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(54) Title: METHOD FOR SYSTEMICALLY INFLUENCING PROCESSES IN THE MALE MEIOCYTE

Fig. 3
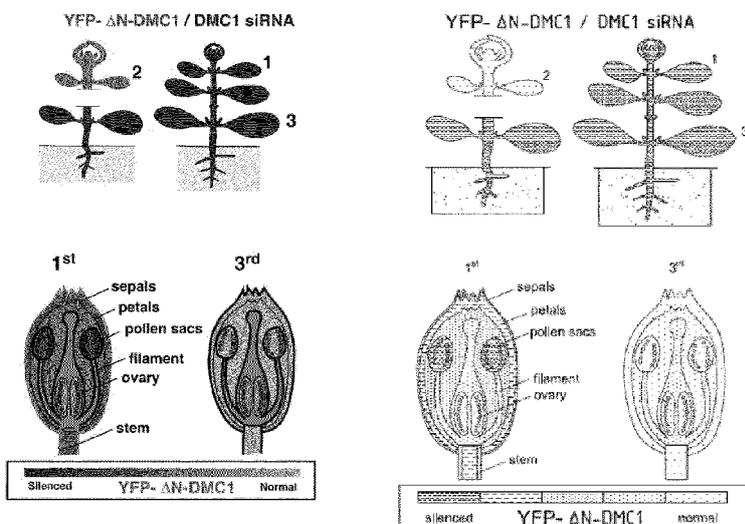
(57) **Abstract:** The invention relates to a method for interfering with processes in the male meiocytes of a plant, by combining a first plant comprising a rootstock, which transgenically harbours a nucleic acid sequence, with a scion from a second plant grafted onto the rootstock of the said first plant, whereby the said transgenic nucleic acid sequence generates polynucleotide molecules in the rootstock of the first plant, which polynucleotide molecules are transported systemically across the graft junction to enter the male meiocytes produced by the scion of the second plant, and wherein the said systemically transported polynucleotide molecules are at least partially complementary to a transcript expressed in the male meiocytes produced by the scion of the second plant.

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METHOD FOR SYSTEMICALLY INFLUENCING PROCESSES IN THE MALE MEIOCYTE

FIELD OF THE INVENTION

5   This invention relates to a method for interfering with processes in the male meiocytes of a plant.

BACKGROUND OF THE INVENTION

          Sexual reproduction is arguably the most
10   fundamental process in a flowering plant's life cycle. It provides a next generation of organisms, and through recombination (reshuffling) of the genetic features of the father and mother organism it allows the creation of genetic diversity. This diversity in the next generation allows
15   plants to e.g. adapt better and more readily to changes in their biotic and abiotic environment.

          Biological studies have led to a thorough understanding of the processes and factors that play important roles in sexual reproduction in angiosperms.
20   Essential stages are:

          - the formation and ripening of male gametes (microspores, pollen grains) and female gametes (egg cell inside an embryo sac inside an ovule) from diploid meiocytes. The formation of haploid gametes occurs via two
25   meiotic divisions, during which the two parental genomes of the gamete-producing organism are recombined;

          - pollination (the deposition of male gametes on the female reproductive organs/stigma), which can e.g. be mediated by wind, insects or other specialised pollinators,
30   or simply by gravity, proximity or active pollination mechanisms when it concerns a self-fertilising species;

          - the germination of pollen grains and the subsequent transfer of male nuclei (sperm cells) towards

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receptive and chemically attracting egg cells through a
pollen tube;

        - the release of sperm nuclei inside a receptive
embryo sac, leading to double fertilisation, in which one
5   sperm cell fuses with the egg cell to give rise to a diploid
embryo, and the other sperm cell fuses with the diploid
central cell, to give rise to triploid endosperm;

        - mitotic divisions and developmental patterning
of the zygote, leading to the formation of an embryo.

10   For the purpose of this invention the first stage
is most important: the formation of gametes, in particular
male gametes, from meiocytes. In angiosperms the male
gametes or microspores are formed inside anthers. They
originate from diploid pollen mother cells (a type of
15 meiocyte) that undergo meiosis, which encompasses two rounds
of meiotic division. The final result of meiosis is a set of
four haploid microspores for each pollen mother cell, and
these microspores each develop further by undergoing two
mitotic divisions. The first of these divisions is
20 asymmetric, and it gives rise to a vegetative cell and a
generative cell, while the second mitosis concerns the
division of the generative cell. The result is a pollen
grain that contains two sperm cells, in a unique
constellation, as the sperm cells are both located inside
25 the cytoplasm of the vegetative cell.

        The timing of the second mitotic division varies
greatly in the plant kingdom, and the second pollen mitosis
can either occur prior to anthesis (as is the case in e.g.
Arabidopsis and rice), or after anthesis during pollen tube
30 growth (e.g. in tomato and tobacco). Depending on the
species, angiosperm flowers can thus shed either tricellular
(containing one vegetative nucleus plus two generative

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nuclei) or bicellular pollen grains (containing one
vegetative nucleus plus one generative nucleus).

Meiotic cell division is of fundamental importance
during gamete formation, as it provides a means for
5   reshuffling the parental genetic information so that new
genetic combinations arise, and a means for "undoubling" the
genome in haploid gametes. Meiocytes have received
considerable research attention, because if one would be
able to influence the molecular, physiological and/or
10   developmental processes that take place inside a meiocyte,
one could potentially have a huge impact on sexual
reproduction, on the transmission of genetic material to the
next generation, and even on evolution.

It is known that in plants RNA-based silencing
15   signals spread systemically to distant organs. For long-
distance transport of such RNA-based signals, flowering
plants use a specialized vascular system named phloem. The
phloem facilitates the directional transfer of small
molecules such as ions, sugars, nucleotides, hormones, amino
20   acids, and proteins from source (producing) tissues to sink
(consuming) tissues. In a number of plant species small
polynucleotide molecules (typically RNA molecules with a
size of 18 to 27 nucleotides), derived from specific gene
products, were shown to be present in the phloem sap and
25   suggested to relocate following the source-sink flow.

It is known from the literature that a long-
distance transport pathway allows plants to induce systemic
post-transcriptional gene silencing (PTGS) associated with
small interfering RNA (siRNA) molecules.

30   PTGS - associated with siRNA - is reset by an
unknown mechanism and is re-established in the following
generation. PTGS-resetting seems to take place during

meiosis and can be established again in gametes, as shown in studies on transposon activity in male gametophytes.

siRNAs targeting transposable element transcripts are activated in the vegetative nucleus and transferred into fertilizing sperm cells, where they down-regulate transposon activity. Thus, within pollen grains and fertilizing pollen tubes an siRNA-mediated silencing machinery is active. The PTGS mechanism is also functional in sporophytic tissues, as anther-specific genes could be silenced. Studies conducted with transgenic GFP-expressing lines, in which siRNA-associated spread of silencing was induced by over-expression of transcripts containing GFP sequences, suggested systemic spread of siRNA signals into strong sink tissues such as newly forming leaves.

However, it seems that not all sink tissues can receive PTGS signals. Systemic siRNA-mediated GFP-silencing was reported to be very limited in growing flower buds, and silencing of GFP in the meiotic (sporogenic) tissues of anthers or carpels has never been observed.

In the prior art it is thus presumed that meiocytes are inaccessible for long-distance mobile signals, and that a barrier exists that protects the future microspores in their earliest stages of development.

In the research that led to the present invention it was however found how meiocytes can be targeted with systemic polynucleotide signals from a transgenic rootstock, without rendering transgenic the shoot grafted thereon (scion), or the meiocytes or microspores produced by the scion, or plants derived from these microspores.

The present inventors found how to interfere with (molecular) processes in the male meiocytes, through the said polynucleotide signals that are transported into the male meiocytes as a systemic signal.

In the prior art it is also known that the over-expression of RNAi constructs with constitutive promoters can lead to silencing of gene expression in the male meiocytes (Dirks R et al, 2009, *Plant Biotechnol J* 7: 837-
5   845; Reverse Breeding: WO03/017753). However, a considerable drawback of this approach is the fact that the organism producing the male meiocytes, as well as at least 50% of the microspores produced after meiosis, are transgenic. For regulatory, efficiency and practical reasons it is very
10   desirable to develop a method for producing transgene-free plants from spores that originated from meiocytes in which molecular recombination processes have been interfered with. Such microspores can e.g. be used for the production of transgene-free doubled haploid plants (DHs).
15   The prior art discloses various methods for the production of haploid plants from microspores, such as *in vitro* microspore culture (regeneration of haploid plantlets from microspores, and subsequent genome doubling with e.g. colchicine), and the method disclosed in WO2011044132, which
20   involves pollination of a haploid-inducer line, which after fertilisation eliminates the maternal chromosomes from the zygote, leading to the formation of viable seeds containing a haploid embryo that is genetically identical to the microspore used for pollination and fertilisation.
25   In the research leading to this invention it was thus surprisingly found that siRNA signals, generated in a mature rootstock, can systemically penetrate and be active in apical sporogenic cells, in particular meiocytes. This was demonstrated by grafting a non-transgenic scion onto a
30   transgenic rootstock harbouring an RNAi construct and producing siRNA molecules, and by observing silencing phenotypes of genes in the male meiocytes formed by the non-transgenic scion. Such systemic siRNA molecules targeting

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meiotic tissue can thus affect gene expression in the
progeny.

The siRNA molecules, generated from the RNAi
construct in the transgenic rootstock, can thus be
5   transmitted through long-distance transport across a graft
junction, and throughout the scion. The current research
demonstrated that the polynucleotide (siRNA) molecules
generated from these constructs can subsequently be forced
to enter into the male meiocytes and effect gene silencing
10   therein, or interfere in another way with transcripts or
genomic material in these meiocytes. Until now it was deemed
impossible to import molecules into early developing
meiocytes or microspores through long-distance transport.
Surprisingly, within a flower the siRNA molecules are
15   preferentially targeted towards the anthers and the
meiocytes therein, whereas the female meiocytes (megaspore
mother cells contained in the ovary) do not receive the
siRNA signal.

This invention can be applied to interfere with
20   all molecular processes that take place in male meiocytes,
such as meiosis, chromosome recombination, and homologous
recombination, and also to interfere with the physiology of
microspores, such as e.g. the responsiveness to androgenetic
stimuli of microspores derived from these meiocytes.
25   "Interference" can either refer to suppression or blocking
of these processes, or to enhancement thereof. It also
allows specific alteration of the genetic material that is
transferred to the next generation, e.g. through site-
directed mutagenesis (during the stage in which homologous
30   recombination takes place spontaneously in meiocytes) in
genes and/or regulatory (cis-acting) sequences.

Importantly, application of this invention does
not render the male meiocytes, the microspores developing

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therefrom or the scion transgenic, as only the root stock
harbours a transgene. The microspores and the scion only
contain transiently expressed transgene-derived molecules,
which are not passed on to the next generation. Only the

5   molecular effects brought about by the transgene-derived
molecules are transmitted to the next generation. The use of
transgenes expressing dominant-negative factors that can
systemically move across graft junctions into transgene-free
plant material allows researchers to take full advantage of

10   transgene technology to impose novel traits onto crop
plants, without rendering them stably transgenic.

Specific mutations can be introduced into a plant
genome through homologous recombination (Paszkowski J et al,
1988, *EMBO J* 7: 4021-4026; Mengiste T and Paszkowski J,

15   1999, *Biol Chem* 380: 749-758; Vergunst AC and Hooykaas PJJ,
1999, *Crit Rev Plant Sci* 18: 1-31) or through
oligonucleotide-based mutation induction (Oh TJ and May GD,
2001, *Curr Opin Biotechnol* 12: 169-172; KeyBase technology:
WO2010074562, EP2167660, WO2007073149).

20   The method of this invention can be used for plant
breeding purposes. Current breeding methods are slow and
take >5-10 years for cultivar development. One of the
disadvantages is the inefficiency of trait selection in
early generations. This problem can be overcome by inducing

25   doubled-haploid production in non-transgenic scions by
grafting them onto compatible transgenic root stocks, as
illustrated by example 3 below.

The present invention can be applied to all plant
species that can be grafted and transformed. Crop species on

30   which this invention can be practised include e.g. tobacco,
poplar, maize, wheat, barley, rice, sorghum, sugar beet,
oilseed rape, ryegrass, sunflower, soybean, tomato,
cucumber, gherkin, corn salad, spinach, pepper, petunia,

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potato, eggplant, melon, carrot, radish, lettuce, vegetable *Brassica* species (cabbage, cauliflower, broccoli, kohlrabi, Brussels sprouts), leek, bean, pea, endive, chicory, onion, strawberry, fennel, table beet, celery, celeriac, asparagus,

5 sunflower, and grape vine.

        The present invention thus relates to a method for interfering with processes in the male meiocytes of a plant, by combining a first plant comprising a rootstock, which transgenically harbours a nucleic acid sequence, with a

10 scion from a second plant grafted onto the rootstock of the said first plant, whereby the said transgenic nucleic acid sequence generates polynucleotide molecules in the rootstock of the first plant, which polynucleotide molecules are transported systemically across the graft junction to enter

15 the male meiocytes produced by the scion of the second plant, and wherein the said polynucleotide molecules are at least partially complementary to a transcript expressed in the male meiocytes produced by the scion of the second plant.

20   Expression of the transgenic nucleic acid sequence in the first plant is achieved by operably linking the said nucleic acid sequence to a promoter sequence that confers a ubiquitous or systemic expression profile, or a tissue- or cell-type-specific expression profile onto the said

25 transgenic nucleic acid sequence. For this purpose the transgenic nucleic acid sequence is cloned into a transgenic construct, downstream (at the 3' end) of a promoter sequence that has the ability to drive the expression of the said nucleic acid sequence in plants. Promoter sequences differ

30 in their expression patterns in plant tissues, and some have the ability to drive gene expression in all tissues (ubiquitous expression), while others have a (spatio-temporally) more narrow expression pattern, or a very

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specific expression pattern that is restricted to a specific tissue type or cell type.

Examples of promoter sequences that confer a ubiquitous expression profile onto a transgenic nucleic acid
5   sequence are e.g. the cauliflower mosaic virus 35S promoter (CaMV 35S), selected ubiquitin promoters, selected actin promoters, and many others. Examples of tissue- or cell-type-specific promoters are also known to the skilled person.

10   For the purpose of this invention, the promoter sequence confers onto the said transgenic nucleic acid sequence an expression profile that encompasses the rootstock. This is desired because only the rootstock is used from the said first plant harbouring the transgenic
15   nucleic acid sequence, and therefore the said nucleic acid sequence should be expressed at least in the rootstock of the said first plant. The rootstock encompasses the source tissues from which phloem sap is transported to the sink tissues, and more specifically to the sink tissues of the
20   scion. A rootstock comprises the roots and may in addition comprise a part of the stem and some (mature) leaves. According to the invention, the expression product of the transgenic nucleic acid sequence can be produced in the complete rootstock or in a part thereof, for example by
25   using a root-, leaf- or stem-specific promoter.

In one embodiment the expression of the transgenic nucleic acid sequence in the rootstock of the first plant is constitutive. In another embodiment, the expression of the transgenic nucleic acid sequence in the rootstock of the
30   first plant is transient. Transient expression can e.g. be achieved by use of an inducible promoter, selected from the group comprising heat-inducible promoters, chemical-inducible promoters, steroid-inducible promoters and

alcohol-inducible promoters. Transient expression can be
achieved by using an inducible promoter or by transiently
introducing the transgenic construct (with a strong
promoter) into the root stock instead of stably integrating
5   the transgene in the rootstock genome. Such transient
transformation can for example be achieved by *Agrobacterium*
infiltration via the stomata of the leaves that remain
attached to the rootstock after grafting of the scion onto
the rootstock.

10   In one embodiment, interfering with processes in
the male meiocytes consists of the suppression or blocking
of processes in the male meiocytes. In another embodiment,
interfering with processes in the male meiocytes consists of
the enhancement of processes in the male meiocytes. When
15   interfering consists of the suppression or blocking of
processes in the male meiocytes, this can be achieved by
destabilizing the target gene mRNA by means of
polynucleotide molecules that are at least partially
complementary to the target gene mRNA or transcript and that
20   have been generated from the transgenic nucleic acid
sequence. The polynucleotide molecules can be selected from
the group comprising miRNA, antisense RNA, RNAi molecules,
siRNA molecules, Virus-Induced Gene Silencing (VIGS)
molecules, co-suppressor molecules or RNA oligonucleotides.

25   In one embodiment the said transgenic nucleic acid
sequence is stably integrated into the genome of the first
plant. In another embodiment the said transgenic nucleic
acid sequence is transiently present in the rootstock of the
first plant.

30   In one embodiment the said polynucleotide
molecules are generated and expressed constitutively in the
first plant, preferably in an expression domain that
comprises the roots. This implies that no external treatment

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is required for the generation and expression of the polynucleotide molecules. The properties of the promoter sequence to which the transgenic nucleic acid sequence is operably linked determine the temporal and spatial pattern

5   of the generation and expression of the polynucleotide molecules.

In another embodiment the said polynucleotide molecules are generated and expressed transiently in the first plant, preferably in an expression domain that

10   comprises the roots. This implies that external factors or treatments can be used to induce and/or modulate the temporal and/or spatial pattern of the generation and expression of the polynucleotide molecules, by virtue of the effect these external factors or treatments have on the

15   activity of the promoter sequence to which the transgenic nucleic acid sequence is operably linked.

In this embodiment the transient expression of the polynucleotide molecules in the first plant is achieved by use of an inducible promoter, selected from the group

20   comprising heat-inducible promoters, chemical-inducible promoters, steroid-inducible promoters and alcohol-inducible promoters. Treatment of the rootstock with the agent(s) and/or condition(s) that induce the activity of the inducible promoter (such as a heat shock or heat treatment,

25   specific chemicals, specific steroids such as dexamethasone, or alcohol) activates the inducible promoter, which results in the transient expression of the polynucleotide molecules in the first plant, preferably in an expression domain that comprises the roots.

30   The polynucleotide molecules derived from the transgenic nucleic acid sequence are at least partially complementary to a transcript expressed in the male meiocytes produced by the scion of the second plant. In one

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embodiment, the polynucleotide molecules are at least partially complementary to the transcript (transcribed nucleic acid sequence or mRNA) of a gene that is involved in chromosome recombination. In this embodiment, the gene is

5   suitably selected from the group comprising *SPO11*, *MER1*, *MER2*, *MRE2*, *MEI4*, *REC102*, *REC104*, *REC114*, *MEK1/MRE4*, *RED1*, *HOP1*, *RAD50*, *MRE11*, *XRS2*, *PRD1*, *PRD2*, *PRD3*, *PHS1*, *NBS1*, *COM1*, *RHD54/TID1*, *DMC1*, *SAE3*, *RED1*, *HOP1*, *HOP2*, *MND1*, *REC8*, *MEI5*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RPA1*, *SMC3*, *SCC1*,

10   *MSH2*, *MSH3*, *MSH6*, *PMS1*, *SOLODANCERS*, *HIM6*, *CHK2*, *SGS1*, *MSH4*, *MSH5*, *MER3/RCK*, *ZIP1*, *ZIP2*, *ZIP3*, *ZIP4*, *PTD*, *SHOC1*, *ZYP1*, *MLH1*, *MLH3*, or their functional homologues.

In another embodiment, the polynucleotide molecules that are generated by the transgenic nucleic acid

15   sequence in the rootstock of the first plant and that are transported systemically across the graft junction to enter the male meiocytes produced by the scion of the second plant, are DNA polynucleotides. These DNA polynucleotides typically have the same size as the RNA polynucleotides of

20   the previous embodiments, i.e. between about 18 nucleotides and about 27 nucleotides. These DNA polynucleotides can be derived from nuclear DNA, from plasmid DNA, from chloroplast DNA, from mitochondrial DNA, or from other DNA molecules.

This invention further relates to a method for

25   specifically altering the genome sequence of male meiocytes of a plant, by combining a first plant comprising a rootstock, which transgenically harbours a nucleic acid sequence, with a scion from a second plant grafted onto the rootstock of the said first plant, whereby the said

30   transgenic nucleic acid sequence generates polynucleotide molecules in the rootstock of the first plant, which polynucleotide molecules are transported systemically across the graft junction to enter the male meiocytes produced by

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the scion of the second plant, and wherein the said polynucleotide molecules are at least partially homologous to a DNA fragment contained in the genome of the second plant.

5   In this embodiment advantage is taken of homologous recombination in the male meiocyte, to effect targeted nucleotide exchange in the genome sequence of the male meiocyte. This can e.g. be achieved through the presence of small DNA polynucleotides in the male meiocytes
10 during the stage in which chromosome recombination occurs, and in which the chromosomes are more easily accessible. Specific mutations can be introduced into a plant genome through homologous recombination (Paszkowski J et al, 1988, *EMBO J* 7: 4021-4026; Mengiste T and Paszkowski J, 1999, *Biol*
15 *Chem* 380: 749-758; Vergunst AC and Hooykaas PJJ, 1999, *Crit Rev Plant Sci* 18: 1-31) or through oligonucleotide-based mutation induction (Oh TJ and May GD, 2001, *Curr Opin Biotechnol* 12: 169-172; KeyBase technology: WO2010074562, EP2167660, WO2007073149). The polynucleotides used for this
20 purpose are essentially homologous to a genomic sequence, except for one or more mismatches located essentially in the middle of the targeted sequence in the genome intended to create a targeted nucleotide-exchange or point-mutation in the genome.

25   The invention will be further illustrated in the Examples that follow and that are not intended to limit the invention in any way. In the Examples, reference is made to the following figures.

          Figure 1: (**a**) Representative photograph of a
30 grafted tobacco plant. A non-transgenic scion was grafted onto a transgenic rootstock, and all leaves were removed from the scion, to force the phloem-mobile silencing signal from source tissues (the rootstock) into the scion's apical

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region (sink tissues). **(b)** A schematic drawing of the graft
combinations used in the cytometric (FACS) analysis of
pollen harvested from grafted plants. The histograms
indicate the size (scattered light) and fluorescence
intensity of Propidium Iodide-stained pollen coat and
nuclei. The numbers obtained by control grafts (wild
type/wild type) are highly reproducible. In contrast, the
size and DNA content of pollen from *DMC1* siRNA transgenic
scions (reverse graft) or wild-type scions grafted on *DMC1*
siRNA lines (true graft) is significantly altered. The
numbers refer to representative images of scion anthers
forming: male sterile flowers producing no or low quantities
of aberrant pollen (numbers 1 and 2), and anthers with
regular pollen production (number 3).

   Figure 2: (Upper panel) Microscopic images of DAPI
stained nuclei (blue) in microspore tetrads isolated from
scion flowers. Note the appearance of unbalanced tetrads
(arrowhead) on *DMC1* siRNA-producing scions (middle image)
and on wild-type plants grafted onto *DMC1* silencing lines
(right image). **(Lower panel)** Scanning electron microscopy
images of pollen harvested from scion flowers. Pollen grains
from control grafts (wild type/wild type) were normal in
shape and size. In contrast, wild-type scions grafted on
transgenic *DMC1* silencing lines produced misshaped pollen
grains that varied in size. Note that within one shoot the
number of misshaped pollen was higher in primary flowers
than in late flowers. This observation correlated with the
sterile anther phenotype appearing on the first flower
formed on wild-type scions grafted on *DMC1* siRNA lines.

   Figure 3: Schematic drawing of the observed spread
of the *DMC1* silencing signal in 35S::YFP-ΔN *DMC1* flower
buds, both in color (left) and black-and-white (right).Green
or dotted parts indicate parts of the plant where no

silencing of the YFP reporter construct was observed. Blue
or striped parts indicate the presence of *DMC1* silencing
(=disappearance of YFP signals). *DMC1* silencing
(blue/dotted) was observed in the epidermis of sepals and
5   carpels, and in the sporogenic tissue of anthers. No
silencing of the YFP reporter construct was observed in
female organs such as pistils, carpels or ovules
(green/striped), or in later forming flower buds on an
inflorescence. Note that the observed spread of *DMC1*
10 silencing reiterated within primary flower buds appearing in
inflorescences emerging from secondary shoots.

EXAMPLES

EXAMPLE 1

15 *Stable transformation of tobacco plants*

Transgenic *Nicotiana tabacum* var. SR1 plants were
obtained by leaf disc transformation with the *Agrobacterium
tumefaciens* strain LB4004, harbouring a binary vector, with
the method described by Horsch RB et al, 1985, *Science* 227:
20 1229-1231.

EXAMPLE 2

Suppression of chromosome recombination in vivo *through
long-distance transport of siRNA across a graft junction in*
25 *tobacco*

An important example of a process that typically
occurs in the male meiocytes of a plant is meiotic
chromosome recombination. This invention allows the
interference with meiotic chromosome recombination by means
30 of a transgene in a rootstock that generates short
interfering RNA (siRNA) molecules, which are delivered into
the male meiocytes that are produced by a scion grafted on
top of the transgenic rootstock.

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To provide evidence for this, transgenic tobacco
plants (*Nicotiana tabacum* var. SR1) harbouring an
ectopically expressed RNAi hairpin construct for the *DMC1*
gene (*DISRUPTED MEIOTIC cDNA1*) from *Arabidopsis thaliana*
5   (TAIR: AT3G22880; expression driven by a constitutive
promoter) were produced following the method of example 1.
DMC1 is a meiotic cell-cycle-specific factor and a member of
the highly conserved RecA-type recombinase family (DNA
dependent ATP-ases) present in meiocytes, such as pollen
10   mother cells in anthers and megaspore mother cells in ovules
(Doutriaux MP et al, 1998, *Mol Gen Genet* 257: 283-291). *DMC1*
was shown to be crucial for inter-homolog recombination and
bivalent formation, and, consequently, for proper pollen and
ovule development. Lack of a functional *DMC1* gene product
15   induces achiasmatic meiosis, resulting in formation of
anomalous pollen grains, which contain an aberrant number of
chromosomes. The chromosome number in spores depends on an
orderly meiotic separation of sister chromatids occurring in
the second meiotic phase.
20   The plants were grown in soil in greenhouse
conditions (16 hours of light, 25°C day temperature and 20°C
night temperature), and three transgenic lines clearly
showing the silencing phenotype of *DMC1* were selected for
further experiments. This silencing phenotype comprises
25   consistently low levels of endogenous *DMC1* mRNA, the
formation of unbalanced microspore tetrads after meiosis, at
least partial male sterility and the production of
irregularly shaped (aneuploid) pollen, which are indicative
for a dysfunctional meiosis, caused by the presence of siRNA
30   molecules targeting the *DMC1* transcript in the male
meiocytes.

Scions from wildtype plants were grafted onto 3-
to 4-week-old plants from these selected transgenic lines

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(with the first leaf being about 10 cm in length). Two
plants are thus combined, in which the bottom part
(transgenic rootstock) of the one plant supports the upper
part (wildtype scion) of the other plant. The graft

5   incisions were made at an angle of 45° with a razor blade,
usually between the third and fourth leaf. During the
transfer the scion stem was shortly submerged in water,
until the graft junction was aligned. A toothpick served as
space holder between the parafilm and wrapped stem to assist

10   some evaporation from the graft junction.

After grafting, newly emerging leaves on the scion
and secondary shoots appearing on the rootstock were cut
off, to force the delivery of phloem-mediated molecules
towards the apical region of the scion. A successful graft

15   namely leads to the formation of a vascular connection
between the rootstock and the scion, and phloem transport is
directed towards sink tissues (such as meristematic regions
and newly emerging leaves). By ensuring that the apical
region of the scion is the only remaining above-ground sink

20   tissue in the plant, phloem transport can be effectively
directed towards that apical region, where flowers will be
formed. Control grafts were included in the experiment,
namely wildtype scions grafted onto wildtype rootstocks, and
transgenic scions grafted onto transgenic or wildtype

25   rootstocks.

Bolting of the scions occurred approximately 3
weeks after grafting. Preliminary examination of the anthers
revealed a deficiency in pollen production in all grafts in
which either the scion or the rootstock were transgenic

30   (**Figure 1B**). This phenotype was consistent with the
silencing phenotype of *DMC1*. In the former case this was not
surprising, as the entire scion was transgenic, and inside
the meiocytes siRNA molecules directed against *DMC1* were

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thus produced. In the latter case, however, it implied that - as taught by the current invention - siRNA molecules generated by the RNAi hairpin construct directed against *DMC1* were mobilized from the transgenic rootstock and

5   transported systemically into the scion with the phloem sap, and delivered into the male meiocytes that were produced inside sporogenic tissues of the flower buds of the scion.

In control grafts in which the scion itself was transgenic this male sterility was observed in all flowers,

10   while in grafts in which the scion was wildtype (grafted onto a transgenic rootstock) the effect was limited to the first flowers. First flowers were nearly sterile, while later flowers were fully fertile. The phloem-mediated siRNA signal thus preferentially entered the initially produced

15   flowers, being the first sink tissue the phloem sap encountered on its way towards the scion's apex. The male sterility phenotype was also observed in the first flowers that developed on secondary shoots.

A more thorough examination of the first flowers

20   from wildtype scions grafted onto a transgenic rootstock was conducted, and - based on a highly reproducible relation between size distribution and fluorescence emission from wildtype pollen - fluorescence-activated cell sorting (FACS) analysis revealed a much higher variability in pollen size

25   (irregular distribution), as compared to pollen from wildtype flowers (**Figure 1B**).

This observation was confirmed with scanning electron microscopy, and pollen produced by the first flowers of wildtype scions supported by a *DMC1* siRNA-

30   producing transgenic rootstock appeared misshapen and smaller. Pollen from later flowers was normal (**Figure 2**).

Table 1 Statistics of *DMC1* siRNA phenotypes observed after grafting.

Stock / scion	*DMC1* phenotype/Grafts tested [a]
wild type/wild type	0/13 (0%)
wild type/*DMC1* siRNA	15/15 (100%)
DMC1 siRNA/wild type	6/13 (46%)
wild type/*YFP-ΔN DMC1*	0/8 (0%)
YFP-ΔN DMC1/DMC1 siRNA	5/5 (100%)
DMC1 siRNA/*YFP-ΔN DMC1*	3/5 (60%) [b]

[a] evaluated by sterility, aberrant pollen production, appearance of unbalanced
tetrads, FACS, cytological inspection of meiosis, qPCR, and presence of *DMC1*-
specific siRNA molecules.
[b] verified by lack of *YFP-ΔN DMC1* fluorescence in scion leaves and primary buds.

To further address this phenotype, an earlier
stage in pollen development was investigated. In control
grafts (wildtype scion onto wildtype rootstock) microspore
tetrads were all normal-looking and balanced (n=100). In
wildtype scions grafted onto transgenic rootstocks, however,
the overall number of tetrads was lower in first (early)
flowers, and a significant number of these tetrads (up to
10%, with n>100) was unbalanced, as indicated by the
appearance of a small nucleus in one tetradic cell (figure
2). Unbalanced tetrad formation is one of the typical
phenotypes associated with the loss of *DMC1* activity during
meiosis.

While *DMC1* is also active in the female
reproductive organs, no evidence of *DMC1* silencing was
apparent in the female reproductive organs of wildtype
scions grafted onto transgenic rootstocks. When pollinated
with wildtype pollen they produced a normal number of viable
seeds, indicating a normal female fertility.

To further investigate the *DMC1* silencing in male
meiocytes through systemic transport of RNAi signals,

20

another set of transgenic tobacco plants was generated, using the method described in Example 1. These plants harboured a *35S::YFP:ΔN-DMC1* transgenic reporter construct, containing a dysfunctional, truncated *DMC1* cDNA of

5   *Arabidopsis thaliana* lacking the first 276 bp (i.e. leading to deletion of 92 amino acids at the N-terminus), fused to the Yellow Fluorescent Protein (YFP) which serves as a visual reporter, and driven by the constitutive cauliflower mosaic 35S promoter (CaMV 35S).

10   Shoots from these transgenic plants – which normally show a ubiquitous fluorescent signal due to the overexpression of the fusion protein - were subsequently grafted onto transgenic rootstocks harbouring the *DMC1* silencing construct. The flower buds emerging on the scion

15   were examined, and in the first (early) flower buds the YFP fluorescence signal was strong in carpels and stigma, but absent inside anthers. This demonstrated that in primary flower buds the systemic silencing signal is preferably delivered into anthers (**Figure 3**).

20   Thus, this example teaches how chromosome recombination can be efficiently suppressed in male meiocytes of tobacco, without rendering these meiocytes or the male gametes resulting thereof transgenic. This invention allows the application of Reverse Breeding

25   (WO03017753) in a preferred embodiment, namely with non-transgenic progeny. This concept is further explained in Example 3.

             While in this example the *DMC1* gene was targeted, any other gene that is functionally involved in any of the

30   steps of meiotic chromosome recombination could be targeted with comparable results.

EXAMPLE 3

Non-transgenic Reverse Breeding in Brassica, mediated by systemic silencing signals

5   The experimental approach outlined in Example 2 was applied to *Brassica oleracea*, resulting in transgenic rootstocks harbouring an ectopically expressed hairpin RNAi silencing construct against the *SDS (SOLO DANCERS)* gene. Onto these transgenic rootstocks wildtype hybrid scions were grafted from an interspecific F1 hybrid (*Raphanus sativus* x

10   *Brassica oleracea*), and the silencing signal was forced into male meiocytes by removing all scion leaves, as described in Example 2. The first flower buds produced by the scions contained a significant proportion of aberrant microspore tetrads, and these flowers were largely male sterile.

15   When the first flower buds produced on the scion were about 3 mm in size, the microspores developing therein had reached the optimal stage for microspore regeneration *in vitro* (androgenesis). Microspores were purified from these buds, and they were given a stress treatment of two days at

20   32°C, which is optimal to induce sporophytic development of the haploid spores. Balanced (euploid) microspores were regenerated into haploid plantlets using methods known to the person skilled in the art (for example Swanson EB et al., *Plant Cell Rep* 6: 94-97; Takahata Y and Keller WA,

25   *Plant Sci* 74: 235-242), and colchicine treatment was subsequently used for genome doubling, leading to haploid or doubled haploid (DH) plantlets with a full complement of chromosomes.

    Due to the suppression of chromosome recombination

30   by *DMC1* silencing in the male meiocytes, the chromosomes in the DH plants obtained by this method had not been recombined, and they had the exact genetic constitution as the chromosomes of the parental lines of the hybrid plant

that had been used as a scion for grafting. This was
confirmed with molecular markers (two per chromosome, i.e.
one per chromosome arm) that are polymorphic between
Raphanus sativus and *Brassica oleracea*, the parents of the
5   F1 hybrid that was used as a scion in the graft.

Using the same set of molecular markers, DH plants
regenerated from microspores collected from anthers of the
scion were screened to identify individuals that were
genetically complementary, i.e. containing sets of parental
10   chromosomes that when combined in an F1 correspond to the
exact set of chromosomes present in the hybrid used as a
scion for grafting. Crossing of these complementary
individuals resulted in the exact reconstruction of the
hybrid genotype (*Raphanus sativus* x *Brassica oleracea*).

15   This example thus illustrates how the current
invention can be used to exactly reconstruct a hybrid
genotype. Importantly, this was achieved without rendering
the reconstructed hybrid plant transgenic, as suppression of
chromosome recombination prior to DH production had been
20   achieved through a transient siRNA signal in the male
meiocytes.

The approach outlined in the previous examples can
also be applied in other plant species. It is also
25   particularly useful to apply it in crop species with long
life cycles, such as trees (for example poplar, and fruit
trees such as apple, pear, cherry, peach, plum, banana,
citrus fruits, papaya, fig, etc.) and grape vines. This
invention could be used to seed-propagate specific traits in
30   such crops, and transgene-free elite lines can be produced
much faster, and several years of traditional breeding
efforts can be avoided.

23

CLAIMS

1. Method for interfering with processes in the
male meiocytes of a plant, by combining a first plant
5   comprising a rootstock, which transgenically harbours a
nucleic acid sequence, with a scion from a second plant
grafted onto the rootstock of the said first plant, whereby
the said transgenic nucleic acid sequence generates
polynucleotide molecules in the rootstock of the first
10   plant, which polynucleotide molecules are transported
systemically across the graft junction to enter the male
meiocytes produced by the scion of the second plant, and
wherein the said systemically transported polynucleotide
molecules are at least partially complementary to a
15   transcript expressed in the male meiocytes produced by the
scion of the second plant.

2. Method as claimed in claim 1, wherein
expression of the transgenic nucleic acid sequence in the
first plant is achieved by operably linking the said nucleic
20   acid sequence to a promoter sequence that confers a
ubiquitous expression profile, or a tissue- or cell-type-
specific expression profile, onto the said transgenic
nucleic acid sequence.

3. Method as claimed in claim 2, wherein the
25   promoter sequence confers onto the said transgenic nucleic
acid sequence an expression profile that encompasses roots.

4. Method as claimed in claims 1-3, wherein
interfering with processes in the male meiocytes consists of
the suppression or blocking of processes in the male
30   meiocytes.

5. Method as claimed in claim 4, wherein the
suppression or blocking of processes in the male meiocytes
is achieved by destabilizing the target gene mRNA by means

of polynucleotide molecules that are at least partially
complementary to the target gene mRNA or transcript and that
have been generated from the transgenic nucleic acid
sequence.

5   6. Method as claimed in claim 5, wherein the
polynucleotide molecules are selected from the group
comprising miRNA, antisense RNA, RNAi molecules, siRNA
molecules, Virus-Induced Gene Silencing (VIGS) molecules,
co-suppressor molecules or RNA oligonucleotides.

10   7. Method as claimed in claims 1-3, wherein
influencing of processes in the male meiocytes of plants
consists of the enhancement of processes in the male
meiocytes of plants.

          8. Method as claimed in claims 1-7, wherein the
15 polynucleotide molecules are transiently expressed in the
first plant.

          9. Method as claimed in claim 8, wherein the
transient expression of the polynucleotide molecules in the
first plant is achieved by use of an inducible promoter,
20 selected from the group comprising heat-inducible promoters,
chemical-inducible promoters, steroid-inducible promoters
and alcohol-inducible promoters.

          10. Method as claimed in claim 8, wherein the
transient expression of the polynucleotide molecules is
25 effected by expression from a construct encoding the
polynucleotide molecules that is not integrated in the
genome of the rootstock cells.

          11. Method as claimed in claim 10, wherein
construct is delivered to the rootstock cells by
30 infiltration of leaves on the rootstock with *Agrobacterium*.

          12. Method as claimed in claims 1-11, wherein the
polynucleotide molecules are at least partially

complementary to the transcript of a gene that is involved in chromosome recombination.

13. Method as claimed in claim 12, wherein the gene is selected from the group comprising *SPO11*, *MER1*, *MER2*, *MRE2*, *MEI4*, *REC102*, *REC104*, *REC114*, *MEK1/MRE4*, *RED1*, *HOP1*, *RAD50*, *MRE11*, *XRS2*, *PRD1*, *PRD2*, *PRD3*, *PHS1*, *NBS1*, *COM1*, *RHD54/TID1*, *DMC1*, *SAE3*, *RED1*, *HOP1*, *HOP2*, *MND1*, *REC8*, *MEI5*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RPA1*, *SMC3*, *SCC1*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, *SOLODANCERS*, *HIM6*, *CHK2*, *SGS1*, *MSH4*, *MSH5*, *MER3/RCK*, *ZIP1*, *ZIP2*, *ZIP3*, *ZIP4*, *PTD*, *SHOC1*, *ZYP1*, *MLH1*, *MLH3*, or their functional homologues.

14. Method as claimed in any one of the claims 1-13, wherein the polynucleotide is a DNA polynucleotide.

15. Method for specifically altering the genome sequence of male meiocytes of a plant, by combining a first plant comprising a rootstock, which transgenically harbours a nucleic acid sequence, with a scion from a second plant grafted onto the rootstock of the said first plant, whereby the said transgenic nucleic acid sequence generates polynucleotide molecules in the rootstock of the first plant, which polynucleotide molecules are transported systemically across the graft junction to enter the male meiocytes produced by the scion of the second plant, and wherein the said polynucleotide molecules are at least partially homologous to a DNA fragment contained in the genome of the second plant.
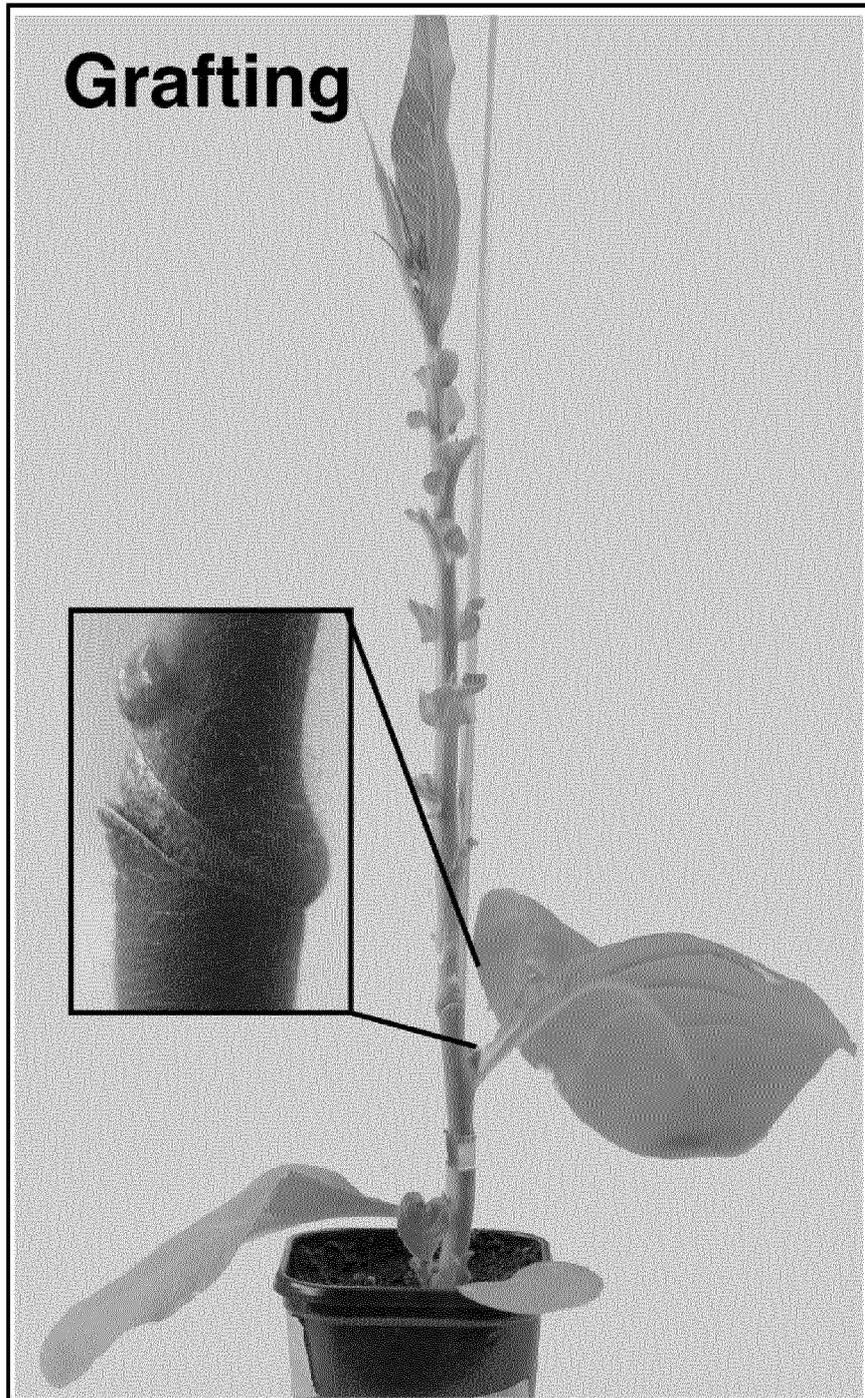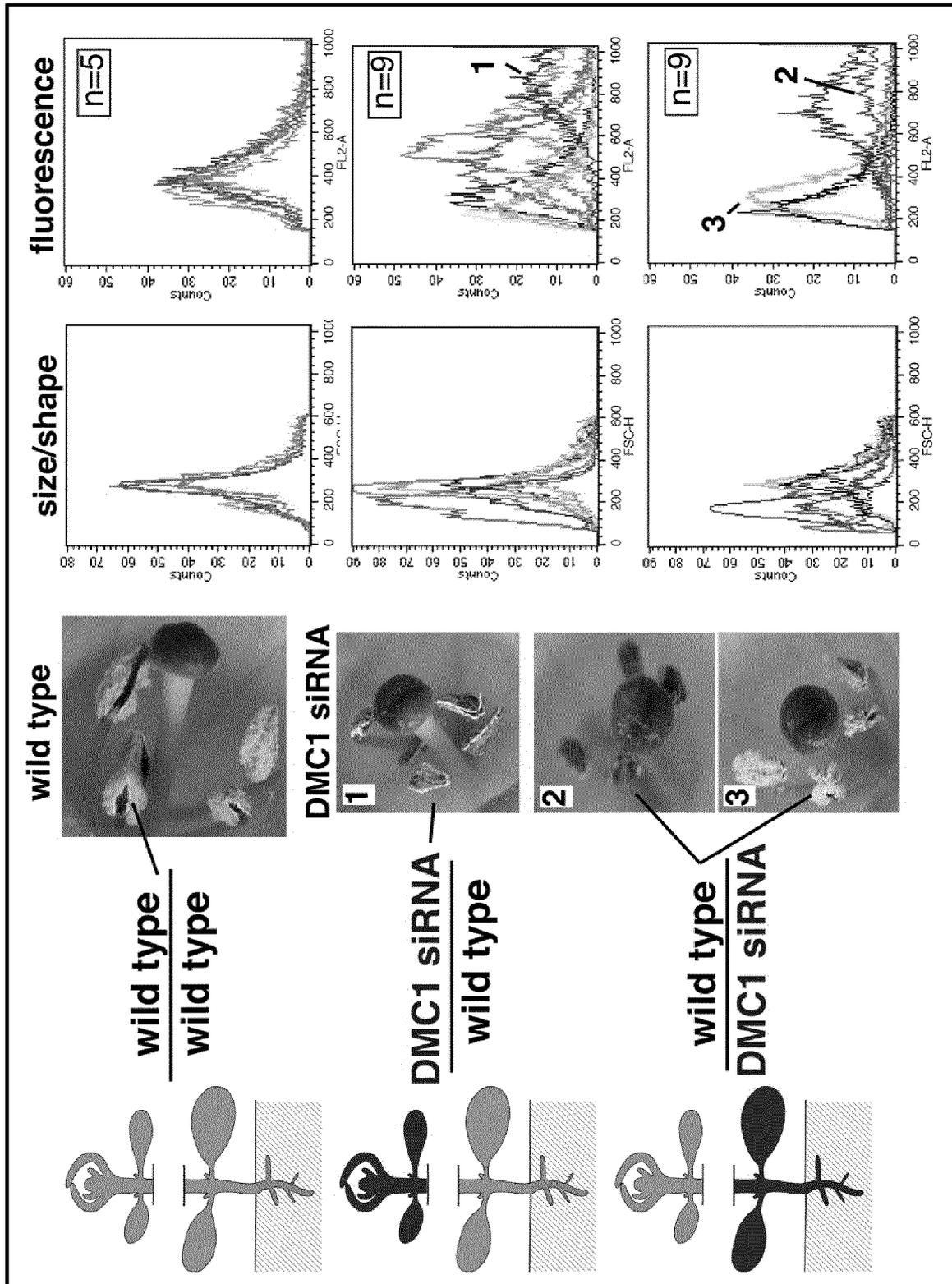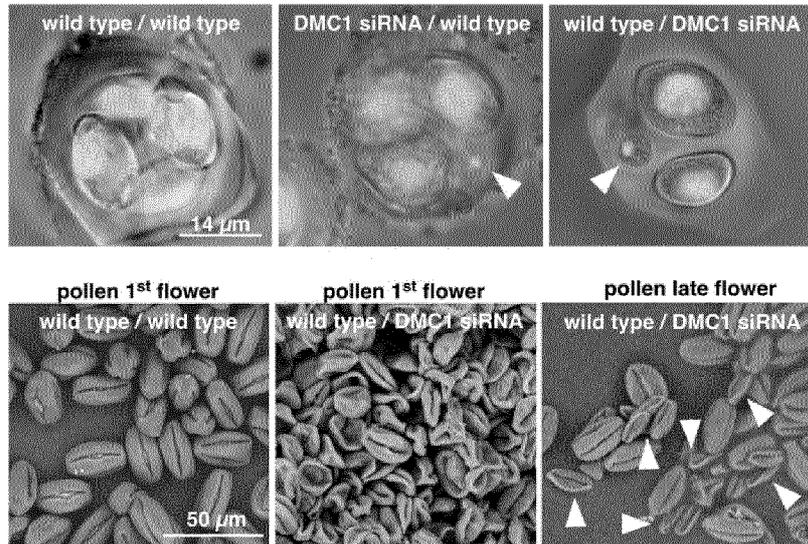
Fig. 1A

2/4

Fig. 1B

Fig. 2

4/4

Fig. 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A01H5/00   A01H5/02   C12N15/01   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, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHU-YUN DENG ET AL: "OsDMC1 is required for homologous pairing in Oryza sativa", PLANT MOLECULAR BIOLOGY, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 65, no. 1-2, 12 June 2007 (2007-06-12), pages 31-42, XP019532703, ISSN: 1573-5028, DOI: 10.1007/S11103-007-9195-2 the whole document ----- -/--	1-13

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 2012	25/10/2012

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Bilang, Jürg

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WIJNKER E ET AL: "Managing meiotic recombination in plant breeding", TRENDS IN PLANT SCIENCE, ELSEVIER SCIENCE, OXFORD, GB, vol. 13, no. 12, 1 December 2008 (2008-12-01), pages 640-646, XP025694256, ISSN: 1360-1385, DOI: 10.1016/J.TPLANTS.2008.09.004 [retrieved on 2008-11-26] the whole document -----	1-13
A	J. KEHR ET AL: "Long distance transport and movement of RNA through the phloem", JOURNAL OF EXPERIMENTAL BOTANY, vol. 59, no. 1, 18 December 2007 (2007-12-18), pages 85-92, XP055013845, ISSN: 0022-0957, DOI: 10.1093/jxb/erm176 the whole document -----	1-13
A	M N UDDIN ET AL: "Non-cell-autonomous RNA silencing spread in plants", BOTANICAL STUCIES, vol. 52, 1 April 2011 (2011-04-01), pages 129-136, XP055013922, the whole document -----	1-13
A	KRAGLER ET AL: "RNA in the phloem: A crisis or a return on investment?", PLANT SCIENCE, ELSEVIER IRELAND LTD, IE, vol. 178, no. 2, 1 February 2010 (2010-02-01), pages 99-104, XP026876947, ISSN: 0168-9452 [retrieved on 2010-01-27] the whole document -----	1-13
A	YOO B-C ET AL: "A Systemic Small RNA Signaling System in Plants", THE PLANT CELL, AMERICAN SOCIETY OF PLANT BIOLOGISTS, US, vol. 16, 1 August 2004 (2004-08-01), pages 1979-2000, XP002997405, ISSN: 1040-4651, DOI: 10.1105/TPC.104.023614 the whole document -----	1-13

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARADA ET AL: "Grafting and RNA transport via phloem tissue in horticultural plants", SCIENTIA HORTICULTURAE, ELSEVIER SCIENCE PUBLISHERS, XX, vol. 125, no. 4, 26 July 2010 (2010-07-26), pages 545-550, XP027130979, ISSN: 0304-4238 [retrieved on 2010-06-19] the whole document -----	1-13
A	DINANT S ET AL: "The phloem pathway: New issues and old debates", COMPTES RENDUS - BIOLOGIES, ELSEVIER, PARIS, FR, vol. 333, no. 4, 1 April 2010 (2010-04-01), pages 307-319, XP026991617, ISSN: 1631-0691 [retrieved on 2010-04-01] the whole document -----	1-13
A	DAVID TWELL: "Male gametogenesis and germline specification in flowering plants", SEXUAL PLANT REPRODUCTION, SPRINGER, BERLIN, DE, vol. 24, no. 2, 20 November 2010 (2010-11-20), pages 149-160, XP019906743, ISSN: 1432-2145, DOI: 10.1007/S00497-010-0157-5 the whole document -----	1-13

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