber consists of cellulose, a natural polymer composed of many molecules of the sugar glucose. Its unique structure is ideally suited for textile production. Each fiber is basically a hollow tube a few centimetres in length that, when spun and woven, provides the very special characteristic “feel” of cotton. Natural cellulose containing fibers, however, do not possess the chemical versatility of synthetic fibers, due to the relative inert nature of the cellulose consisting of β -1-4 linked glucose monomers.

[07] WO06/136351, WO11/089021 and WO12/048807 all describe methods and means for altering cell wall reactivity in fibers of fiber producing plants such as cotton, by inclusion of positively charged oligosaccharides or polysaccharides into the cell wall. To this end, N-acetylglucosamine transferases, including chitin synthases, are expressed in fibers of the plants, and optionally also glutamine:fructose-6-phosphate amidotransferase. Although, chitin could be efficiently produced in cotton plant cell walls, it was also observed that the transgenic plants usually exhibited some reduced growth which may be attributed to a negative effect of the expression of the transgene in cotton outside of the cotton fibers.

[08] It would thus be advantageous to be able to increase the tissue-selectivity of the expression of transgenes in fiber-producing plants, in particular to be able to increase the selectivity of expression in fibers and/or fiber-developing cells, while expression in

other cells of the fiber-producing plant is substantially reduced or abolished. This could be conveniently achieved by including into the recombinant DNA construct of interest, a target site for a microRNA, preferably an endogenous miRNA, differentially expressed between fibers and the rest of the plant, preferably absent in fibers and fiber-developing cells and ubiquitously expressed in all other parts of the plant, so that expression of the miRNA in cells other than fiber or fiber-developing cells directs the post-transcriptional cleavage of any messenger RNA originating by adventitious transcription of the recombinant DNA construct of interest incorporating the miRNA target site, in cells outside the fiber developing cells or fibers. A mirroring selectivity profile (ie. expression limited to cells outside of the fiber developing cells) can also be envisaged.

[09] Incorporation of miRNA target sequences in chimeric constructs has also been described as a trigger for the production of so-called tasiRNAs (trans-acting siRNAs) see e.g. WO 2006/138638 or WO2007/039454.

[10] WO2006/111512 describes improved methods controlling gene expression in the field of genetics, especially plant genetics, and provides agents capable of controlling gene expression. The document specifically provides sequences of naturally occurring, tissue-specifically expressed microRNAs. The patent application further provides for transgenic expression constructs comprising sequences interacting with said microRNAs. By incorporation of the microRNA encoding sequence the expression from said expression construct is specifically silenced in the tissue where the naturally occurring microRNA is naturally expressed. Thereby the expression profile resulting from the promoter is modulated and leakiness is reduced. The document further provides for a method for modulating transgenic expression by incorporating sequences encoding said microRNAs into transgenic expression constructs. The compositions and methods of the invention can be used to enhance performance of agricultural relevant crops and for therapy, prophylaxis, research and diagnostics in diseases and disorders, which afflict mammalian species.

[11] WO2007/047016 describes methods for producing non-natural hybrid seed. Also disclosed are miRNAs and miRNA recognition sites useful for conferring inducible sterility on a crop plant, and recombinant DNA construct including such exogenous miRNA recognition sites.

[12] WO2008/133643 discloses novel microRNAs and their precursors, and recombinant DNA constructs including such novel miRNAs, miRNA precursors, miRNA promoters, and miRNA recognition sites corresponding to the miRNAs. Included are novel miRNA and miRNA precursors that exhibit nutrient-responsive expression. Also disclosed are miRNA decoy sequences. Further provided are non-natural transgenic plant cells, plants, and seeds containing in their genome a recombinant DNA construct as described in the patent application and methods of controlling gene expression using such recombinant DNA constructs.

[13] WO2009/003078 provides molecular constructs and methods for the temporally specific control of gene expression in plants or in plant pests or pathogens. More specifically, this patent application provides plant miRNA genes having novel circadian expression patterns that are useful for designing recombinant DNA constructs for temporally specific expression of at least one gene. Also provided are non-natural transgenic plant cells, plants, and seeds containing in their genome a recombinant DNA construct of this invention.

[14] Kwak et al. (BMC Genomics 2009, 10:457 entitled "Enrichment of a set of microRNAs during the cotton fiber development" describes a deep sequencing approach to investigate global expression and complexity of small RNAs during cotton fiber initiation and development. Two small RNA libraries were prepared and analyzed from wild-type and fuzz/lintless cotton ovules. The study demonstrated significant differences in expression abundance of miRNAs between the wild-type and mutant and suggests that these differentially expressed miRNAs potentially regulate transcripts distinctly involved in cotton fiber development.

[15] Wang et al. (Molecular Plant 2012, Volume 5 Number 4, pages 889-900 entitled “ A comparative miRNAome analysis reveals seven fiber initiation-related and 36 novel miRNAs in developing cotton ovules” describes high throughput sequencing combined with computational analysis to characterize miRNAomes from the ovules of wild-type upland cotton and a fibreless mutant during fiber initiation.

[16] The art does remains deficient in describing microRNA molecules with the appropriate expression profile, in particular microRNAs which are expressed and processed in some or all parts of fiber producing plants, particularly cotton plants, except for fibers or fiber developing cells, and whose target sequences could be used to increase the specificity of expression of transgenes (including N-acetylglucosamine transferase and/or glutamine:fructose-6-phosphate amidotransferase) in fibers or fiber-developing cells (compared to the rest of the plant). These and other problems are solved as described hereinafter in the different embodiments, examples and claims.

Summary of the invention

[17] In one embodiment of the invention, a recombinant gene for spatially selective expression in a fiber-producing plant is provided comprising the following operably linked elements:

- (a) a plant-expressible promoter, such as a constitutive promoter, an inducible-promoter, a tissue-specific promoter, a developmentally regulated promoter or such as a fiber-preferential or fiber-selective promoter, including a promoter selected from the group of a promoter from cotton from a fiber-specific β - tubulin gene, a promoter from cotton from a fiber-specific actin gene, a promoter from a fiber specific lipid transfer protein gene from cotton, a promoter from an expansin gene from cotton, or a promoter from a chitinase gene in cotton; a promoter from cotton from a glucanase gene, a promoter of the cotton FS18 gene, a promoter of the SCW-PRP gene from cotton, a promoter of the FB8-like gene from cotton;
- (b) a region encoding a biologically active RNA molecule;
- (c) optionally a 3' transcription termination and polyadenylation region

characterized in that the recombinant gene further comprises a target sequence recognized by a miRNA, such as a microRNA endogenous to the plant, the miRNA being differentially expressed in cells leading to fibers in the fiber producing plant compared to cells of the fiber-producing plants other than the cells leading to the fibers. Preferably, the target sequence recognized by the miRNA is heterologous to at least one of the elements of the recombinant gene and may be located in a 3' untranslated region of the recombinant gene. The target sequence may be additionally introduced in the recombinant gene or may be obtained by modification of the sequence of one of the operably linked elements, preferably one of the operably linked elements other than the plant-expressible promoter.

[18] In one aspect of the invention, the miRNA is less abundantly or substantially not expressed in cells leading to fibers in the fiber producing plants compared to cells of the fiber-producing plants other than the cells leading to the fibers and may be selected from the group consisting of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230 and Ghi_miR398 or have a nucleotide sequence selected from the nucleotide sequence of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 20, SEQ ID No. 21 or SEQ ID No. 22. The target sequence recognized by the miRNA may thus have the nucleotide sequence of SEQ ID No. 5, SEQ ID No. 5 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 6, SEQ ID No. 6 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 7 or SEQ ID No. 8, SEQ ID No. 23, SEQ ID No. 23 from nucleotide position 4 to 24, SEQ ID No. 23 from nucleotide position 11 to 32, SEQ ID No. 23 from nucleotide positions 4 to 32 or may have a nucleotide sequence which is complementary to the nucleotide sequence of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230, Ghi_miR398, SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, or SEQ ID No. 4, SEQ ID No 20, SEQ ID No 21 or SEQ ID No 22 whereby one or more of the following mismatches may occur:

- (a) a mismatch between the nucleotide at the 5' end of the miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (b) a mismatch between any one of the nucleotides in position 1 to position 9 of the miRNA and the corresponding nucleotide sequence in the target RNA sequence;

- (c) three mismatches between any one of the nucleotides in position 12 to position 21 of the miRNA and the corresponding nucleotide sequence in the target RNA sequence provided that there are no more than two consecutive mismatches; and
- (d) no mismatch is allowed at positions 10 and 11 of the miRNA.

[19] The recombinant gene according to the invention may encode any protein or polypeptide including a polypeptide with N-acetylglucosamine transferase activity such as a NODC-type N-acetylglucosamine transferase, an N-acetylglucosamine transferase activity comprising a signal anchor sequence selected from the signal anchor sequence of a rat sialyl transferase, the signal anchor sequence of a human galactosyl transferase, the signal anchor sequence of the Arabidopsis homologue of the yeast HDEL receptor (AtERD2), the signal anchor sequence of the α -2,6-sialyltransferase, the signal anchor sequence of β 1,2-xylosyltransferase from *Arabidopsis thaliana*, the signal anchor sequence of N-acetylglucosaminyl transferase I from tobacco or the amino acid sequence YYHDL or LKLEI, or chitin synthase 2 of *Saprolegnia monoica*. The polypeptide may also be glutamine:fructose-6-phosphate amidotransferase.

[20] The invention also provides plant cells, particularly plant cells capable of developing into a fiber cell, as well as plants, parts or tissue thereof or seeds of a plant, comprising a recombinant gene or construct according to the invention. An example of such a plant is a cotton plant.

[21] The invention further relates to a method of producing a fiber-producing plant, such as a cotton plant, with spatially selective expression of a recombinant gene, comprising the steps of

- (a) Introducing a recombinant gene according to the invention into at least one cell of the fiber producing plant; and
- (b) Optionally, regenerating a fiber-producing plant from the at least one cell comprising the recombinant gene.

[22] In yet another aspect of the invention, microRNA from cotton is provided selected from the group of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230, or Ghi_miR398 or having the nucleotide sequence of any one of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 20, SEQ ID No. 21 or SEQ ID No. 22.

[23] The invention also provides target sites recognized by a microRNA according to the invention including target sites having the nucleotide sequence of any one of SEQ ID Nos 5 to 8, SEQ ID 8 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 6 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 8 from nucleotide position 4 to 24, SEQ ID No. 23, SEQ ID No. 23 from nucleotide position 4 to 24, SEQ ID No. 23 from nucleotide position 11 to 32, SEQ ID No. 23 from nucleotide positions 4 to 32, or a nucleotide sequence complementary to a miRNA having a nucleotide sequence according to any one of SEQ ID Nos 1 to 4, SEQ ID No. 20, SEQ ID No. 21 or SEQ ID No. 22 whereby one or more of the following mismatches may occur:

- (a) a mismatch between the nucleotide at the 5' end of the miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (b) a mismatch between any one of the nucleotides in position 1 to position 9 of the miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (c) three mismatches between any one of the nucleotides in position 12 to position 21 of the miRNA and the corresponding nucleotide sequence in the target RNA sequence provided that there are no more than two consecutive mismatches; and
- (d) no mismatch is allowed at positions 10 and 11 of the miRNA.

[24] In yet another embodiment of the invention, use of a micro RNA and/or a target site according to the invention is provided to increase the selectivity of fiber-selective expression of a recombinant gene in a fiber-producing cell of a fiber-producing plant, such as a cotton plant.

[25] In still another embodiment of the invention, pre-microRNAs from cotton are provided having a nucleotide sequence of any one of SEQ ID NOs: 9, 10, 11 or 12 or a nucleotide sequence essentially similar or identical thereto wherein the microRNA region and optionally the region complementary to the microRNA region (microRNA* region) have been altered.

[26] The invention also provides a method for producing fibers from a fiber-producing plant, such as cotton, comprising the steps of growing a plant comprising a recombinant gene according to the invention and harvesting fibers from the grown plants as well as plant fibers, such as cotton fibers obtained by such method.

Brief description of the figures

[27] The following Examples, not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference.

[28] **Figure 1.** Experimental validation of spatial and temporal expression pattern for various miRNA in cotton (*Gossypium hirsutum* L. var. FM958) showing a differential pattern between fibers and other parts of the plant. The following sample numbers apply to all panels: 1) Root sampled at 2-leaf stage ; 2) cotyledon sampled at seedling stage; 3) Leaf sampled at 2-leaf stage; 4) Leaf sampled at flowering stage; 5) Stem sampled at 2-leaf stage; 6) Stem sampled at flowering stage; 7) Shoot apical meristems (SAM) sampled during vegetative growth; 8) Developing fiber 8 days post-anthesis (dpa); 9) Developing fiber 15dpa; 10) Developing fiber 20 dpa; 11) Developing fiber 25 dpa; 12) Developing fiber 30 dpa.

[29] **Panel A.** Spatial and temporal expression pattern for Ghi_miR403 as determined by small RNA deep sequencing. Y-axis: miRNA abundance (reads/million). X-axis: Sample number.

[30] **Panel B.** Spatial and temporal expression pattern for Ghi_miR403 as determined by Northern blot assays. Shown in the upper panel is a northern blot probed for miR403. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for endogenous small RNAs. The arrow indicates mature miR403. The lower panel shows the ethidium bromide-stained gel section corresponding to the 5S rRNA and tRNA zone. Sample numbers are indicated below panels.

[31] **Panel C.** Spatial and temporal expression pattern for Ghi_miR408 as determined by small RNA deep sequencing. Y-axis: miRNA abundance (reads/million). X-axis: Sample number.

[32] **Panel D.** Spatial and temporal expression pattern for Ghi_miR408 as determined by Northern blot assays. Shown in the upper panel is a northern blot probed for miR408. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for endogenous small RNAs. The arrow indicates mature miR408. The lower panel shows the ethidium bromide-stained gel section corresponding to the 5S rRNA and tRNA zone. Sample numbers are indicated below panels.

[33] **Panel E.** Spatial and temporal expression pattern for Ghi_miR398 as determined by Northern blot assays. Shown in the upper panel is a northern blot probed for miR398. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for endogenous small RNAs. The arrow indicates mature miR398. The lower panel shows the ethidium bromide-stained gel section corresponding to the 5S rRNA and tRNA zone. Sample numbers are indicated below panels.

[34] **Panel F.** Spatial and temporal expression pattern for Ghi_miRcan1230 as determined by small RNA deep sequencing. Y-axis: miRNA abundance (reads/million). X-axis: Sample number.

[35] **Panel G.** Spatial and temporal expression pattern for Ghi_miRcan1230 as determined by Northern blot assays. Shown in the upper panel is a northern blot probed

for miR1230. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for endogenous small RNAs. The arrow indicates miRcan1230. The lower panel shows the ethidium bromide-stained gel section corresponding to the 5S rRNA and tRNA zone. Sample numbers are indicated below panels.

[36] **Figure 2.** Validation of miR403-mediated repression of LUC reporter gene expression in an *Agrobacterium*-mediated transient assay system on *Nicotiana benthamiana*. The numbers refer to the following experimental set-up: 1): injection of *Agrobacterium* containing control vector only; 2) injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter; 3) injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a miR403 target site in its 3'UTR; 4) injection of *Agrobacterium* containing T-DNA vector expressing a firefly luciferase (LUC) based reporter under control of a CaMV35S promoter engineered with a mutated miR403 interacting site in its 3'UTR; 5) Co-injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a miR403 target site in its 3'UTR and *Agrobacterium* containing a control T-DNA vector; 6) Co-injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC) based reporter under control of a CaMV35S promoter engineered with a miR403 target site in its 3'UTR and a *Agrobacterium* containing a T-DNA vector expressing miR403 pre-miRNA under control of a CaMV35S promoter; 7) Co-injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a mutated miR403 interacting site in its 3'UTR and a *Agrobacterium* containing a control T-DNA vector; 8) Co-injection of *Agrobacterium* containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a mutated miR403-interacting site in its 3'UTR and a *Agrobacterium* containing a T-DNA vector expressing miR403 pre-miRNA under control of a CaMV35S promoter.

[37] **Panel A** and **Panel H**. LUC activity detected from *Nicotiana benthamiana* leaf tissues sampled at 2 day post injection (dpi) (panel A) or 3 dpi (panel H). Y-axis: Relative LUC activity ($\times 10^7$ unit/mg Fresh Weight); X-axis: sample number.

[38] **Panel B** and **Panel I**. Accumulation of miR403 in *Nicotiana benthamiana* leaf tissues sampled at (B) 2 and (I) 3 days post injection as detected by Northern blots. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for small RNAs.

[39] **Panel C** and **Panel J**. Northern blots of panels B and I, probed with U6 small nuclear RNA as control.

[40] **Panel D** and **Panel K**. Ethidiumbromide-stained gel section corresponding to the 5S rRNA and tRNA zone from *Nicotiana benthamiana* leaf tissues sampled at 2 day post injection (dpi) (panel D) or 3 dpi (panel K).

[41] **Panel E** and **Panel L**. Detection of luciferase protein by immunoblot assay of *Nicotiana benthamiana* leaf tissues sampled at 2 day post injection (panel E) or 3 dpi (panel L) using monoclonal anti-Luciferase antibody produced in mouse.

[42] **Panel F** and **Panel M**. Detection of elongation factor 1α protein by immunoblot assay of *Nicotiana benthamiana* leaf tissues sampled at 2 day post injection (panel F) or 3 dpi (panel M) using polyclonal anti-E1F serum antibody produced in rabbit.

[43] **Panel G** and **Panel N**. Accumulation of miR168 in *Nicotiana benthamiana* leaf tissues sampled at (G) 2 and (N) 3 days post injection as detected by Northern blots.

[44] **Panel O** and **Panel P**. Accumulation of LUC mRNA detected from *Nicotiana benthamiana* leaf tissues sampled at 3 days post injection. Shown in the panel O are the RT-PCR products resolved in an ethidium bromide-stained agarose gel. The relative band intensity reflects the level of intact LUC mRNAs that did not undergo miR403-directed cleavage. Panel P shows the products of control RT-PCR reactions for mRNAs

encoding *N. benthamiana* heat shock protein 70-like. Numbers underneath panel O indicate the relative signal intensity as measured by Image Quant.

[45] **Figure 3.** Nucleotide sequence of miR403 and miR403-interacting site (target site) (sequences are included in the sequence listing as SEQ ID No 1 for the Ghi_miR403 and Ath_miR403; as SEQ ID No. 18 for the target nucleotide sequence in Arabidopsis AGO2 mRNA; as SEQ ID No. 19 for the miR403 form reproduced under the target site; as SEQ ID No. 5 for the miR403 engineered target site; as SEQ ID NO 5 for Ath_miR403_R; and as SEQ ID No. 1 for Ghi_miR403_R).

[46] **Figure 4.** Nucleotide sequence of major and minor forms of mature miR408 detected in cotton and of a miR408 target sequence.(Sequences are included in the sequence listing as SEQ ID No. 2 for ghi-miR408.1; as SEQ ID No. 20 for ghi-miR408.2; as SEQ ID No. 6 for miR408-target and as SEQ ID No. 2 for ath-MiR408_R).

[47] **Figure 5.** Nucleotide sequence of target sequence (top) and miRNA sequence for the following miRNA detected in cotton: (A) Ghi_miRcan1230- three forms; (B) Ghi_miR398. (Sequences are included in the sequence listing as SEQ IDs No. 10 and 23 for the target sequence of miRcan1230(1) and combined for miRcan1230(2) (3) respectively; as SEQ ID No. 3 for miRcan1230 (1) as SEQ ID 21 for miRcan1230(2) , as SEQ ID 22 for miRcan1230(3); as SEQ ID NO. 4 for miR398 and as SEQ ID No. 8 for the target sequence of miR398. The target sequence for miRcan1230(3) corresponds to SEQ ID No. 23 from nucleotide 11 to 35, preceded by 5'-a g a-3'.

[48] **Figure 6.** Validation of miRcan1230-mediated repression of LUC reporter gene expression in an Agrobacterium-mediated transient assay system on *Nicotiana benthamiana*. The numbers refer to the following experimental set-up: 1): injection of Agrobacterium containing control vector only; 2) injection of Agrobacterium containing a T-DNA vector expressing a miRcan1230 pre-microRNA under control of a CaMV35S promoter; 3) injection of Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter; 4) injection of

Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a miRcan1230 target site in its 3'UTR; 5) injection of Agrobacterium containing T-DNA vector expressing a firefly luciferase (LUC) based reporter under control of a CaMV35S promoter engineered with a mutated miRcan1230 interacting site in its 3'UTR; 6) Co-injection of Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a miRcan1230 target site in its 3'UTR and Agrobacterium containing a control T-DNA vector; 7) Co-injection of Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC) based reporter under control of a CaMV35S promoter engineered with a miRcan1230 target site in its 3'UTR and a Agrobacterium containing a T-DNA vector expressing miRcan1230 pre-miRNA under control of a CaMV35S promoter; 8) Co-injection of Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC)-based reporter under control of a CaMV35S promoter engineered with a mutated miRcan1230 interacting site in its 3'UTR and a Agrobacterium containing a control T-DNA vector; 8) Co-injection of Agrobacterium containing a T-DNA vector expressing a firefly luciferase (LUC)- based reporter under control of a CaMV35S promoter engineered with a mutated miRcan1230-interacting site in its 3'UTR and a Agrobacterium containing a T-DNA vector expressing miRcan1230 pre-miRNA under control of a CaMV35S promoter.

[49] **Panel A.** LUC activity detected from *Nicotiana benthamiana* leaf tissues sampled at 3 day post injection (dpi). Y-axis: Relative LUC activity ($\times 10^7$ unit/mg Fresh Weight); X-axis: sample number.

[50] **Panel B.** Accumulation of miRcan1230 in *Nicotiana benthamiana* leaf tissues sampled at 3 days post injection as detected by Northern blots. GFP-derived, 21- and 24-nt synthetic RNA oligos were used as size markers for small RNAs.

[51] **Panel C.** Northern blots of panel B, probed with U6 small nuclear RNA as control.

[52] **Panel D.** Ethidiumbromide-stained gel section corresponding to the 5S rRNA and tRNA zone from *Nicotiana benthamiana* leaf tissues sampled at 3 day post injection (dpi).

[53] **Panel E and Panel F.** Accumulation of LUC mRNA detected from *Nicotiana benthamiana* leaf tissues sampled at 3 days post injection . Shown in the panel E and F are the RT-PCR products resolved in an ethidium bromide-stained agarose gel. The relative band intensity reflects the level of 5' region of LUC mRNAs that did not undergo miRcan1230-directed cleavage (panel E) or the 3' region of LUC mRNAs (panel F).

[54] **Panel G** shows the products of control RT-PCR reactions for mRNAs encoding *N. benthamiana* heat shock protein 70-like. Numbers underneath panel E and F indicate the relative signal intensity as measured by Image Quant.

Detailed description of preferred embodiments

[55] The current invention is based on the fortuitous identification of miRNA molecules (and pre-miRNA molecules as well as target recognitions sites) from cotton (*Gossypium hirsutum*) which are not expressed in developing cotton fiber cells while present in most if not all other cotton plant parts. Such miRNAs, and/or their target sites may be conveniently used to enhance tissue selectivity of expression in a fiber-producing plant, such as cotton.

[56] Thus, in a first embodiment, the invention provides a method for spatially selective expression in a fiber-producing plant, such as cotton, comprising the steps of introducing a recombinant gene into at least one cell of the fiber producing plant, such as cotton, and optionally regenerating a plant from said at least one cell comprising the recombinant construct or gene, whereby the recombinant construct or gene comprises the following operably linked elements (such as DNA elements):

- (a) a plant-expressible promoter;
- (b) a region encoding a biologically active RNA molecule;

(c) optionally a 3' transcription termination and polyadenylation region and wherein the recombinant gene or construct further comprises a target sequence recognized by a miRNA which is differentially expressed in cells leading to fibers in the fiber producing plant compared to cells of the fiber-producing plants other than said cells leading to said fibers.

[57] As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

[58] As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S, the subterranean clover virus promoter No 4 or No 7, or T-DNA gene promoters and the like.

[59] It will be clear that constitutive plant-expressible promoters may be suitable for the invention. In such cases, the spatial selectivity or tissue-specificity will be provided by the expression profile of the microRNA, which will in turn determine in which tissues or plants or parts, the transcription products of the recombinant gene incorporating a target sequence for the miRNA, will be processed post-transcriptionally by the miRNA (and associated proteins) to cleave that transcript and thus suppress or prevent further activity of the biologically active RNA (e.g. prevent translation of a peptide or protein encoded by the biologically active RNA).

[60] It is also clear that inducible promoters, such as a temperature inducible or a chemically inducible promoter or a promoter which is responsive to developmental cues, may be used in accordance with the invention. The resulting expression profile of the

recombinant gene will be a combined expression profile, mirroring the spatial distribution of the miRNA in the plant and the response to the inducing or repressing factors of the promoter used.

[61] Tissue selective promoters may also be used. In a preferred embodiment of the invention a fiber-preferential or fiber-selective promoter is used.

[62] The term “fiber specific” or “fiber cell specific” or “fiber-selective”, with respect to the expression of a gene or with respect to a promoter, refers to, for practical purposes, the highly specific, expression of a gene or expression directed by a promoter, in fiber cells of plants, such as cotton plants. In other words, transcript levels of a DNA in tissues different of fiber cells is either below the detection limit or very low (less than about 0.2 picogram per microgram total RNA).

[63] The term “fiber-preferential” or “fiber-cell preferential” with respect to the expression of a DNA in accordance with this invention, refers to an expression pattern whereby the DNA is expressed predominantly in fiber cells or fibers, but expression can be identified in other tissues of the plant. Preferably, the expression in fiber cells is about 2 to about 10 times higher in the fiber cells than in other tissues.

[64] Such promoters (all herein incorporated by reference) include the promoter from cotton from a fiber-specific β - tubulin gene (as described in WO0210377), the promoter from cotton from a fiber-specific actin gene (as described in WO0210413), the promoter from a fiber specific lipid transfer protein gene from cotton (as described in US5792933), a promoter from an expansin gene from cotton (WO9830698) or a promoter from a chitinase gene in cotton (US2003106097) or the promoters of the fiber specific genes described in US6259003 or US6166294. Fiber selective promoters as described in WO08/083969 (from cotton glucanase genes), WO12/093032 (from cotton FS18 or SCW-PRP gene) or in US application 13/630,119 (from cotton FB8-like genes) are also suitable plant-expressible promoters. Also suitable for the invention is

the promoter comprising the nucleotide sequence of SEQ ID 13 from nucleotide position 61 to nucleotide position 1499.

[65] As used herein, a “miRNA” is an RNA molecule of about 20 to 22 nucleotides in length which can be loaded into a RISC complex and direct the cleavage of another RNA molecule, wherein the other RNA molecule comprises a nucleotide sequence essentially complementary to the nucleotide sequence of the miRNA molecule whereby one or more of the following mismatches may occur: a mismatch between the nucleotide at the 5' end of said miRNA and the corresponding nucleotide sequence in the target RNA molecule; a mismatch between any one of the nucleotides in position 1 to position 9 of said miRNA and the corresponding nucleotide sequence in the target RNA molecule; three mismatches between any one of the nucleotides in position 12 to position 21 of said miRNA and the corresponding nucleotide sequence in the target RNA molecule provided that there are no more than two consecutive mismatches. no mismatch is allowed at positions 10 and 11 of the miRNA (all miRNA positions are indicated starting from the 5' end of the miRNA molecule).

[66] A miRNA is processed from a “pre-miRNA” molecule by proteins, such as DCL proteins, present in any plant cell and loaded onto a RISC complex where it can guide the cleavage of the target RNA molecules.

[67] As used herein, a “pre-miRNA” molecule is an RNA molecule of about 100 to about 200 nucleotides, preferably about 100 to about 130 nucleotides which can adopt a secondary structure comprising a double stranded RNA stem and a single stranded RNA loop and further comprising the nucleotide sequence of the miRNA (and its complement sequence) in the double stranded RNA stem. Preferably, the miRNA and its complement are located about 10 to about 20 nucleotides from the free ends of the miRNA double stranded RNA stem. The length and sequence of the single stranded loop region are not critical and may vary considerably, e.g. between 30 and 50 nt in length. Preferably, the difference in free energy between unpaired and paired RNA structure is between -20 and -60 kcal/mole, particularly around -40 kcal/mole. The complementarity between the

miRNA and the miRNA* need not be perfect and about 1 to 3 bulges of unpaired nucleotides can be tolerated. The secondary structure adopted by an RNA molecule can be predicted by computer algorithms conventional in the art such as mFOLD. The particular strand of the double stranded RNA stem from the pre-miRNA which is released by DCL activity and loaded onto the RISC complex is determined by the degree of complementarity at the 5' end, whereby the strand which at its 5' end is the least involved in hydrogen bonding between the nucleotides of the different strands of the cleaved dsRNA stem is loaded onto the RISC complex and will determine the sequence specificity of the target RNA molecule degradation. However, if empirically the miRNA molecule from a particular synthetic pre-miRNA molecule is not functional (because the "wrong" strand is loaded on the RISC complex, it will be immediately evident that this problem can be solved by exchanging the position of the miRNA molecule and its complement on the respective strands of the dsRNA stem of the pre-miRNA molecule. As is known in the art, binding between A and U involving two hydrogen bonds, or G and U involving two hydrogen bonds is less strong than between G and C involving three hydrogen bonds.

[68] One set of miRNA suitable for methods according to the invention includes miRNAs which are less abundantly expressed in cells leading to fibers in fiber producing plants compared to cells of those fiber-producing plants other than cells leading to said fibers. Especially suited are miRNAs which are substantially not expressed in cells leading to fibers in a fiber-producing plant but ubiquitously expressed in cells other than said cells leading to fibers.

[69] Such miRNAs include the following microRNAs isolated from *Gossypium hirsutum*: Ghi_miR403, Ghi_miR408, Ghi_miRcan1230 and Ghi_miR398. These microRNAs comprise a nucleotide sequence selected from SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, or SEQ ID No. 4 (see also figures 3, 4 and 5). They are processed in *Gossypium hirsutum* from pre-miRNAs comprising the nucleotide sequence of SEQ ID Nos 9, 10, 11 or 12, or a nucleotide sequence essentially similar thereto.

[70] The indication miRcanXXXX refers to the provisional number allocation for new microRNAs, in correspondence with the naming conventions for microRNA.

[71] It will be clear that orthologs of these miRNA or pre-miRNA can be isolated, or identified and used, also in other plants, particularly fiber-producing plants.

[72] “A target sequence recognized by a miRNA” refers to the nucleotide sequence within an RNA molecule towards which the miRNA guides the RISC complex with which it is associated, so that the RNA molecule is cleaved by the ribonuclease activity of an Argonaute protein (AGO) central to the RISC complex. The target sequence is essentially complementary, or complementary to the miRNA nucleotide sequence. As used herein, a nucleotide sequence which is complementary to another nucleotide sequence is a nucleotide sequence with opposite polarity in the phosphor-ribose backbone as the other sequence and which has a nucleotide sequence which allows base-pairing between the bases of the sequence and the other sequence. Base-pairing in this context includes G:U base-pairs as well as standard Watson-Crick base-pairing. It will also be clear that when RNA sequences are the nucleotide sequences to be essentially complementary with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

[73] It has been observed that one or more of the following mismatches may occur in the base-pairing between the nucleotides of the target site and the nucleotides of the miRNA:

- (a) a mismatch between the nucleotide at the 5' end of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (b) a mismatch between any one of the nucleotides in position 1 to position 9 of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (c) three mismatches between any one of the nucleotides in position 12 to position 21 of the miRNA and the corresponding nucleotide sequence in the target RNA sequence provided that there are no more than two consecutive mismatches; and
- (d) no mismatch is allowed at positions 10 and 11 of the miRNA.

[74] Thus target sites suitable for the invention may comprise a nucleotide sequence which is complementary to the nucleotide sequence of any of the nucleotide sequences of SEQ ID Nos 1 to 4 or which is essentially similar to those nucleotide sequences, and wherein one or more of the above mentioned mismatches occur. Examples of target sites recognized by miRNAs suitable for the invention can be found in figures 3, 4 and 5 and in the sequence listing under entries SEQ ID Nos 5 to 8 and SEQ ID 23 for an engineered target sequence recognized by microRNA Ghi_miRcan1230(2) and (3) respectively. The core target sequence recognized by microRNA Ghi_miRcan1230(2) corresponds to SEQ ID 23 from nucleotide 4 to 24. The core target sequence recognized by microRNA Ghi_miRcan1230(3) corresponds to SEQ ID 23 from nucleotide 11 to 32.

[75] The use of a target site or sequence complementary to the miRNA nucleotide sequence (so-called core target site) will generally be sufficient, but it may be advantageous to include one, two, three or more nucleotides which are flanking the core target site in the nucleotide sequence of the natural target RNA molecule. An example thereof can be found in SEQ ID No 5 where the nucleotide sequence from nucleotide position 4 to nucleotide position 24 corresponds to the core target sequence of miR403, while the additional nucleotide sequence 1 to 3 and 25-27 correspond to the sequences flanking the core target sequence in the natural target RNA molecule. Similarly, an example thereof can be found in SEQ ID No 6 where the nucleotide sequence from nucleotide position 4 to nucleotide position 24 corresponds to the core target sequence of miR408, while the additional nucleotide sequence 1 to 3 and 25-27 correspond to the sequences flanking the core target sequence in the natural target RNA molecule.

[76] Exogenous target site for miRNA may be introduced in the recombinant construct of interest in various manners. The target site nucleotide sequence may be inserted as additional nucleotide sequence. In another embodiment, the miRNA target site may be engineered by modifying a naturally occurring sequence with a sufficient degree of similarity to the target site, according to the rules mentioned elsewhere, to arrive a nucleotide sequence recognized by the miRNAs according to the invention.

[77] The exogenous target sites for miRNA may be introduced anywhere in the transcribed region of the recombinant construct. However, if the transcribed biologically active RNA molecule encodes a polypeptide, care has to be taken that potential introduction of the target site for miRNA in the coding region, preferably does not disrupt the open reading frame or otherwise alters the amino acid sequence of the encoded polypeptide. The target sites for miRNA can be conveniently included in the untranslated regions of the transcript, such as the 5' untranslated region or the 3' untranslated region.

[78] It will be clear to the skilled artisan that the methods and means described herein can be used to obtain spatially selective expression of any gene of interest. However, the methods of the invention can be used to enhance the specificity of expression of genes coding for potentially deleterious products if and when expressed in cells other than fiber cells (and limit such expression substantially to fiber cells in fiber producing plants).

[79] Examples of such nucleotide sequences have been described and include a nucleic acid encoding a polypeptide with N-acetylglucosamine transferase activity which may be an N-acetylglucosamine transferase of the NODC-type, such as a NODC-type N-acetylglucosamine transferase obtainable from a *Rhizobium* species, an *Azorhizobium* species, a *Bradyrhizobium* species, a *Mesorhizobium* species, a *Ralstonia* species, a *Streptomyces* species, a *Burkholderia* species, a *Cupriavidus* species or a *Sinorhizobium* species. The nucleic acid may also encode an N-acetylglucosamine transferase activity, including chitin synthase, further comprising a signal anchor sequence selected from the signal anchor sequence of a rat sialyl transferase, the signal anchor sequence of a human galactosyl transferase, the signal anchor sequence of the Arabidopsis homologue of the yeast HDEL receptor (AtERD2), the signal anchor sequence of the α -2,6-sialyltransferase, the signal anchor sequence of β 1,2-xylosyltransferase from *Arabidopsis thaliana*, the signal anchor sequence of N-acetylglucosaminyl transferase I from tobacco or the amino acid sequence YYHDL or LKLEI. The N-acetylglucosamine transferase may comprise an amino acid sequence

encoded by the nucleotide sequence of SEQ ID No. 13 from nucleotide position 1503 to nucleotide position 4439. (Also described in WO 06/136351 incorporated herein by reference). The N-acetylglucosamine transferase may also be chitin synthase, preferably chitin synthase 2 of *Saprolegnia monoica* as described in WO11/089021 (herein incorporated by reference).

[80] The biologically active RNA transcribed from the recombinant DNA, gene or construct according to the invention may also encode a glutamine:fructose-6-phosphate amidotransferase, such as a glutamine:fructose-6-phosphate amidotransferase comprising an amino acid sequence encoded by the nucleotide sequence of SEQ ID No. 13 from nucleotide position 6202 to nucleotide position 8031 or a glutamine:fructose-6-phosphate amidotransferase from *Volvariella volvacea* comprising an amino acid sequence as represented in Genbank entry AAT75220 (incorporated herein by reference).

[81] The invention is also directed towards recombinant constructs or genes as herein described in particular towards a recombinant gene for spatially selective expression in a fiber-producing plant comprising the following operably linked elements:

- (a) a plant-expressible promoter;
- (b) a region encoding a biologically active RNA molecule;
- (c) optionally a 3' transcription termination and polyadenylation region

characterized in that the recombinant gene further comprises a target sequence recognized by a miRNA which is differentially expressed in cells leading to fibers in said fiber producing plant compared to other cells of fiber-producing plants.

[82] A “fiber”, such as a “cotton fiber”, as used herein, refers to a seed trichome, more specifically a single cell of a fiber-producing plant, such as cotton, that initiates from the epidermis of the outer integument of the ovules, at or just prior to anthesis. The morphological development of cotton fibers has been well documented (Basra and Malik, 1984, Int Rev of Cytology 89: 65–113; Graves and Stewart, 1988, *supra*; Ramsey and Berlin, 1976, American Journal of Botany 63 (6): 868-876; Ruan and Chourey,

1998, Plant Physiology 118: 399–406; Ruan et al. 2000, Aust. J. Plant Physiol. 27:795–800; Stewart, 1975, Am. J. Bot. 62, 723-730). Cotton fibers, in particular from *Gossypium hirsutum*, undergo four overlapping developmental stages: fiber cell initiation, elongation, secondary cell wall biosynthesis, and maturation. Fiber cell initiation is a rapid process. White fuzzy fibers begin to develop immediately after anthesis and continue up to about 3 days post-anthesis (DPA), which is followed by fiber cell elongation (until about 10 to about 17 DPA). Depending upon growth conditions, secondary cell wall biosynthesis initiates and continues to about 25 to about 45 DPA, followed by a maturation process from about 45 to about 60 DPA. The secondary cell wall synthesis and maturation phase are herein commonly referred to as “fiber strength building phase”. Only about 25 to 30% of the epidermal cells differentiate into the commercially important lint fibers (Kim and Triplett, 2001). The majority of cells does not differentiate into fibers or develop into short fibers or fuzz. During fiber elongation and secondary wall metabolism, the fiber cells elongate rapidly, synthesize secondary wall components, and show dramatic cellular, molecular and physiological changes. Fiber elongation is coupled with rapid cell growth and expansion (Seagull, 1991. In *Biosynthesis and biodegradation of cellulose* (Haigler, C. H. & Weimer, P. J., eds) pp. 143-163, Marcel Dekker, New York) and constant synthesis of a large amount of cell metabolites and cell wall components such as cellulose. About 95% of the dry-weight in mature cotton fibers is cellulose (Pfluger and Zambryski, 2001, Curr Biol 11: R436–R439; Ruan et al., 2001, Plant Cell 13: 47–63). Non-celluloid components are also important to fiber cell development (Hayashi and Delmer, 1988, Carbohydr. Res. 181: 273-277; Huwyler et al., 1979, Planta 146: 635–642; Meinert and Delmer, 1977, Plant Physiol 59: 1088-1097; Peng et al., 2002, Science 295: 147–150). Compared to other plant cells, cotton fibers do not contain lignin in secondary walls but have large vacuoles that are presumably related to rapid cell growth and expansion (Basra and Malik, 1984, *supra*; Kim and Triplett, 2001, Plant Physiology 127: 1361–1366; Mauney, 1984, *supra*; Ruan and Chourey, 1998, *supra*; Ruan et al., 2000, *supra*; Van ‘t Hof, 1999, American Journal of Botany 86: 776–779).

[83] A “fiber-producing plant” refers to a plant species that produces fibers as defined above, such as a cotton plant. Of the *Gossypium* species, the A genome diploid *Gossypium* species and AD genome allotetraploid *Gossypium* species are known to produce spinnable fiber. Botanically, there are three principal groups of cotton that are of commercial importance. The first, *Gossypium hirsutum* (AADD), is native to Mexico and Central America and has been developed for extensive use in the United States, accounting for more than 95 % of U.S. production. This group is known in the United States as American Upland cotton, and their fibers vary in length from about 7/8 to about 1 5/16 inches (about 22 – about 33 mm). Worldwide it accounts for about 90% of the cotton production. A second botanical group, *G. barbadense* (AADD), which accounts for about 5% of U.S. production and about 8% of the worldwide production, is of early South American origin. With fibers varying in length from about 1 1/4 to about 1 9/16 inches (about 32 - about 40 mm), it is known in the United States as American Pima, but is also commonly referred to as Extra Long Staple (ELS) cotton. A third group, *G. herbaceum* (AA) and *G. arboreum* (AA), embraces cotton plants with fibers of shorter length, about 1/2 to about 1 inch (about 13 – about 25 mm), that are native to India and Eastern Asia. None from this group is cultivated in the United States.

[84] The term “gene” means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. into a pre-mRNA, comprising intron sequences, which is then spliced into a mature mRNA, or directly into a mRNA without intron sequences) in a cell, operable linked to regulatory regions (e.g. a promoter). A gene (genomic DNA) may thus comprise several operably linked sequences, such as a promoter, a 5' leader sequence comprising e.g. sequences involved in translation initiation, a (protein) coding region (with introns) and a 3' non-translated sequence comprising e.g. transcription termination sites. “cDNA sequence” refers to a nucleic acid sequence comprising the 5' untranslated region, the coding region without introns and the 3' untranslated region and a polyA tail. “Endogenous gene” is used to differentiate from a “foreign gene”, “transgene” or “chimeric gene”, and refers to a gene from a plant of a certain plant genus, species or variety, which has not been introduced into that plant by transformation (i.e. it is not a “transgene”), but which is normally

present in plants of that genus, species or variety, or which is introduced in that plant from plants of another plant genus, species or variety, in which it is normally present, by normal breeding techniques or by somatic hybridization, e.g., by protoplast fusion. Similarly, an “endogenous allele” of a gene is not introduced into a plant or plant tissue by plant transformation, but is, for example, generated by plant mutagenesis and/or selection, introgressed from another plant species by, e.g., marker-assisted selection, or obtained by screening natural populations of plants.

[85] “Expression of a gene” or “gene expression” refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA molecule. The RNA molecule is then processed further (by post-transcriptional processes) within the cell, e.g. by RNA splicing and translation initiation and translation into an amino acid chain (polypeptide), and translation termination by translation stop codons. The term “functionally expressed” is used herein to indicate that a functional, i.e. biologically active, protein is produced; the term “not functionally expressed” to indicate that a protein with significantly reduced or no functionality (biological activity) is produced or that no or a significantly reduced amount of protein is produced.

[86] Methods to transform plants are well known in the art and are of minor relevance for the current invention. Methods to transform cotton plants are also well known in the art. *Agrobacterium*-mediated transformation of cotton has been described e.g. in US patent 5,004,863 or in US patent 6,483,013 and cotton transformation by particle bombardment is reported e.g. in WO 92/15675.

[87] The chimeric genes according to the invention may be introduced into plants in a stable manner or in a transient manner using methods well known in the art. The chimeric genes may be introduced into plants, or may be generated inside the plant cell as described e.g. in EP 1339859.

[88] The invention is also directed towards fiber-producing plants comprising a recombinant construct according to the invention. Preferred fiber-producing plants include cotton.

[89] “Cotton” as used herein includes *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium arboreum* and *Gossypium herbaceum*. “Cotton progenitor plants” include *Gossypium arboreum*, *Gossypium herbaceum*, *Gossypium raimondii*, *Gossypium longicalyx* and *Gossypium kirkii*.

[90] The methods and means of the current invention may also be employed for other plant species such as hemp, jute, flax and woody plants, including but not limited to *Pinus spp.*, *Populus spp.*, *Picea spp.*, *Eucalyptus spp.* etc.

[91] The plants according to the invention can be used in a conventional breeding scheme to produce more plants with the same characteristics or to introduce the chimeric gene according to the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

[92] The invention is also directed towards a method for producing fibers from fiber-producing plants such as cotton, comprising the steps of a) growing a plant comprising a recombinant construct according to the invention and b) harvesting fibers from the grown plants. Fibers, such as cotton fibers, obtained from these plants are also within the scope of the invention.

[93] The invention also provides novel pre-miRNAs from cotton, in particular pre-microRNAs from cotton having a nucleotide sequence of any one of SEQ ID NOs: 9, 10, 11 or 12. Such pre-miRNAs can be used for any purpose pre-miRNAs are used in the art, including replacing or adapting the microRNA and microRNA* region to obtain a pre-miRNA with a nucleotide sequence essentially similar or identical thereto wherein the microRNA region and optionally the region complementary to the microRNA region (microRNA* region) have been altered and recognize novel target sites. Such

engineered pre-miRNAs may be expressed from their naturally associated promoter region, and the engineered miRNA may thereby gain an expression profile similar to that of the miRNAs (e.g. fiber-specific or expression in all plant parts except fibers).

[94] As used herein “comprising” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region, which is functionally or structurally defined, may comprise additional DNA regions etc.

[95] The following non-limiting examples describe the identification of microRNAs from cotton which have a low or no expression in fibers at different developmental stages and use of target sites corresponding to these microRNAs in transgenes.

[96] Unless states otherwise in the Examples, all recombinant techniques are carried out according to standard protocols as described in “Sambrook J and Russell DW (eds.) (2001) Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press, New York” and in “Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (eds.) (2006) Current Protocols in Molecular Biology. John Wiley & Sons, New York”.

[97] Standard materials and references are described in “Croy RDD (ed.) (1993) Plant Molecular Biology LabFax, BIOS Scientific Publishers Ltd., Oxford and Blackwell Scientific Publications, Oxford” and in “Brown TA, (1998) Molecular Biology LabFax, 2nd Edition, Academic Press, San Diego”. Standard materials and methods for polymerase chain reactions (PCR) can be found in “McPherson MJ and Møller SG (2000) PCR (The Basics), BIOS Scientific Publishers Ltd., Oxford” and in “PCR

Applications Manual, 3rd Edition (2006), Roche Diagnostics GmbH, Mannheim or www.roche-applied-science.com“.

[98] In the description and examples, reference is made to the following sequences:

SEQ ID No. 1:	nucleotide sequence of microRNA Ghi_miR403
SEQ ID No. 2:	nucleotide sequence of microRNA Ghi_miR408
SEQ ID No. 3:	nucleotide sequence of microRNA Ghi_miRcan1230(1)
SEQ ID No. 4:	nucleotide sequence of microRNA Ghi_miR398
SEQ ID No. 5:	nucleotide sequence of engineered target sequence recognized by microRNA Ghi_miR403
SEQ ID No. 6:	nucleotide sequence of engineered target sequence recognized by microRNA Ghi_miR408
SEQ ID No. 7:	nucleotide sequence of target sequence recognized by microRNA Ghi_miRcan1230(1) or (2)
SEQ ID No. 8:	nucleotide sequence of target sequence recognized by microRNA Ghi_miR398
SEQ ID No. 9:	nucleotide sequence of pre-microRNA Ghi_miR403
SEQ ID No. 10:	nucleotide sequence of pre-microRNA Ghi_miR408
SEQ ID No. 11:	nucleotide sequence of pre-microRNA Ghi_miRcan1230
SEQ ID No. 12:	nucleotide sequence of pre-microRNA Ghi_miR398
SEQ ID No. 13:	nucleotide sequence of pTBI312
SEQ ID No. 14:	nucleotide sequence of pTEA1_v2
SEQ ID No. 15:	nucleotide sequence of pEA2
SEQ ID No. 16:	nucleotide sequence of pTEA3
SEQ ID No. 17:	nucleotide sequence of pTEA6
SEQ ID No. 18:	target sequence of the miR403-interacting site in Arabidopsis AGO2 mRNA
SEQ ID No. 19:	miR403 from <i>A. thaliana</i>
SEQ ID No. 20:	minor form of Ghi_miR408

- SEQ ID No. 21: nucleotide sequence of alternative form microRNA
Ghi_miRcan1230(2)
- SEQ ID No. 22: nucleotide sequence of alternative form microRNA
Ghi_miRcan1230(3)
- SEQ ID No. 23: nucleotide sequence of engineered target sequence recognized by
microRNA Ghi_miRcan1230(2) and (3)
- SEQ ID No. 24: nucleotide sequence of pTEA9
- SEQ ID No. 25: nucleotide sequence of pTEA10

Examples

Example 1. Materials and methods

[99] **Total RNA extraction and small RNA fractionation.** Total RNAs including the low molecular weight (LMW) RNA fraction were extracted from plant materials and sRNAs were isolated by 17% polyacrylamide gel electrophoresis (PAGE) essentially as previously described [Qi, X., Bao, F.S., and Xie, Z. (2009). Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. *PLoS One* 4, e4971].

[100] **Small RNA library construction and Illumina sequencing.** PAGE-purified small RNAs were used for custom small RNA library construction essentially as described previously [Qi et al. supra], except that the 5' RNA adapter does not include any custom index sequences. PAGE-purified cDNAs for each small RNA library were submitted for sequencing on Illumina's Hi-Seq2000 instrument.

[101] **Plasmid constructs and transient expression assay.** All binary vectors were constructed using the backbone of pCB302 [Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D.J. (1999). A mini binary vector series for plant transformation. *Plant Mol Biol* 40, 711-717]. The *Agrobacterium* strain GV2260 transformed with desired binary vectors were used for transient expression assay following established procedure [Johansen, L.K., and Carrington, J.C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol* 126, 930-938].

[102] **Luciferase activity assay.** Luciferase activity was measured using a Luciferase Assay Systems (Promega) following the manufacturer's instructions.

[103] **Small RNA detection by northern blot.** LMW RNA blot was done as described previously [Xie, Z., Allen, E., Wilken, A., and Carrington, J.C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 102, 12984-12989]. A locked nucleic acid (LNA) probe (Exiqon) was used for miR403 detection with enhanced sensitivity

[Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S., and Havelda, Z. (2004). Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res* 32, e175].

[104] **Analysis of luciferase mRNA levels by reverse transcription coupled with polymerase chain reaction (RT-PCR).** Total RNA extracts were enriched for poly (A)⁺ mRNAs using the Oligotex (Qiagen) procedure following the manufacturer's instructions. The poly (A)⁺-enriched RNAs (~125ng) were then used for reverse transcription with Superscript III (Invitrogen), following by PCR using gene-specific primers.

[105] **Detection of luciferase protein by immunoblot assay.** Immunoblot assays were done essentially as described previously [Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., and Carrington, J.C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev Cell* 4, 205-217] using a monoclonal anti-Luciferase antibody produced in mouse (Sigma). The control blot for elongation factor 1-alpha was done using polyclonal anti-EF1A serum prepared in rabbit (Agrisera).

Example 2. Identification and Experimental Validation of miRNAs with Desired

Expression Patterns

[106] Utilization of currently available “tissue-specific promoters” in plant genetic engineering does not always lead to satisfactory outcomes due to “leaky” expression of transgenes outside the desired expression domains. To achieve enhanced tissue- and developmental stage-specific expression of transgenes for genetic engineering of cotton fiber, we use endogenous microRNA (miRNA)-based strategy to eliminate the undesired transgene expression outside the developing cotton fiber. Specifically, when a transgene of interest is engineered with an interaction site for an endogenous miRNA, expression of the transgene would be repressed by such a miRNA when their expression domains overlap spatially or temporally. Endogenous miRNAs that are absent in the developing

fiber but are expressed in other tissues could therefore be used as natural agents to knock-down undesired transgene expression outside the fiber.

[107] The recently emerged next-generation DNA sequencing (NGS) technology allows expression profiling of small RNAs using high-throughput platforms in a relatively cost-effective manner. Illumina's Sequencing-by-synthesis (SBS) platform was used to obtain the expression profile of cotton miRNAs among different tissue types as well as developmental stages. A total of 12 sampling point points were included for small RNA expression profiling by deep sequencing (sRNA-seq; Table 1). Over 100 million (M) high quality reads were obtained from each small RNA library. Using an in-house-built computational pipeline, putative miRNAs that are conserved in multiple plant species were first identified. A computational approach for de novo identification of miRNAs that have not been reported from any other plant species was then developed.

Table 1. Tissue Sampling for Small RNA Expression Profiling

Sample No.	Tissue type	Developmental Stage	Samle Designation
1	Root	2-leaf stage	Root
2	Cotyledons	Seedling	Cotyledons
3	Leaf	2-leaf stage	Leaf (1)
4	Leaf	Flowering stage	Leaf (2)
5	Stem	2-leaf stage	Stem (1)
6	Stem	Flowering stage	Stem (2)
7	Shoot apical meristems	Vegetative growth	SAM
8	Developing fiber	8 dpa	Fiber (8 dpa)
9	Developing fiber	15 dpa	Fiber (15 dpa)
10	Developing fiber	20 dpa	Fiber (20 dpa)
11	Developing fiber	25 dpa	Fiber (25 dpa)
12	Developing fiber	30 dpa	Fiber (30 dpa)

[108] The comprehensive small RNA datasets that have been generated allowed extensive and meaningful expression profiling using a computational approach. Several plant miRNAs were identified in cotton which exhibit an interesting expressional pattern and could otherwise be considered as candidates for the purpose of this invention.

[109] However, the majority of these miRNAs turned out to only represent mature miRNAs arising from a subset of paralogous MIRNA loci that form multi-member miRNA families. These miRNAs were removed from the list because certain paralogous MIRNA loci appear to exhibit quite different expression patterns, while the mature miRNAs arising from these loci may be nearly identical in sequence and therefore may share regulatory targets.

[110] The remaining candidates are those miRNAs likely arising from only one or very few paralogous loci. miR403 was present at low abundance in non-fiber samples but was nearly absent in the developing fibers. miR408 which is deeply conserved in land plants, exhibited a similar profile. Others include Ghi_miRcan1230 and Ghi_miR398.

[111] These expression patterns were experimentally validated and confirmed by Northern blot-based assays (Figure 1).

Example 3. Validation of miR403-mediated repression of LUC reporter in a transient assay system

[112] Although multiple miRNAs may be considered for use as a natural agent for clearance of undesired transgene expression in the non-fiber tissues in cotton, we have chosen miR403 as the first subject for further experimental validation due to its conserved nature and well-established interactions with its target in Arabidopsis. Briefly, miR403 is a semi-conserved plant miRNA that has been found only in the dicotyledonous species [Cuperus, J.T., Fahlgren, N., and Carrington, J.C. (2011). Evolution and functional diversification of MIRNA genes. *Plant Cell* 23, 431-442]. In Arabidopsis, miR403 has been shown to target the 3' untranslated region (3' UTR) of mRNA for ARGONAUTE2 (AGO2), one of 10 AGO family proteins in this reference plant species [Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* 57, 19-53; Vaucheret, H. (2008). Plant ARGONAUTES. *Trends Plant Sci* 13, 350-358]. The outcome of the

interaction between miR403 and its target involves cleavage of AGO2 mRNA [Allen, E., Xie, Z., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121, 207-221; Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A., and Carrington, J.C. (2005). Expression of Arabidopsis MIRNA genes. *Plant Physiol* 138, 2145-2154]. The Arabidopsis AGO2 is believed to play a role in defense against certain viral pathogens [Harvey, J.J., Lewsey, M.G., Patel, K., Westwood, J., Heimstadt, S., Carr, J.P., and Baulcombe, D.C. (2011). An antiviral defense role of AGO2 in plants. *PLoS One* 6, e14639; Jaubert, M., Bhattacharjee, S., Mello, A.F., Perry, K.L., and Moffett, P. (2011). ARGONAUTE2 mediates RNA-silencing antiviral defenses against Potato virus X in Arabidopsis. *Plant Physiol* 156, 1556-1564; Wang, X.B., Jovel, J., Udomporn, P., Wang, Y., Wu, Q., Li, W.X., Gasciolli, V., Vaucheret, H., and Ding, S.W. (2011). The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in Arabidopsis thaliana. *Plant Cell* 23, 1625-1638]. However, AGO2 does not appear to play an essential role for normal growth and development, as loss-of-function mutations in AGO2 do not appear to result in any notable defect [Vaucheret, H. (2008). Plant ARGONAUTES. *Trends Plant Sci* 13, 350-358; Mallory, A., and Vaucheret, H. (2010). Form, function, and regulation of ARGONAUTE proteins. *Plant Cell* 22, 3879-3889].

[113] Taking advantage of the well-characterized sequences involved in miR403-target interaction in Arabidopsis, a firefly luciferase (LUC)-based reporter has been designed [Xiong, L., David, L., Stevenson, B., and Zhu, J. (1999). High throughput screening of signal transduction mutant with luciferase imaging. *Plant Molecular Biology Reporter* 17, 159-170] engineered with a miR403 target site in its 3'UTR (designated as 35S::LUC-TAR^{miR403}). The efficacy of miR403-mediated down-regulation of LUC reporter expression was assessed by expressing the 35S::LUC-TAR^{miR403}, either alone or co-expressed with a 35S promoter-driven MIR403-producing construct (designated 35S::miR403) in an Agrobacterium-mediated transient assay system on *Nicotiana benthamina* [Johansen, L.K., and Carrington, J.C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient

expression system. Plant Physiol 126, 930-938]. LUC reporter constructs either lacking the miR403 target site (designated 35S::LUC) or harboring a mutated miR403 interacting site (designated as 35S::LUC-TAR^{miR403m}) were also made to serve as controls.

[114] The following scheme for co-injection was used.

1. Control vector only
2. 35S::LUC alone
3. 35S::LUC-TAR^{miR403} alone
4. 35S::LUC-TAR^{miR403m} alone
5. 35S::LUC-TAR^{miR403} + control vector
6. 35S::LUC-TAR^{miR403} + 35S::miR403
7. 35S::LUC-TAR^{miR403m} + control vector
8. 35S::LUC-TAR^{miR403m} + 35S::miR403

[115] At two days post injection (2 d.p.i.), luciferase activities were readily detectable from leaves infiltrated with 35S::LUC, but not from those infiltrated with the vector control (Fig. 2A). In leaves infiltrated with a miR403-targetable version of the LUC construct (35S::LUC-TAR^{miR403}), however, substantially lower luciferase activities were detected when compared to the 35S::LUC (Fig 2A), presumably due to a repression caused by the endogenous miR403 naturally expressed in the *N. benthamiana* leaves. Small RNA Northern blot assays confirmed that this was indeed the case, as low levels of miR403 accumulation were detected from the leaves infiltrated with the vector control (Fig. 2B and I). Consistent with an efficient posttranscriptional “knock down” of 35S::LUC-TAR^{miR403} expression by the endogenous miR403 from the *N. benthamiana* leaves, mutations that disrupt the miR403 interaction site in the engineered LUC reporter restored the luciferase activity to a level that is comparable to that detected from the 35S::LUC (Fig 2A; see the sample labeled with 35S::LUC-TAR^{miR403m}). Such a general trend in luciferase activity was also observed in samples taken at 3d.p.i. (Fig. 2B). To examine the effect of exogenous miR403 on the expression of LUC reporter, we also included in our experiment the “two-component” Agrobacterium injection

treatments where the LUC reporter is co-expressed with transgenic miR403. As shown in Fig 2, substantially elevated levels of miR403 accumulation were observed whenever the 35S::MIR403 construct was included in the co-infiltration (Fig 2B and I). When the miR403-targetable version of the LUC construct (35S::LUC-TAR^{miR403}) was co-injected with 35S::MIR403, a further reduction in the luciferase activity was observed when compared with the control co-injection with the vector (Fig 2A and H), an indication that transgenically expressed miR403 further reinforced the silencing effect of endogenous miR403 upon its targets. As expected, regardless of co-injection with either the 35S::MIR403 or the vector control, the LUC reporter harboring a mutated miR403 interaction site (35S::LUC-TAR^{miR403m}) exhibited elevated luciferase activities when compared with its miR403 targetable counterpart (Fig 2A and H). These data were consistent with, and further substantiated by, the levels of full-length LUC mRNAs detected by RT-PCR based assays (Figure 2 O and P).

[116] In summary, consistent results were obtained showing that the expression of 35S::LUC-TAR^{miR403} can be effectively repressed by the *N. benthamiana* endogenous miR403, while the expression of a non-targeted LUC (35S::LUC) or the LUC with a mutated miR403 target (35S::LUC-TAR^{miR403m}) was not affected. The expression of 35S::LUC-TAR^{miR403} can be further down-regulated when it is co-expressed with 35S::MIR403 which provides additional mature miR403.

Example 4. Validation of miRcan1230-mediated repression of LUC reporter in a transient assay system

[117] Using the candidate target sequences involved in miRcan1230-target interaction in Arabidopsis, a firefly luciferase (LUC)-based reporter has been designed [Xiong, L., David, L., Stevenson, B., and Zhu, J. (1999). High throughput screening of signal transduction mutant with luciferase imaging. *Plant Molecular Biology Reporter* 17, 159-170] engineered with a miRcan1230 target site in its 3'UTR (designated as 35S::LUC-TAR^{miRcan1230}). The efficacy of miRcan1230-mediated down-regulation of LUC reporter

expression was assessed by expressing the 35S::LUC-TAR^{miRcan1230}, either alone or co-expressed with a 35S promoter-driven MIRcan1230-producing construct (designated 35S::miRcan1230) in an Agrobacterium-mediated transient assay system on *Nicotiana benthamiana* [Johansen, L.K., and Carrington, J.C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiol* 126, 930-938]. LUC reporter constructs either lacking the miRcan1230 target site (designated 35S::LUC) or harboring a mutated miRcan1230 interacting site (designated as 35S::LUC-TAR^{miRcan1230m}) were also made to serve as controls.

[118] The following scheme for co-injection was used.

1. Control vector only
2. 35S::LUC-TAR^{miRcan1230} alone
3. 35S::LUC alone
4. 35S::LUC-TAR^{miRcan1230} alone
5. 35S::LUC-TAR^{miRcan1230m} alone
6. 35S::LUC-TAR^{miRcan1230} + control vector
7. 35S::LUC-TAR^{miRcan1230} + 35S::miRcan1230
8. 35S::LUC-TAR^{miRcan1230m} + control vector
9. 35S::LUC-TAR^{miRcan1230m} + 35S::miRcan1230

[119] At three days post injection (3 d.p.i.), luciferase activities were readily detectable from leaves infiltrated with 35S::LUC, but not from those infiltrated with the vector control (Fig. 6A lanes 3 and 1). In leaves infiltrated with a miRcan1230-targetable version of the LUC construct alone (lane 4) (35S::LUC-TAR^{miRcan1230}), luciferase activities were detected comparable to the 35S::LUC (Fig 6A, lane 3), as there appears to be no repression which can be caused by endogenous miRcan1230 in the *N. benthamiana* leaves, as cotton miRcan1230 is not conserved in other species. Small RNA Northern blot assays indicated that miRcan1230 accumulation was detected only from the leaves infiltrated with the 35S::miRcan1230 construct only (Figs. 6B, lanes 2, 7 and 9).

[120] To examine the effect of exogenously expressed miRcan1230 on the expression of LUC reporter, we included in our experiment the “two-component” Agrobacterium injection treatments where the LUC reporter is co-expressed with transgenic miRcan1203. As shown in Fig 6, substantially elevated levels of miRcan1230 accumulation were observed whenever the 35S::MIRcan1230 construct was included in the co-infiltration and only then (Fig 6B). When the miRcan1230-targetable version of the LUC construct (35S::LUC-TAR^{miRcan1230}) was co-injected with 35S::MIRcan1230, a significant reduction in the luciferase activity was observed when compared with the control co-injection with the vector (Fig 6A), an indication that transgenically expressed miRcan1230 exercised the silencing effect of miRcan1230 upon its targets. As expected, regardless of co-injection with either the 35S::MIRcan1230 or the vector control, the LUC reporter harboring a mutated miRcan1230 interaction site (35S::LUC-TAR^{miRcan1230m}) exhibited elevated luciferase activities when compared with its miRcan1203 targetable counterpart (Fig 6A).

[121] These data were consistent with, and further substantiated by, the levels of LUC mRNAs detected by RT-PCR based assays (Figure 6 E and F). The RT-PCR based assays distinguished between detection of the 5' part of the LUC mRNA (region A) upstream of the miRcan1230 target site or the 3' part of the LUC mRNA (region B), downstream of the miRcan1230 target site. Region A cannot be detected when no 35S::LUC vector (with or without target site) has been injected (lanes 1 and 2) but is also significantly reduced when the 35S::LUC TAR^{miRcan1230} is coexpressed with 35S::MIRcan1230. Region B can always be detected except when no 35S::LUC vector (with or without target site) has been injected.

[122] In summary, consistent results were obtained showing that the expression of 35S::LUC-TAR^{miRcan1230} can be effectively repressed by the *N. benthamiana* only when it is co-expressed with 35S::MIRcan1230 which provides mature miRcan1230.

Example 5. miR403-mediated or miR408-mediated repression of chitin synthase and/or glutamine:fructose-6-phosphate amidotransferase in cotton fibers

[123] Using recombinant DNA techniques the following recombinant genes were constructed by operably linking the following DNA elements:

[124] Recombinant construct chitin synthase + miR408 binding site

- The Pscw-prpr promoter
- A DNA region coding for golgi-targeting signal from XylT35 fused to the coding sequence from chitin synthase from *Neurospora crassa*
- A sequence including the binding site of microRNA408
- The 3'UTR fragment from the CaMV 35S gene

Further details are included in Table 2 referring to SEQ ID No. 15.

[125] Recombinant glutamine:fructose-6-phosphate amidotransferase + miR408 binding site

- The Pscw-prpr promoter
- A DNA region coding for the glutamine:fructose-6-phosphate amidotransferase of *Escherichia coli*
- A sequence including the binding site of microRNA408
- 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana*

Further details are included in Table 3 referring to SEQ ID No. 14.

[126] Recombinant construct chitin synthase + miR403 binding site

- The Pscw-prpr promoter
- A DNA region coding for golgi-targeting signal from XylT35 fused to the coding sequence from chitin synthase from *Neurospora crassa*
- A sequence including the binding site of microRNA403
- The 3'UTR fragment from the CaMV 35S gene

Further details are included in Table 4 referring to SEQ ID No. 13.

[127] Recombinant glutamine:fructose-6-phosphate amidotransferase + miR403 binding site

- The Pscw-prpr promoter
- A DNA region coding for the glutamine:fructose-6-phosphate amidotransferase of *Escherichia coli*
- A sequence including the binding site of microRNA403
- 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana*

Further details are included in Table 4 referring to SEQ ID No. 13.

[128] The recombinant constructs were inserted in various combinations in T-DNA vectors together with a selectable marker, such as a plant-expressible glyphosate tolerance gene. Examples of such T-DNA vectors include pTEA1 (comprising recombinant glutamine:fructose-6-phosphate amidotransferase followed by miRNA408 binding site; SEQ ID No. 14) and pTDBI312 (comprising recombinant glutamine:fructose-6-phosphate amidotransferase followed by miRNA403 binding site and recombinant chitin synthase followed by miRNA403 binding site; SEQ ID No. 13) pTEA6 (containing glutamine:fructose-6-phosphate amidotransferase with a miR403 target site in its 3' UTR and chitin synthase with a miR408 target site in its 3' UTR; Table 6 and SEQ ID No. 17) or pTEA3 (containing glutamine:fructose-6-phosphate amidotransferase and chitin synthase both with a miR408 target site in their 3' UTR; Table 5 and SEQ ID No. 16).

[129] These T-DNA vectors were introduced into *Agrobacterium* strains containing a helper Ti-plasmid and used in cotton transformation essentially as described in WO00/71733.

[130] Transgenic cotton plants were regenerated and the expression of glutamine:fructose-6-phosphate amidotransferase and chitin synthase in cotton fibers is determined. Transgenic cotton plants comprising recombinant constructs with miRNA

target sites in their 3'UTR show less disadvantageous phenotypes (small plants etc.) than transgenic cotton plants comprising recombinant constructs without miRNA target sites in their 3'UTR.

Example 6. miRcan1230-mediated or miR398-mediated repression of chitin

synthase and/or glutamine:fructose-6-phosphate amidotransferase in
cotton fibers

[131] Using recombinant DNA techniques the following recombinant genes were constructed by operably linking the following DNA elements:

[132] Recombinant construct chitin synthase + miR398 binding site

- The Pscw-prp promoter
- A DNA region coding for golgi-targeting signal from XylT35 fused to the coding sequence from chitin synthase from *Neurospora crassa*
- A sequence including the binding site of microRNA398
- The 3'UTR fragment from the CaMV 35S gene

Further details are included in Table 7 referring to SEQ ID No. 24.

[133] Recombinant glutamine:fructose-6-phosphate amidotransferase + miRcan1230 binding site

- The Pscw-prp promoter
- A DNA region coding for the glutamine:fructose-6-phosphate amidotransferase of *Escherichia coli*
- A sequence including the binding site of microRNA398
- 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana*

Further details are included in Table 7 referring to SEQ ID No. 24.

[134] Recombinant construct chitin synthase + miRcan1230 binding site

- The Pscw-prp promoter
- A DNA region coding for golgi-targeting signal from XylT35 fused to the coding sequence from chitin synthase from *Neurospora crassa*
- A sequence including the binding site of microRNAcan1230
- The 3'UTR fragment from the CaMV 35S gene

Further details are included in Table 8 referring to SEQ ID No. 25.

[135] Recombinant glutamine:fructose-6-phosphate amidotransferase + miRcan1230 binding site

- The Pscw-prp promoter
- A DNA region coding for the glutamine:fructose-6-phosphate amidotransferase of *Escherichia coli*
- A sequence including the binding site of microRNAcan1230
- 3' untranslated region of of the histone H4 gene of *Arabidopsis thaliana*

Further details are included in Table 8 referring to SEQ ID No. 25.

[136] The recombinant constructs were inserted in various combinations in T-DNA vectors together with a selectable marker, such as a plant-expressible glyphosate tolerance gene. Examples of such T-DNA vectors include pTEA9 (comprising recombinant glutamine:fructose-6-phosphate amidotransferase followed by miRNA398 binding site and recombinant chitin synthase followed by miRNA398 binding site; SEQ ID No. 24) and pTEA10 (containing glutamine:fructose-6-phosphate amidotransferase with a miRcan1230 target site in its 3' UTR and chitin synthase with a miRcan1230 target site in its 3' UTR; SEQ ID No. 25).

[137] These T-DNA vectors were introduced into *Agrobacterium* strains containing a helper Ti-plasmid and used in cotton transformation essentially as described in WO00/71733.

[138] Transgenic cotton plants were regenerated and the expression of glutamine:fructose-6-phosphate amidotransferase and chitin synthase in cotton fibers is

determined. Transgenic cotton plants comprising recombinant constructs with miRNA target sites in their 3'UTR show less disadvantageous phenotypes (small plants etc.) than transgenic cotton plants comprising recombinant constructs without miRNA target sites in their 3'UTR.

Table 2: genetic elements of recombinant DNA construct pEA2 comprising chitin synthase and+miR408 target site – nucleotide sequence reference is SEQ ID No. 15

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
Pscw-prp	431	1869	promoter from proline rich cell wall protein from <i>G.hirsutum</i> ;
RPxylTAt	1873	1977	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1978	4809	coding sequence for chitin synthase from <i>Neurospora crassa</i>
tmiR408At-N1	4822	4848	sequence including the binding site of microRNA408 of <i>Arabidopsis thaliana</i>
tmiR408At	4825	4845	Core target sequence of microRNA408 of <i>Arabidopsis thaliana</i>
3'35S	4869	5105	3'UTR fragment from the CaMV 35S gene.

Table 3: genetic elements of T-DNA of vector pTEA1 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase and miR408 target site - reference is SEQ ID No. 14

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	10979	11003	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	11067	12505	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	12509	14338	coding region of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006), adapted to plant codon usage
tmiR408At-N1	14339	44	sequence including the binding site of microRNA408 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR408At	3	41	Core target sequence of microRNA408 of <i>Arabidopsis thaliana</i>
3'histonAt	60	720	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748 ABC	770	1686	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	1736	2198	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	2205	2576	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	2577	3914	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	3938	4598	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	4711	4735	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Table 4: genetic elements of T-DNA of vector pTBDI312 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase and chitin synthase both containing a miR403 target site in their 3' UTR - reference is SEQ ID No. 13

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	1	25	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	61	1499	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
RPxylTAt	1503	1607	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1608	4439	coding sequence of the chitin synthase 2 gene of <i>Neurospora crassa</i> (Din and Yarden, 1994)
tmiR403At-N1	4440	4465	sequence including the binding site of microRNA403 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR403At	4443	4462	Core binding site of microRNA403 of <i>Arabidopsis thaliana</i>
3'35S	4486	4722	sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon et al., 1991)
Pscw-prp	4760	6198	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	6202	8031	coding sequence of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006)
tmiR403At-N1	8038	8063	sequence including the binding site of microRNA403 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR403At	8041	8060	Core binding site of microRNA403 of <i>Arabidopsis thaliana</i>
3'histonAt	8089	8749	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748	8799	9715	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	9749	10229	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	10234	10605	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	10606	11943	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	11967	12627	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	12740	12764	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Table 5: genetic elements of T-DNA of vector pTEA3 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase and chitin synthase both containing a miR408 target site in their 3' UTR - reference is SEQ ID No. 16

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	1	25	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	61	1499	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
RPxylTAt	1503	1607	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1608	4439	coding sequence of the chitin synthase 2 gene of <i>Neurospora crassa</i> (Din and Yarden, 1994)
tmiR408At-N1	4452	4478	sequence including the binding site of microRNA408 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR408At	4455	4475	Core binding site of microRNA408 of <i>Arabidopsis thaliana</i>
3'35S	4499	4735	sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon et al., 1991)
Pscw-prp	4773	6211	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	6215	8044	coding sequence of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006)
tmiR408At-N1	8057	8083	sequence including the binding site of microRNA408 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR408At	8060	8080	Core binding site of microRNA408 of <i>Arabidopsis thaliana</i>
3'histonAt	8105	8765	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748	8815	9731	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	9765	10245	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	10250	10621	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	10622	11959	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	11983	12643	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	12756	12780	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Table 6: genetic elements of T-DNA of vector pTEA6 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase containing a miR403 target site in its 3' UTR and chitin synthase containing a miR408 target site in its 3' UTR - reference is SEQ ID No. 17

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	1	25	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	61	1499	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
RPxylTAt	1503	1607	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1608	4439	coding sequence of the chitin synthase 2 gene of <i>Neurospora crassa</i> (Din and Yarden, 1994)
tmiR408At-N1	4452	4478	sequence including the binding site of microRNA408 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR408At	4455	4475	Core binding site of microRNA408 of <i>Arabidopsis thaliana</i>
3'35S	4499	4735	sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon et al., 1991)
Pscw-prp	4773	6211	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	6215	8044	coding sequence of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006)
tmiR403At-N1	8051	8076	sequence including the binding site of microRNA403 of <i>Arabidopsis thaliana</i> (Harvey et al., 2011)
tmiR403At	8054	8073	Core binding site of microRNA403 of <i>Arabidopsis thaliana</i>
3'histonAt	8102	8762	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748	8812	9728	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	9762	10242	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	10247	10618	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	10619	11956	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	11980	12640	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	12753	12777	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Table 7: genetic elements of T-DNA of vector pTEA9 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase containing a miR398 target site in its 3' UTR and chitin synthase containing a miR398 target site in its 3' UTR - reference is SEQ ID No. 24

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	1	25	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	61	1499	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
RPxylTAt	1503	1607	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1608	4439	coding sequence of the chitin synthase 2 gene of <i>Neurospora crassa</i> (Din and Yarden, 1994)
tmiR398Gh-N1	4446	4472	sequence including the binding site of microRNA398 of <i>Gossypium hirsutum</i>
tmiR398At	4449	4469	Core binding site of microRNA398 of <i>Gossypium hirsutum</i>
3'35S	4484	4708	sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon et al., 1991)
Pscw-prp	4759	6197	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	6201	8030	coding sequence of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006)
tmiR398Gh_N1	8043	8069	sequence including the binding site microRNA398 of <i>Gossypium hirsutum</i>
tmiR398Gh	8046	8066	Core binding site of microRNA398 of <i>Gossypium hirsutum</i>
3'histonAt	8081	8741	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748	8791	9707	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	9757	10219	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	10226	10597	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	10598	11935	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	11959	12619	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	12732	12756	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Table 8: genetic elements of T-DNA of vector pTEA10 comprising recombinant construct containing glutamine:fructose-6-phosphate amidotransferase containing a miRcan1230 target site in its 3' UTR and chitin synthase containing a miRcan1230 target site in its 3' UTR - reference is SEQ ID No. 25

<u>Name</u>	<u>Start</u>	<u>End</u>	<u>Description</u>
RB	1	25	Right border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)
Pscw-prp	61	1499	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
RPxylTAt	1503	1607	coding sequence for the Golgi retention peptide of the beta-1,2-xylosyltransferase gene of <i>Arabidopsis thaliana</i> (Pagny et al., 2003)
chs2Nc-1Pc	1608	4439	coding sequence of the chitin synthase 2 gene of <i>Neurospora crassa</i> (Din and Yarden, 1994)
tmiRcan1230 Gh-N1	4446	4479	sequence including the binding site of microRNAcan1230 of <i>Gossypium hirsutum</i>
tmiR408At	4449	4476	Core binding site of microRNAcan1230 of <i>Gossypium hirsutum</i>
3'35S	4491	4715	sequence including the 3' untranslated region of the 35S transcript of the Cauliflower Mosaic Virus (Sanfaçon et al., 1991)
Pscw-prp	4766	6204	sequence including the promoter region of a proline-rich cell wall protein gene of <i>Gossypium hirsutum</i> (cotton)
gfaEc-1Pb	6208	8037	coding sequence of the glutamine:fructose-6-phosphate amidotransferase gene of <i>Escherichia coli</i> (Frohberg and Essigmann, 2006)
tmiRcan1230 Gh-N1	8050	8083	sequence including the binding site of microRNAcan1230 of <i>Gossypium hirsutum</i>
tmiRcan1230 Gh	8053	8080	Core binding site of microRNAcan1230 of <i>Gossypium hirsutum</i>
3'histonAt	8095	8755	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
Ph4a748	8805	9721	sequence including the promoter region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
intron1 h3At	9771	10233	sequence including the first intron of gene II of the histone H3.III variant of <i>Arabidopsis thaliana</i> (Chaubet et al., 1992)
TPotp C	10240	10611	coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of <i>Zea mays</i> (corn) and <i>Helianthus annuus</i> (sunflower) (Lebrun et al., 1996)
2mepsps	10612	11949	coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of <i>Zea mays</i> (corn) (Lebrun et al., 1997)
3'histon At	11973	12633	sequence including the 3' untranslated region of the histone H4 gene of <i>Arabidopsis thaliana</i> (Chabouté et al., 1987)
LB	12746	12770	Left border repeat from the T-DNA of <i>Agrobacterium tumefaciens</i> (Zambryski, 1988)

Claims

1. A recombinant gene for spatially selective expression in a fiber-producing plant comprising the following operably linked elements:

- (a) a plant-expressible promoter;
- (b) a region encoding a biologically active RNA molecule;
- (c) optionally a 3' transcription termination and polyadenylation region

characterized in that said recombinant gene further comprises a target sequence recognized by a miRNA, said miRNA being differentially expressed in cells leading to fibers in said fiber producing plant compared to cells of said fiber-producing plants other than said cells leading to said fibers.

2. The recombinant gene of claim 1, whereby said miRNA is a microRNA endogenous to said plant.

3. The recombinant gene of claim 1 or 2, whereby said target sequence recognized by said miRNA is heterologous to at least one of the elements of said recombinant gene.

4. The recombinant gene of any one of claims 1 to 3, wherein said recombinant gene comprises a 3' untranslated region comprising said target sequence.

5. The recombinant gene of any one of claims 1 to 4, wherein said target sequence is heterologous with regard to one or more of said operably linked elements.

6. The recombinant gene of any one of claims 1 to 5, wherein said target sequence is additionally introduced in said recombinant gene.

7. The recombinant gene of any one of claims 1 to 6, wherein said target sequence is obtained by modification of the sequence of one of the operably linked elements, preferably one of the operably linked elements other than the plant-expressible promoter.

8. The recombinant gene of any one of claims 1 to 7, wherein said plant expressible promoter is selected from a constitutive promoter, an inducible-promoter, a tissue-specific promoter, a developmentally regulated promoter.
9. The recombinant gene of any one of claims 1 to 8, wherein said plant-expressible promoter is a fiber-preferential or fiber-selective promoter.
10. The recombinant gene of any one of claims 1 to 9, wherein said plant-expressible promoter is selected from the group of a promoter from cotton from a fiber-specific β -tubulin gene, a promoter from cotton from a fiber-specific actin gene, a promoter from a fiber specific lipid transfer protein gene from cotton, a promoter from an expansin gene from cotton, or a promoter from a chitinase gene in cotton; a promoter from cotton from a glucanase gene, a promoter of the cotton FS18 gene, a promoter of the SCW-PRP gene from cotton, a promoter of the FB8-like gene from cotton.
11. The recombinant gene of any one of claims 1 to 10, wherein said plant-expressible promoter comprises the nucleotide sequence of SEQ ID No. 13 from nucleotide position 61 to nucleotide position 1499.
12. The recombinant gene of any one of claims 1 to 11, wherein said miRNA is less abundantly expressed in cells leading to fibers in said fiber producing plants compared to cells of said fiber-producing plants other than said cells leading to said fibers.
13. The recombinant gene of claim 12, wherein said miRNA is substantially not expressed in cells leading to said fibers of said fiber-producing plant but ubiquitously expressed in cells of said fiber-producing plant other than said cells leading to said fibers.
14. The recombinant gene of claim 12 or 13, wherein said miRNA is selected from the group consisting of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230 and Ghi_miR398.

15. The recombinant gene of any one of claims 12 to 14, wherein said miRNA has a nucleotide sequence selected from the nucleotide sequence of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 20, SEQ ID No. 21 or SEQ ID No. 22.

16. The recombinant gene of any one of claims 12 to 15, wherein said target sequence has the nucleotide sequence of SEQ ID No. 5, SEQ ID No. 5 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 6, SEQ ID No. 6 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 8 from nucleotide position 4 to 24, SEQ ID No. 23, SEQ ID No. 23 from nucleotide position 4 to 24, SEQ ID No. 23 from nucleotide position 11 to 32, or SEQ ID No. 23 from nucleotide positions 4 to 32.

17. The recombinant gene of any one of claims 12 to 16, wherein said target sequence has a nucleotide sequence which is complementary to the nucleotide sequence of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230, Ghi_miR398, SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 20, SEQ ID No. 21 or SEQ ID No. 22 whereby one or more of the following mismatches may occur:

- (a) a mismatch between the nucleotide at the 5' end of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (b) a mismatch between any one of the nucleotides in position 1 to position 9 of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (c) three mismatches between any one of the nucleotides in position 12 to position 21 of said miRNA and the corresponding nucleotide sequence in the target RNA sequence provided that there are no more than two consecutive mismatches; and
- (d) no mismatch is allowed at positions 10 and 11 of the miRNA.

18. The recombinant gene of any one of claims 1 to 17, wherein said biologically active RNA encodes a polypeptide with N-acetylglucosamine transferase activity.

19. The recombinant gene of claim 18 wherein said N-acetylglucosamine transferase is a NODC-type N-acetylglucosamine transferase, such as a NODC-type N-acetylglucosamine transferase obtainable from a *Rhizobium* species, an *Azorhizobium* species, a *Bradyrhizobium* species, a *Mesorhizobium* species, a *Ralstonia* species, a *Streptomyces* species, a *Burkholderia* species, a *Cupriavidus* species or a *Sinorhizobium* species.

20. The recombinant gene of claim 18, wherein said N-acetylglucosamine transferase activity comprises a signal anchor sequence selected from the signal anchor sequence of a rat sialyl transferase, the signal anchor sequence of a human galactosyl transferase, the signal anchor sequence of the Arabidopsis homologue of the yeast HDEL receptor (AtERD2), the signal anchor sequence of the α -2,6-sialyltransferase, the signal anchor sequence of β 1,2-xylosyltransferase from *Arabidopsis thaliana*, the signal anchor sequence of N-acetylglucosaminyl transferase I from tobacco or the amino acid sequence YYHDL or LKLEI.

21. The recombinant gene of claim 18, wherein said N-acetylglucosamine transferase comprises an amino acid sequence encoded by the nucleotide sequence of SEQ ID No. 13 from nucleotide position 1503 to nucleotide position 4439.

22. The recombinant gene of claim 18, wherein said N-acetylglucosamine transferase is chitin synthase, preferably chitin synthase 2 of *Saprolegnia monoica*.

23. The recombinant gene of any one of claims 1 to 17, wherein said biologically active RNA encodes a glutamine:fructose-6-phosphate amidotransferase.

24. The recombinant gene of claim 23, wherein said glutamine:fructose-6-phosphate amidotransferase comprises an amino acid sequence encoded by the nucleotide sequence of SEQ ID No. 13 from nucleotide position 6202 to nucleotide position 8031.

25. A plant cell comprising a recombinant gene according to any one of claims 1 to 24.

26. The cell of claim 25, wherein said cell is a cell capable of developing into a fiber cell.

27. A plant, part, tissue or seed of a plant comprising a cell according to claim 25 or 26, or consisting essentially of cells according to claim 25 or 26.

28. The plant cell according to claim 25 or claim 26, wherein said plant cell is a cotton plant cell.

29. The plant, part, tissue or seed of a plant according to claim 27 which is cotton.

30. A method of producing a fiber-producing plant with spatially selective expression of a recombinant gene, comprising the step of

- (a) Introducing a recombinant gene according to any one of claims 1 to 29 into at least one cell of said fiber producing plant;
- (b) Optionally, regenerating a fiber-producing plant from said at least one cell comprising said recombinant gene.

31. The method according to claim 30, wherein said fiber-producing plant is cotton.

32. A microRNA from cotton selected from the group of Ghi_miR403, Ghi_miR408, Ghi_miRcan1230 or Ghi_miR398 or having the nucleotide sequence of any one of SEQ ID Nos 1 to 4.

33. A target site recognized by a microRNA according to claim 32.

34. A target site according to claim 33 having the nucleotide sequence of any one of SEQ ID Nos 5 to 8, SEQ ID No. 5 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 6 from nucleotide position 4 to nucleotide position 24, SEQ ID No. 8, SEQ ID No. 8 from nucleotide position 4 to 24, SEQ ID No. 23, SEQ ID No. 23 from nucleotide position 4 to 24, SEQ ID No. 23 from nucleotide position 11 to 32, or SEQ ID No. 23 from nucleotide positions 4 to 32 or a nucleotide sequence complementary to

a miRNA having a nucleotide sequence according to any one of SEQ ID Nos 1 to 4, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22 or SEQ ID No. 23 whereby one or more of the following mismatches may occur:

- (a) a mismatch between the nucleotide at the 5' end of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (b) a mismatch between any one of the nucleotides in position 1 to position 9 of said miRNA and the corresponding nucleotide sequence in the target RNA sequence;
- (c) three mismatches between any one of the nucleotides in position 12 to position 21 of said miRNA and the corresponding nucleotide sequence in the target RNA sequence provided that there are no more than two consecutive mismatches; and
- (d) no mismatch is allowed at positions 10 and 11 of the miRNA.

35. Use of a microRNA according to claim 32 and/or a target site according to claim 33 or claim 34 to increase the selectivity of fiber-selective expression of a recombinant gene in a fiber-producing cell of a fiber-producing plant.

36. Use according to claim 35, wherein said fiber-producing plant is a cotton plant.

37. A pre-microRNA from cotton having a nucleotide sequence of any one of SEQ ID NOs: 9, 10, 11 or 12, or a nucleotide sequence essentially similar or identical thereto wherein the microRNA region and optionally the region complementary to the microRNA region (microRNA* region) have been altered.

38. A method for increasing tissue selective expression in a fiber-producing plant comprising the step of using a recombinant gene according to any one of claims 1 to 24.

39. A method for producing fibers from a fiber-producing plant comprising the steps of

- (a) growing a plant according to claim 29 or a plant obtained by the process of claim 30;
- (b) harvesting fibers from said grown plants.

40. The method according to claim 39, wherein said fiber-producing plant is cotton.

41. Plant fibers obtained by the method of claim 39 or claim 40.

Figure 1

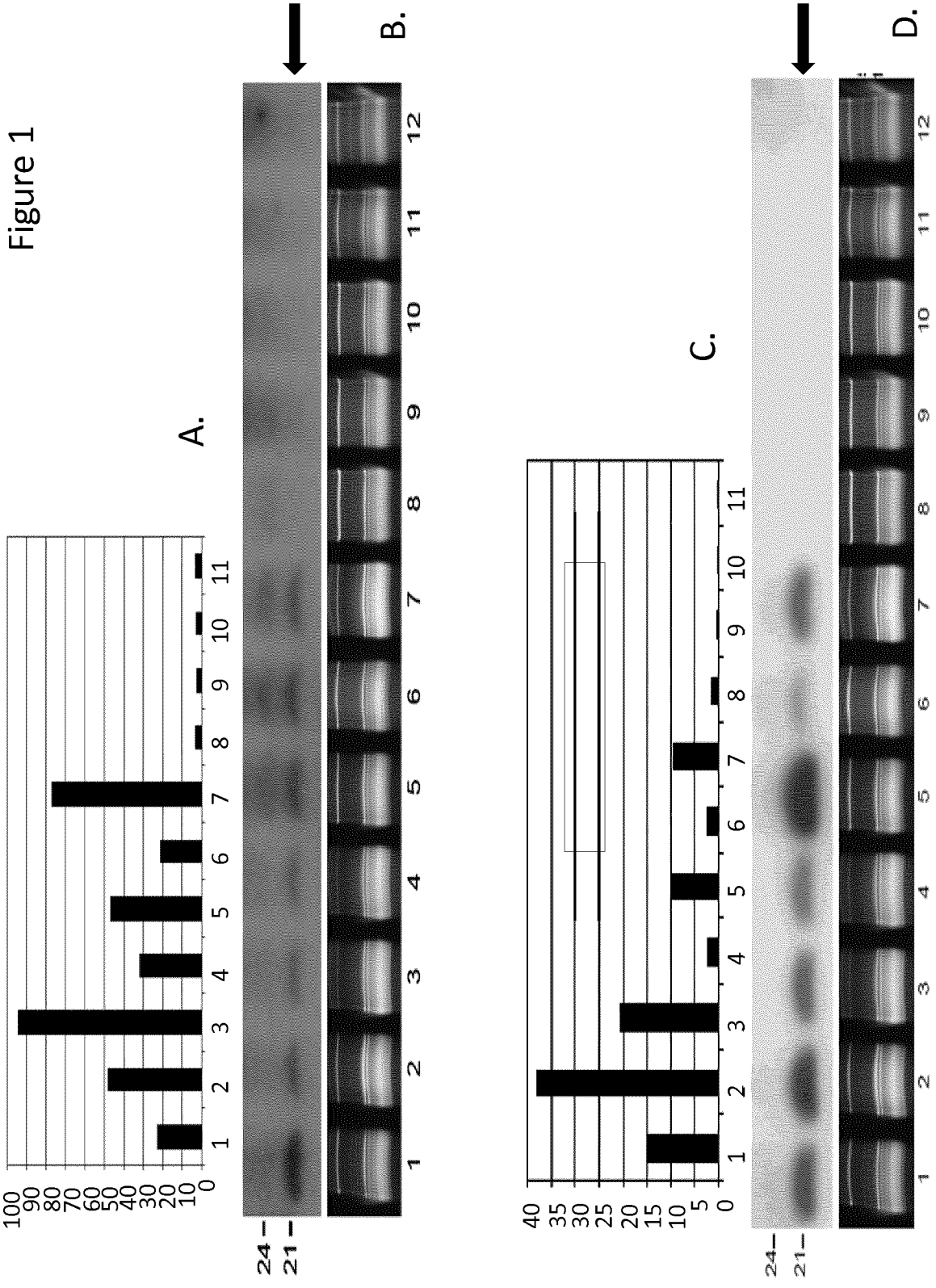
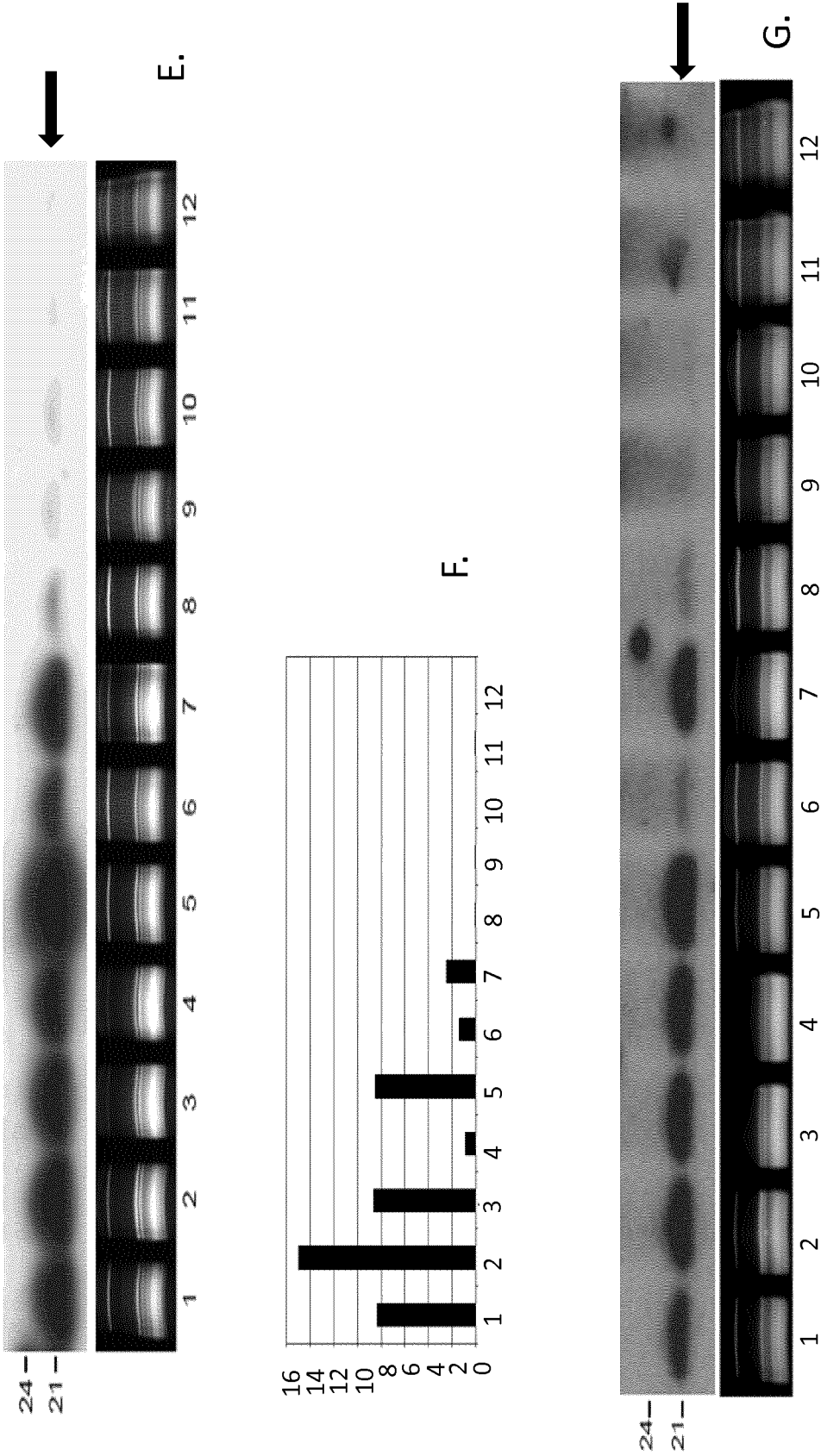
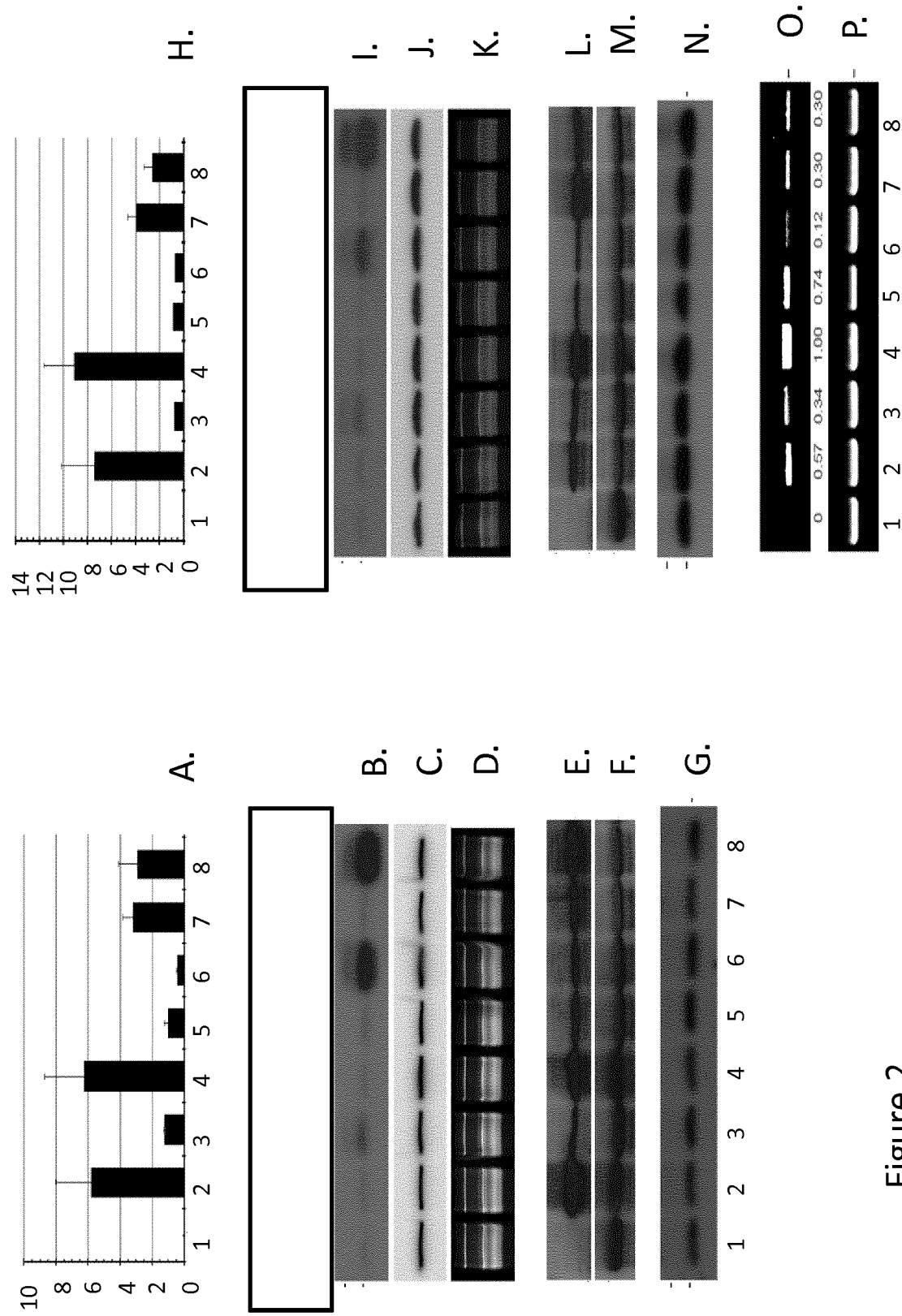
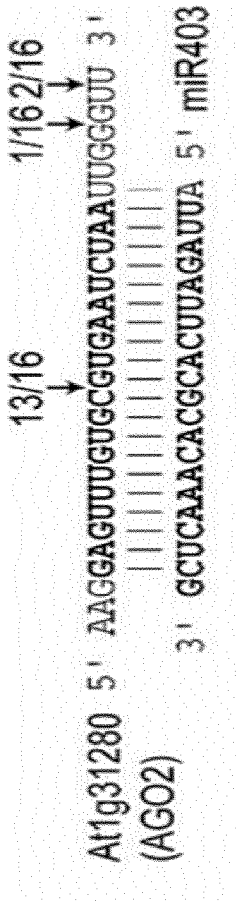


Figure 1 continued





- **miR403 in *Arabidopsis thaliana* and *Gossypium hirsutum***
>Ghi_miR403 TTAGATTTCACGCACAAACTCG
>Ath_miR403 TTAGATTTCACGCACAAACTCG
- **miR403-interacting site in *Arabidopsis AGO2* (At1g31280)mRNA**



- **miR403 target sequence for engineered REPORTER**
>Ath_miR403 target 5'-AAGGAGTTTGTCGTAATCTAATTG-3'
>Ath_miR403 R 3'-----GCTCAACACGCACCTTAGATTa----5'
>Ghi_miR403 R 3'-----GCTCAACACGCACCTTAGATT-----5'

Figure 3

```

1. Ghi_mir408 in FM958 (major & minor forms detected)

>ghi-mir408.1      5'-AUGCACUGCCUCUCCCCUGGC_-3'
>ghi-mir408.2      5'_UGCACUGCCUCUCCCCUGGCU-3'

2. Proposed artificial mir408 target sequence
   (To be engineered into the 3'UTR of the transgene of choice)

>mir408_Target      5'-gacGCCGGTGAAAGAGGCCAGTGCAAgac-3'
>ath_mir408_R        ...o.x.....x
                        3'-CGGTCCCTTCTCCGTCACGTA-5'

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Figure 4

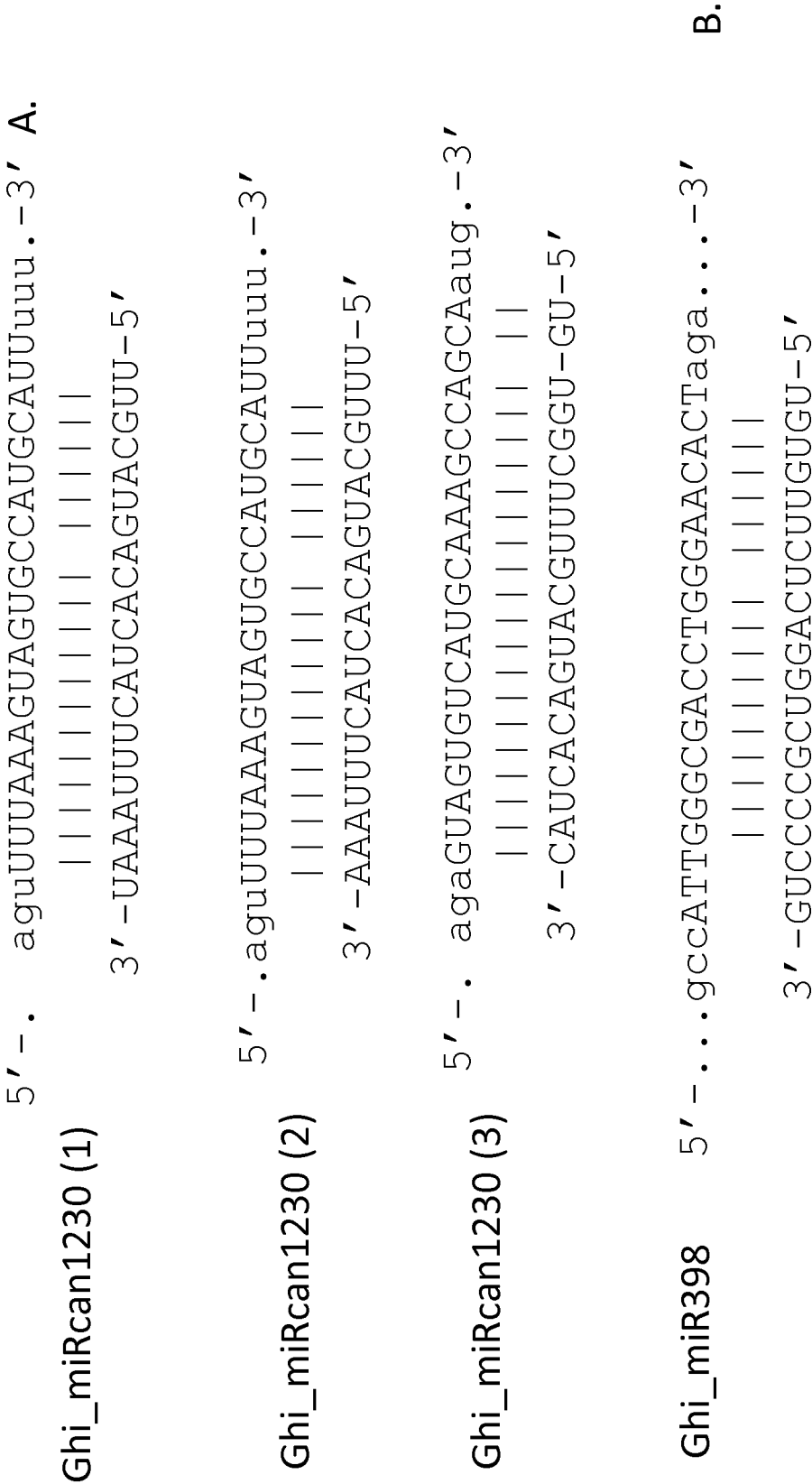


Figure 5

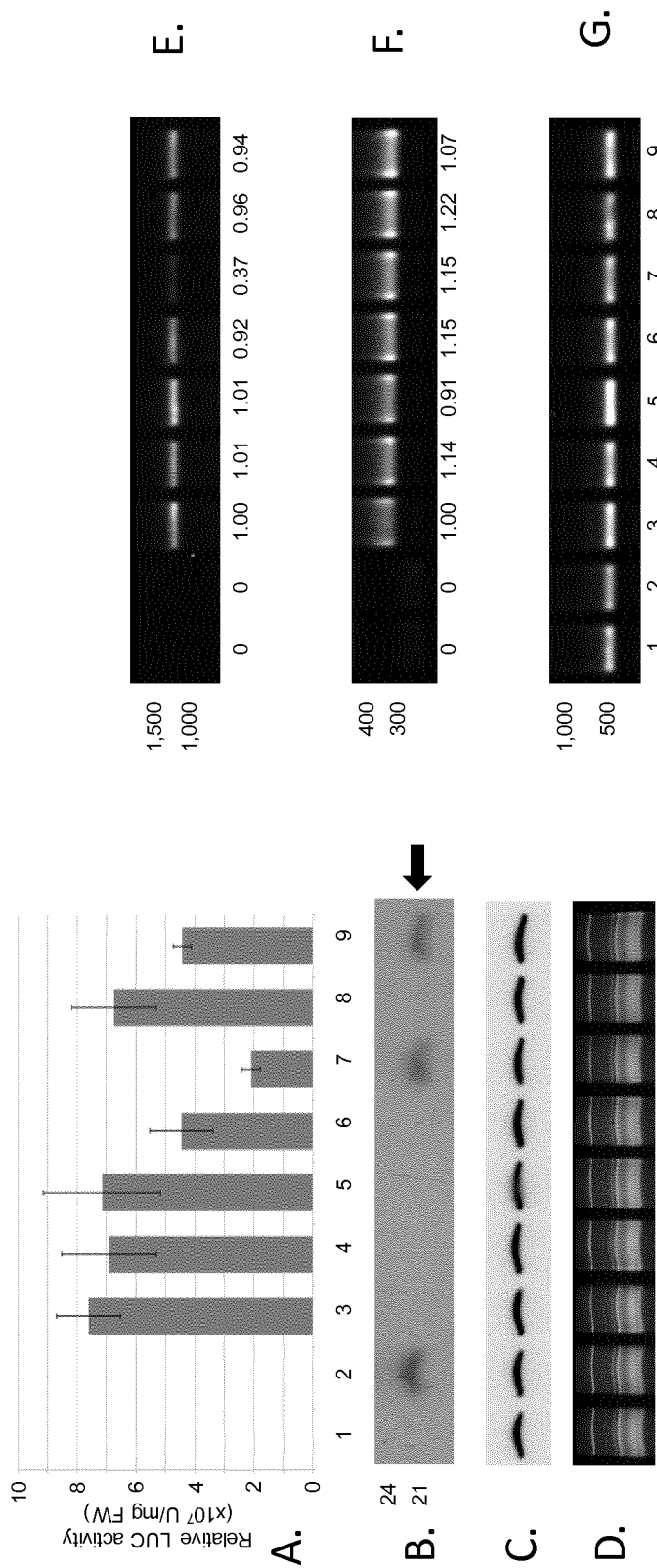


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2014/059778

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-41(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/059778

A. CLASSIFICATION OF SUBJECT MATTER

INV. A01H5/10 C12N9/10 C12N15/11 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01H C12N

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/111512 A1 (BASF PLANT SCIENCE GMBH [DE]; REN PEIFENG [US]; SONG HEE-SOOK [US]; MC) 26 October 2006 (2006-10-26) cited in the application abstract page 6, line 20 - line 32 page 7, line 5 - line 11 page 10, line 28 - line 37 page 51, line 22 - line 29 page 52, line 1 - line 13 page 61, line 1 - line 3 ----- -/-	1-41



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search

18 July 2014

Date of mailing of the international search report

13/10/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Mundel, Christophe

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/059778

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009/003078 A2 (MONSANTO TECHNOLOGY LLC [US]; ALLEN EDWARDS [US]; HEISEL SARA E [US];) 31 December 2008 (2008-12-31) cited in the application abstract paragraph [0010] paragraph [0011] paragraph [0028] -----	1-41
Y	WO 2007/047016 A2 (MONSANTO TECHNOLOGY LLC [US]; ALLEN EDWARDS [US]; GILBERTSON LARRY A []) 26 April 2007 (2007-04-26) abstract paragraph [0009] paragraph [0015] - paragraph [0017] table 2 sequence 164 -----	1-41
Y	WO 2010/042575 A1 (MONSANTO TECHNOLOGY LLC [US]; ALLEN EDWARDS [US]; DENG MOLIAN [US]; HA) 15 April 2010 (2010-04-15) abstract paragraph [0007] paragraph [0043] - paragraph [0044] table 2 sequences 10, 11 -----	1-41
X	KWAK PIETER BAS ET AL: "Enrichment of a set of microRNAs during the cotton fiber development", BMC GENOMICS, BIOMED CENTRAL LTD, LONDON, UK, vol. 10, no. 1, 29 September 2009 (2009-09-29), page 457, XP021062145, ISSN: 1471-2164, DOI: 10.1186/1471-2164-10-457 cited in the application -----	32
Y	abstract page 3, right-hand column, line 40 - page 4, left-hand column, line 1 figure 3 -----	1-41
X	WANG ZHENG-MING ET AL: "A comparative miRNAome analysis reveals seven fiber initiation-related and 36 novel miRNAs in developing cotton ovules.", MOLECULAR PLANT JUL 2012, vol. 5, no. 4, July 2012 (2012-07), pages 889-900, XP002727433, ISSN: 1752-9867 -----	32
Y	abstract page 894, left-hand column, line 23 - line 30 figure 3 -----	1-41
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/059778

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANG MINGXIONG ET AL: "Genome-wide analysis reveals rapid and dynamic changes in miRNA and siRNA sequence and expression during ovule and fiber development in allotetraploid cotton (<i>Gossypium hirsutum</i> L.)", GENOME BIOLOGY, BIOMED CENTRAL LTD., LONDON, GB, vol. 10, no. 11, 4 November 2009 (2009-11-04), page R122, XP021065366, ISSN: 1465-6906, DOI: 10.1186/GB-2009-10-11-R122 abstract page R122.2, right-hand column, line 17 - line 23 page R122.8, right-hand column, line 4 - page R122.10, left-hand column, line 2 page R122.17, left-hand column, line 3 - line 13 -----	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/059778

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006111512 A1	26-10-2006	AU 2006237317 A1	26-10-2006
		BR PI0608829 A2	15-03-2011
		CA 2604807 A1	26-10-2006
		CN 101203611 A	18-06-2008
		EP 1874936 A1	09-01-2008
		US 2009293148 A1	26-11-2009
		WO 2006111512 A1	26-10-2006
		ZA 200709897 A	25-03-2009
WO 2009003078 A2	31-12-2008	US 2007300329 A1	27-12-2007
		WO 2009003078 A2	31-12-2008
WO 2007047016 A2	26-04-2007	AR 055446 A1	22-08-2007
		AU 2006302969 A1	26-04-2007
		BR PI0617224 A2	19-07-2011
		CA 2625031 A1	26-04-2007
		EA 200801074 A1	30-10-2008
		EG 26093 A	13-02-2013
		EP 1934354 A2	25-06-2008
		US 2007199095 A1	23-08-2007
		US 2009235388 A1	17-09-2009
		US 2013074213 A1	21-03-2013
		WO 2007047016 A2	26-04-2007
WO 2010042575 A1	15-04-2010	US 2011247094 A1	06-10-2011
		US 2013298281 A1	07-11-2013
		WO 2010042575 A1	15-04-2010

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-41(partially)

A recombinant gene for spatially selective expression in a fiber-producing plant comprising (a) a plant expressible promoter and (b) a region encoding a biologically active RNA molecule wherein the recombinant gene further comprises a target sequence recognized by the Ghi_mir403 miRNA. A plant cell, plant part or plant comprising said recombinant gene. A Ghi_mir403 miRNA. A target site recognised by the Ghi_mir403 miRNA. A pre-microRNA of SEQ ID NO: 9. Use of the Ghi_mir403 miRNA to increase the selectivity of fiber-selective expression. A method for producing fibers from a fiber-producing plant.

2. claims: 1-41(partially)

A recombinant gene for spatially selective expression in a fiber-producing plant comprising (a) a plant expressible promoter and (b) a region encoding a biologically active RNA molecule wherein the recombinant gene further comprises a target sequence recognized by the Ghi_mir408 miRNA. A plant cell, plant part or plant comprising said recombinant gene. A Ghi_mir408 miRNA. A target site recognised by the Ghi_mir408 miRNA. A pre-microRNA of SEQ ID NO: 10. Use of the Ghi_mir408 miRNA to increase the selectivity of fiber-selective expression. A method for producing fibers from a fiber-producing plant.

3. claims: 1-41(partially)

A recombinant gene for spatially selective expression in a fiber-producing plant comprising (a) a plant expressible promoter and (b) a region encoding a biologically active RNA molecule wherein the recombinant gene further comprises a target sequence recognized by the Ghi_mirCan1230 miRNA. A plant cell, plant part or plant comprising said recombinant gene. A Ghi_mirCan1230 miRNA. A target site recognised by the Ghi_mirCan1230 miRNA. A pre-microRNA of SEQ ID NO: 11. Use of the Ghi_mirCan1230 miRNA to increase the selectivity of fiber-selective expression. A method for producing fibers from a fiber-producing plant.

4. claims: 1-41(partially)

A recombinant gene for spatially selective expression in a fiber-producing plant comprising (a) a plant expressible promoter and (b) a region encoding a biologically active RNA molecule wherein the recombinant gene further comprises a target sequence recognized by the Ghi_mir398 miRNA. A plant

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

cell, plant part or plant comprising said recombinant gene.
A Ghi_mir398 miRNA. A target site recognised by the
Ghi_mir398 miRNA. A pre-microRNA of SEQ ID NO: 12. Use of
the Ghi_mir398 miRNA to increase the selectivity of
fiber-selective expression. A method for producing fibers
from a fiber-producing plant.
