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(54) Titre : CONSERVATION DE PLANTES MALE-STERILES
(54) Title: MAINTENANCE OF MALE-STERILE PLANTS

(57) **Abrégé/Abstract:**

Novel transgenic plants that have, stably integrated into their nuclear genome, a maintainer gene comprising a fertility-restorer gene and a pollen-lethality gene. The plants can be used to maintain a homogeneous population of male-sterile plants.





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(54) Title: MAINTENANCE OF MALE-STERILE PLANTS (57) Abstract Novel transgenic plants that have, stably integrated into their nuclear genome, a maintainer gene comprising a fertility-restorer gene and a pollen-lethality gene. The plants can be used to maintain a homogeneous population of male-sterile plants.		

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MAINTENANCE OF MALE-STERILE PLANTS

This invention relates to a process for maintaining male-sterile plant lines that can be used for the production of hybrid seed of a crop, to maintainer plants that can be used in such a process, and to maintainer genes that can be used to produce such maintainer plants.

Background of the Invention

In many, if not most, plant species, the development of hybrid cultivars is highly desired because of their generally increased productivity due to heterosis: the superior performance of hybrid individuals compared with their parents (see, e.g., Fehr (1987) "Principles of Cultivar Development, Volume 1: Theory and Technique", MacMillan Publishing Company, New York; Allard (1960) "Principles of Plant Breeding", John Wiley and Sons, Inc., New York).

The development of hybrid cultivars of various plant species depends upon the capability to achieve almost complete cross-pollination between parents. This is most simply achieved by rendering one of the parent lines male-sterile (i.e., with pollen being absent or nonfunctional), for example, by manually removing the one parent's anthers or by providing the one parent with naturally occurring cytoplasmic or nuclear genes that prevent anther and/or pollen development and/or function, using classical breeding techniques (for a review of the genetics of male-sterility in plants, see Kaul (1988) "Male Sterility in Higher Plants", Springer Verlag, New York).

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For hybrid plants where the seed is the harvested product (e.g., corn and oilseed rape), it is, in most cases, also necessary to ensure that fertility of the hybrid plants is fully restored. In plants in which the male-sterility is under genetic control, this requires the use of genes that can restore male-fertility. Hence, the development of hybrid cultivars is mainly dependent on the availability of suitable and effective sterility and restorer genes.

Endogenous nuclear loci are known for most plant species that contain genotypes which effect male-sterility, and generally, such loci need to be homozygous for particular recessive alleles in order to result in a male-sterile phenotype. The presence of a dominant male-fertile allele at such loci results in male-fertility.

Recently, it has been shown that male-sterility can be induced in a plant by providing the plant with a nuclear male-sterility genotype that includes a chimaeric male-sterility gene comprising a DNA sequence (or male-sterility DNA) coding, for example, for a cytotoxic product (such as an RNase) and under the control of a promoter which is predominantly active in selected tissue of the plant's male reproductive organs. In this regard, tapetum-specific promoters, such as the promoter of the TA29 gene of Nicotiana tabacum, have been shown to be particularly useful for this purpose (Mariani et al (1990) Nature 347:737; European patent publication ("EP") 0,344,029). By providing the nuclear genome of the plant with such a male-sterility gene, an artificial nuclear male-sterility locus is created containing the artificial male-sterility genotype that results in a male-sterile plant.

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Examples of male-sterility DNAs include DNA encoding: RNases such as RNase T1 (which degrades RNA molecules by hydrolyzing the bond after any guanine residue) and Barnase; DNases such as an endonuclease (e.g., EcoRI); or
5 proteases such as a papain (e.g., papain zymogen and papain active protein); enzymes which catalyse the synthesis of phytohormones, such as: isopentenyl transferase which is an enzyme that catalyzes the first step in cytokinin biosynthesis and is encoded by gene 4 of Agrobacterium T-DNA;
10 and the enzymes involved in the synthesis of auxin and encoded by gene 1 and gene 2 of Agrobacterium T-DNA; glucanases; lipases such as phospholipase A₂ (Verheij et al (1981) Rev. Biochem. Pharmacol. 91, 92-203); lipid peroxidases; or plant cell wall inhibitors; proteins toxic to
15 plants cells, such as a bacterial toxin (e.g., the B-fragment of diphtheria toxin or botulin). Still another example of a male-sterility DNA is an antisense DNA which encodes a strand of DNA complementary to a strand of DNA that is naturally transcribed in the plant's stamen cells under the control of
20 an endogenous promoter. Such an antisense DNA can be transcribed into an RNA sequence capable of binding to the coding and/or non-coding portion of an RNA, naturally produced in the stamen cell, so as to inhibit the translation of the naturally produced RNA. An example of such an
25 antisense DNA is the antisense DNA of the TA29 gene which is naturally expressed, under the control of the TA29 promoter, in tapetum cells of the anthers of plants. A further example of a male-sterility DNA encodes a specific RNA enzyme (i.e., a so-called "ribozyme"), capable of highly specific cleavage
30 against a given target sequence, as described by Haseloff and Gerlach (1988) Nature 334, 585-591. Such a ribozyme is, for example, the ribozyme targeted against the RNA encoded by the TA29 gene. Still other examples of male-sterility DNAs encode products which can render the stamen cells susceptible

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to specific diseases, such as fungus infections. Such a male-sterility DNA can be used in a plant wherein all other cells, in which the male-sterility DNA is not expressed, are resistant to the specific disease.

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In addition, it has been recently shown that male-fertility can be restored to such a nuclear male-sterile plant with a chimaeric fertility-restorer gene comprising another DNA sequence (or fertility-restorer DNA) that codes, for example, for a protein that inhibits the activity of the cytotoxic product or otherwise prevents the cytotoxic product from being active at least in the selected tissue of the plant's male reproductive organs (EP 0,412,911). For example, the barnase gene of Bacillus amyloliquefaciens codes for an RNase (Barnase) which can be inhibited by a protein (Barstar) that is encoded by the barstar gene of B. amyloliquefaciens. Hence, the barnase gene can be used for the construction of a chimaeric male-sterility gene while the barstar gene can be used for the construction of a chimaeric fertility-restorer gene. Experiments in different plant species (e.g., oilseed rape) have shown that such a chimaeric barstar gene can fully restore the male-fertility of male-sterile lines in which the male-sterility was due to the presence of such a chimaeric barnase gene (EP 0,412,911: Mariani et al (1991) Proceedings of the CCIRC Rapeseed Congress, July 9-11, 1991 Saskatoon, Saskatchewan, Canada; Mariani et al (1992) Nature 357:384). By coupling a marker gene, such as a dominant herbicide resistance gene (for example, the bar gene coding for phosphinothricin acetyl transferase (PAT) that converts herbicidal phosphinothricin to a non-toxic compound [De Block et al (1987) EMBO J. 6:2513]), to the chimaeric male-sterility and/or fertility restorer gene, breeding systems can be implemented to select for uniform populations of male-sterile plants (EP 0,344,029; EP 0,412,911).

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The production of hybrid seed of any particular cultivar of a plant species requires the: 1) maintenance of small quantities of pure seed of each inbred parent and 2) the preparation of larger quantities of seed of each inbred parent. Such larger quantities of seed would normally be obtained by several (usually two) seed-multiplication rounds, starting from a small quantity of pure seed ("basic seed") and leading, in each multiplication round, to a larger quantity of seed of the inbred parent and finally to a stock of seed of the inbred parent ("parent seed" or "foundation seed") which is of sufficient quantity to be planted to produce the desired quantities of hybrid seed. Of course, in each seed-multiplication round, larger planting areas (fields) are required.

In order to maintain and enlarge a small stock of seeds of male-sterile plants, it has been necessary to cross the parent male-sterile plants with normal pollen-producing parent plants. The offspring of such a cross will, in all cases, be a mixture of male-sterile and male-fertile plants, and the latter have to be removed from the former. With male-sterile plants containing an artificial male-sterility locus as described above, such removal can be facilitated by genetically linking the chimaeric male-sterility gene to a suitable marker gene, such as the bar gene, which allows the easy identification and removal of the male-fertile plants. EP 0,198,288 and US Patent 4,717,219, by comparison, describe methods for linking such marker genes (which can be visible markers or dominant conditional markers) to endogenous nuclear loci containing male-sterility genotypes.

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However, even when suitable marker genes are linked to male-sterility genotypes, the maintenance of parent male-sterile plants still requires the removal from the field of a substantial number of plants. For instance, in systems using a herbicide resistance gene (e.g., the bar gene) linked to a chimaeric male-sterility gene, only half of the parent stock will result in male-sterile plants, thus requiring the removal of the male-fertile plants by herbicide spraying prior to flowering. In any given field, the removal of male-fertile plants effectively reduces the potential yield of hybrid seed or the potential yield of male-sterile plants during each round of seed multiplication for producing of parent seed. This is economically unattractive for many important crop species such as corn and oilseed rape. In order to minimize the number of male-fertile plants which have to be removed, male-fertile maintainer plants have been sought which, when crossed with a male-sterile parent plant, produce a minimum, preferably no, male-fertile offspring, thereby minimizing or avoiding altogether the need to remove such male-fertile offspring. To solve an analogous problem, US Patents 3,710,511 and 3,861,079 have described procedures for producing and maintaining a homogenous population of male-sterile plants by using specific chromosomal abnormalities that are differentially transmitted to the egg and the sperm in the plants.

Summary of the Invention

In accordance with this invention, a cell of a transgenic plant ("the maintainer plant") is provided, in which the nuclear genome contains stably integrated therein: 1) at a first locus or male-sterility locus, a male-sterility genotype in

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homozygous condition; and 2) at a second locus or maintainer locus, a maintainer gene in heterozygous condition; the male-sterility locus and the maintainer locus preferably being unlinked; the maintainer gene being a foreign DNA sequence, preferably a foreign chimaeric DNA sequence, containing:

- a) a fertility-restorer gene that comprises at least:
 - i) a fertility-restorer DNA encoding a restorer RNA and/or protein or polypeptide which, when produced or overproduced in some or all of the cells, preferably stamen cells, of the plant, prevents phenotypic expression of the nuclear male-sterility genotype that would render the plant male-sterile in the absence of expression of the fertility-restorer DNA in the some or all stamen cells and
 - ii) a restorer promoter capable of directing expression of the fertility-restorer DNA at least in the some or all of the cells, preferably stamen cells, of the plant, so that the phenotypic expression of the nuclear male-sterility genotype is prevented, the fertility-restorer DNA being in the same transcriptional unit as, and under the control of, the restorer promoter and
- b) a pollen-lethality gene that is selectively expressed in microspores and/or pollen of the plant to produce nonfunctional pollen and that comprises at least:
 - iii) a pollen-lethality DNA coding for a pollen-lethality RNA and/or protein or polypeptide that, when produced or overproduced in the microspores and/or pollen, significantly

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disrupts their metabolism, functioning and/or development and

- iv) a pollen-specific promoter capable of directing expression of the pollen-lethality DNA selectively in the microspores and/or pollen of the plant, the pollen-lethality DNA being in the same transcriptional unit as, and under the control of, the pollen promoter.

The cell of the maintainer plant of this invention preferably also comprises, especially in the maintainer locus, at least one first marker gene which comprises at least:

- v) a first marker DNA encoding a first marker RNA and/or protein or polypeptide which, when present at least in a first specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the first marker RNA, protein or polypeptide encoded by the first marker DNA at least in the first specific tissue or specific cells and
- vi) a first marker promoter capable of directing expression of the first marker DNA at least in the first specific tissue or specific cells, the first marker DNA being in the same transcriptional unit as, and under the control of, the first marker promoter.

The male-sterility genotype in the cell of the maintainer plant of this invention can be foreign or endogenous but is preferably a foreign, especially chimaeric, male-sterility gene which comprises:

- 1) a male-sterility DNA encoding a sterility RNA and/or protein or polypeptide which, when produced or overproduced in a stamen cell of the plant in the absence of the restorer RNA, protein or polypeptide,

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significantly disturbs the metabolism, functioning and/or development of the stamen cell and

2) a sterility promoter capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter.

The male-sterility genotype in the maintainer plant cell of this invention preferably comprises, especially in the male-sterility locus, at least one second marker gene which comprises at least:

3) a second marker DNA encoding a second marker RNA and/or protein or polypeptide which, when present at least in the second specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the second marker RNA, protein or polypeptide encoded by the second marker DNA at least in the second specific tissue or specific cells and

4) a second marker promoter capable of directing expression of the second marker DNA at least in the second specific tissue or specific cells, the second marker DNA being in the same transcriptional unit as, and under the control of, the second marker promoter.

Also in accordance with this invention are provided the maintainer plants, the seeds of such plants, and plant cell cultures, all of which consist essentially of the cells of this invention.

Further in accordance with this invention are provided the maintainer gene and plasmids containing the maintainer gene, as well as bacterial host cells (e.g., E. coli or Agrobacterium) containing such plasmids.

Still further in accordance with this invention is provided a process for producing, preferably

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enlarging, a homogeneous population of male-sterile plants and their seed that contain a nuclear male-sterility gene in homozygous condition, the process comprising the step of crossing the male-sterile plants with the maintainer plants
5 of this invention. The seed from the resulting male-sterile plants can be harvested and grown into the male-sterile plants. Hybrid seed can then be produced by crossing the male-sterile plants with male-fertile plants of another inbred parent line used as pollinators.

10 According to another aspect of the present invention, there is provided a process for maintaining a male-sterile line of a plant species, said process comprising the steps of: 1) crossing: a) a male-sterile line to be maintained comprising male-sterile parent plants which
15 comprise a male-sterility gene, at a first genetic locus; wherein said male-sterility gene is homozygous at said first genetic locus; with b) a maintainer line comprising male-fertile parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which
20 further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and ii) a pollen-lethality gene comprising,
25 under the control of a first promoter that directs expression selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent
30 plants, significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; wherein said foreign DNA is heterozygous at said second genetic locus; and 2) harvesting seeds from said male-sterile parent

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plants, wherein said seeds grow into a new generation of male-sterile parent plants.

According to another aspect, there is provided a process for maintaining a male-sterile line of a plant species, said process comprising the steps of: 1) obtaining a maintainer line comprising male-fertile parent plants which comprise a male-sterility gene at a first genetic locus, wherein said male-sterility gene is homozygous at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: a) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and b) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants, significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; wherein said foreign DNA is heterozygous at said second genetic locus; and 2) crossing said maintainer line with a male-sterile line to be maintained comprising male-sterile parent plants which comprise said homozygous male-sterility gene at said first genetic locus; and 3) harvesting seeds from said male-sterile parent plants, wherein said seeds grow into a new generation of male-sterile parent plants.

According to another aspect, there is provided a process for obtaining seed of male-sterile parent plants which comprise a homozygous male-sterility gene at a first genetic locus, said process comprising the steps of: 1) crossing said male-sterile line with a maintainer line

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comprising male-fertile parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: a) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and b) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants, significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; and 2) harvesting seeds from said male-sterile parent plants.

According to still another aspect of the present invention, there is provided a cell of a male-fertile parent plant for use in maintaining male-sterile parent plants which comprise a homozygous male-sterility gene at a first genetic locus, wherein said male-fertile parent plant comprises said homozygous male-sterility gene at said first genetic locus and further comprises, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: (i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and (ii) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore and/or pollen cell of said male-fertile parent plant, a first DNA encoding a first RNA, protein or polypeptide which when produced in said microspore or pollen cell of said male-fertile parent plant significantly disrupts the

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metabolism, functioning or development of said microspore or pollen cell; wherein said foreign DNA is heterozygous at said second genetic locus, and wherein said male-fertile parent plant can be crossed with said male-sterile parent plants to produce, on said male-sterile parent plants, seeds which grow into a new generation of male-sterile parent plants.

According to yet another aspect of the present invention, there is provided use of a male-fertile parent plant for maintaining male-sterile parent plants which comprise a homozygous male-sterility gene at a first genetic locus, wherein said male-fertile parent plant comprises said homozygous male-sterility gene at said first genetic locus and further comprises, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: (i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and (ii) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore or pollen cell of said male-fertile parent plant, a first DNA encoding a first RNA, protein or polypeptide which when produced in said microspore or pollen cell of said male-fertile parent plant significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; wherein said foreign DNA is heterozygous at said second genetic locus, and wherein said male-fertile parent plant can be crossed with said male-sterile parent plants to produce, on said male-sterile parent plants, seeds which grow into a new generation of male-sterile parent plants.

According to a further aspect of the present invention, there is provided use of a kit for maintaining a male-sterile line of a plant species, said kit comprising:

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a) a male-sterile line comprising male-sterile parent plants which comprise a male-sterility gene at a first genetic locus, wherein said male-sterility gene is homozygous at said first genetic locus; and, b) a maintainer line
5 comprising male-fertile parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising: (i) a restorer gene which, upon
10 expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and (ii) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore and/or pollen cell of said male-fertile parent plants, a first DNA
15 encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; wherein said foreign DNA is heterozygous at said
20 second genetic locus; and wherein said male-sterile and male-fertile parent plants can be crossed to produce, on said male-sterile plants, seed which grow into a new generation of male-sterile parent plants.

Yet further in accordance with this invention is
25 provided a process for producing, preferably enlarging, a population of the maintainer plants, comprising the step of selfing the maintainer plants.

Detailed Description of the Invention

A male-sterile plant of this invention is a plant
30 of a given species with a nuclear male-sterility genotype.

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A restorer plant of this invention is a plant of the same plant species containing, within its nuclear genome, a fertility-restorer gene that is able to restore the male-fertility in offspring which are obtained from a
5 cross between the male-sterile plant and the restorer plant and which contain both the male-sterility genotype and the fertility-restorer gene.

A restored plant of this invention is a plant of the same species that is male-fertile and that contains,
10 within its nuclear genome, the male-sterility genotype and the fertility-restorer gene.

A parent plant or parent of this invention is a plant that can be used for the production of hybrid seed. The female or seed parent plant is the parent from which the
15 hybrid seed is harvested. For the purposes of this invention, the female parent will

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always be a male-sterile plant. The male or pollen parent is the parent that is used to fertilize the female parent. In many cases, the male parent will also be a restorer plant.

A line is the progeny of a given individual plant.

The male-sterility genotype of this invention is the genotype of at least one locus, preferably only one locus, in the nuclear genome of a plant (i.e., the male-sterility locus), the allelic composition of which can result in male-sterility in the plant. A male-sterility genotype can be endogenous to the plant, but it is generally preferred that it be foreign to the plant. Preferred foreign male-sterility genotypes are those in which the allele responsible for male-sterility contains a foreign male-sterility gene that comprises:

- 1) a male-sterility DNA encoding a sterility RNA and/or protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell and
- 2) a sterility promoter capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter.

Such a male-sterility gene is always a dominant allele at a foreign male-sterility locus. The recessive allele corresponds to the absence of the male-sterility gene in the nuclear genome of the plant.

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Preferred foreign male-sterility DNAs and sterility promoters that can be used in the male-sterility genes in female parent plants and maintainer plants of this invention have been described in EP 0,344,029. A particularly useful male-sterility DNA codes for Barnase (Hartley (1988) J.Mol. Biol. 202:913). Particularly useful sterility promoters are tapetum-specific promoters such as: the promoter of the TA29 gene of Nicotiana tabacum (EP 0,344,029) which can be used in tobacco, oilseed rape, lettuce, chicory, corn and other plant species; the PT72, the PT42 and PE1 promoters from rice, the sequences of which are given in SEQ ID no. 7, SEQ ID no. 8, and SEQ ID no. 9, respectively, of the Sequence Listing and which can be used in rice and other plant species (PCT application PCT/EP 92/00274); and the PCA55 promoter from corn, the sequence of which is given in SEQ ID No. 10, which can be used in corn and other plant species (PCT application PCT/EP 92/00275).

A preferred endogenous male-sterility genotype is one in which a recessive allele ("m") in homozygous condition (m/m) at a male-sterility locus produces male-sterility. At a male-sterility locus, male-fertility would otherwise be encoded by a corresponding dominant allele ("M"). Such a male-sterility genotype is known in many plant species (see Kaul (1988) supra; and 1992 issues of Maize Genetics Cooperation Newsletter, published by the Department of Agronomy and U.S. Department of Agriculture, University Of Missouri, Columbia, Missouri, U.S.A.). The DNA sequences in the nuclear genome of a plant corresponding to m and M alleles can be identified by gene tagging, i.e., by insertional mutagenesis using transposons, or by

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means of T-DNA integration (see, e.g., Wienand and Saedler (1987) In "Plant DNA Infectious Agents", Ed. by T.H.Hohn and J.Schell, Springer Verlag, New York, p. 205; Shepherd (1988) In "Plant Molecular Biology: a Practical Approach", IRL Press, p. 187; Teeri et al (1986) EMBO J. 5:1755).

Fertility-restorer DNAs and restorer promoters that can be used in the maintainer genes of this invention with a foreign male-sterility genotype have been described in EP 0,412,911. In this regard, fertility-restorer genes in which the fertility-restorer DNA encodes Barstar (Hartley (1988) J.Mol. Biol. 202:913) and is under control of tapetum-specific promoters, such as those described above as sterility promoters, are of particular use. In particular, it is believed that a fertility-restorer DNA coding for a mutant of Barstar, in which the cysteine residue at its position 40 is replaced by serine (Hartley (1989) TIBS 14:450), functions better in restoring the fertility in the restored plants of some species.

When an endogenous male-sterility genotype is homozygous for a recessive allele m, it is preferred that the fertility-restorer gene be the dominant allele M of that male-sterility genotype, preferably under the control of its own promoter. The DNA corresponding to such a dominant allele, including its natural promoter, can be isolated from the nuclear genome of the plant by means of gene tagging as described above.

The pollen-lethality DNAs that are used in the pollen-lethality genes of this invention preferably encode an RNA and/or a protein or polypeptide that, when expressed in microspores or pollen, significantly disrupts their metabolism, functioning

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and/or development. In this regard, the pollen-lethality DNAs can encode RNAs, proteins or polypeptides such as are encoded by the male-sterility DNAs described in EP 0,344,029. Of particular interest are male-sterility DNAs that encode ribonucleases (EP 0,344,029) such as RNase T1 from Aspergillus oryzae (Quaas et al (1988) Eur. J. Biochem. 173:617) or Barnase from Bacillus amyloliquefaciens (Hartley (1988) J.Mol.Biol. 202:913).

So that the pollen-lethality DNA is expressed selectively in microspores or pollen of the maintainer plant, it is preferred that the pollen-specific promoter, which controls the pollen-lethality DNA in the pollen-lethality gene, be a promoter capable of directing gene expression selectively in the microspores and/or pollen of the plant. Such a pollen-specific promoter can be an endogeneous promoter or a foreign promoter and can be from the nuclear genome or from the mitochondrial or chloroplast genome of a plant cell, but in any event, the pollen-specific promoter is foreign in the nuclear genome of the plant being transformed. Preferably the pollen-specific promoter causes the pollen-lethality DNA to be expressed only in the microspores and/or pollen, i.e., after meiosis of the microsporocytes in the anthers. The pollen-specific promoter can be selected and isolated in a well known manner from a plant species, preferably the plant species to be rendered male-sterile, so that the pollen-specific promoter directs expression of the pollen-lethality DNA selectively in the microspores and/or pollen so as to kill or disable the microspores and/or pollen in which the pollen-lethality gene is expressed. The pollen-specific

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promoter is preferably also selected and isolated so that it is effective to prevent expression of the pollen-lethality DNA in other tissues of the plant. For example, a suitable endogeneous pollen-specific promoter can be identified and isolated in a plant, to be rendered male-sterile, by :

1. searching for an mRNA which is only present in the plant during the development of its microspores and/or pollen;
2. optionally isolating the microspore- and/or pollen-specific mRNA;
3. preparing and isolating a cDNA from the microspore- and/or pollen-specific mRNA;
4. using this cDNA as a probe to identify regions in the plant genome which contain DNA coding for the corresponding microspore- and/or pollen-specific DNA or alternatively using inverse polymerase chain reactions for the geometric amplification of the DNA sequences which flank, upstream and downstream, a chosen core region of the genomic DNA corresponding to the sequence of the microspore- and/or pollen-specific cDNA; and
5. identifying the portion of the plant genome that is upstream (i.e. 5') from the DNA coding for the microspore- and/or pollen-specific mRNA and that contains the promoter of this DNA.

Examples of such pollen-specific promoters are well known (see MacCormick (1991) TIG 7:298). In this regard, Hamilton et al (1989) Sex. Plant Reprod. 2:208 describes a pollen-specific clone ("Zmg13") from maize inbred line W-22, and the use of the promoter sequences of the clone to direct pollen-specific expression in tobacco has been described by Guerrero et al (1990) Mol.Gen.Genet. 224:161). Other pollen-specific promoters that are likewise believed

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to be useful are: the promoter of the gene corresponding to the Nicotiana tabacum pollen-specific cDNA NTPc303 described by Weterings et al (1992) Plant Mol. Biol. 18:1101; and the promoter of the gene corresponding to the Brassica napus pollen-specific cDNA B54 described by Shen and Hsu (1992) Mol. Gen. Genet. 234:379.

If the fertility-restorer DNA in the fertility-restorer gene of the maintainer gene is also expressed in microspores and/or pollen at the same time as the pollen-lethality DNA is expressed (due for instance to the activity of the restorer promoter in microspores and/or pollen), it is preferred that the pollen-lethality DNA be different from the male-sterility DNA (the expression of which is intended to be prevented by expression of the fertility-restorer DNA of the maintainer gene). For example, if the male-sterility DNA encodes Barnase in the male-sterile plants to be maintained, the fertility-restorer DNA in the maintainer gene should encode Barstar. Thus, if the restorer promoter in the maintainer gene also directs expression of the fertility-restorer DNA in microspores and/or pollen and at the same time as the pollen-lethality DNA is expressed, the pollen-lethality DNA preferably should not encode Barnase but rather, for example, another RNase such as RNase T1.

First and second marker DNAs and first and second marker promoters that can be used in the first and second marker genes of this invention are also well known (EP 0,344,029; EP 0,412,911). In this regard, it is preferred that the first and second marker DNAs be different, although the first and second marker promoters may be the same.

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The fertility-restorer gene, the male-sterility gene, the pollen-lethality gene, and the first and second marker genes in accordance with this invention are generally foreign DNA sequences, preferably foreign chimaeric DNA sequences. Such foreign DNA sequences are preferably provided with suitable 3' transcription regulation sequences and polyadenylation signals, downstream (i.e. 3') from their respective fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, and first and second marker DNAs. In this regard, either foreign or endogenous, transcription termination and polyadenylation signals suitable for obtaining expression of such DNA sequences can be used. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (De Greve et al (1982) J.Mol. Appl. Genet. 1:499; Gielen et al (1983) EMBO J. 3:835; Ingelbrecht et al (1989) The Plant Cell 1:671), the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid (De Picker et al (1982) J.Mol. Appl. Genet. 1:561), the chalcone synthase gene (Sommer and Saedler (1986) Mol. Gen. Genet. 202: 429-434), and the CaMV 19S/35S transcription unit (Mogen et al (1990) The Plant Cell 2:1261-1272), can be used.

By "foreign" with regard to a gene or genotype of this invention is meant that the gene or genotype contains a foreign DNA sequence such as a male-sterility DNA, a fertility-restorer DNA, a pollen-lethality DNA, or a marker DNA and/or a foreign promoter such as a sterility promoter, a restorer promoter, a pollen-specific promoter or a marker promoter. By "foreign" with regard to any DNA sequence, such as a coding sequence or a promoter, in a gene or genotype of this invention is meant that such a DNA is not in the same genomic environment in a plant cell, transformed with such a DNA in

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accordance with this invention, as is such a DNA when it is naturally found in the cell of the plant, bacteria, animal, fungus, virus or the like, from which such a DNA originates. This means, for example, that a foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can be: 1) a nuclear DNA in a plant of origin; 2) endogenous to the transformed plant cell (i.e., from a plant of origin with the same genotype as the plant being transformed); and 3) within the same transcriptional unit as its own endogenous promoter and 3' end transcription regulation signals (from the plant of origin) in the foreign gene or genotype in the transformed plant cell; but 4) inserted in a different place in the nuclear genome of the transformed plant cell than it was in the plant of origin so that it is not surrounded in the transformed plant cell by the genes which surrounded it naturally in the plant of origin. Likewise, a foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a different (i.e., not its own) endogenous promoter and/or 3' end transcription regulation signals in a foreign chimaeric gene or genotype of this invention in a transformed plant cell. A foreign fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a heterologous promoter and/or 3' end transcription regulation signals in a foreign chimaeric gene or genotype of this invention

in a transformed plant cell. A foreign fertility-restorer DNA, a male-sterility DNA, pollen-lethality DNA, or marker DNA can also, for example, be heterologous to the transformed plant cell and in the same transcriptional unit as an endogenous promoter and/or 3' transcription regulation signals (e.g., from the nuclear genome of a plant with the same genotype as the plant being transformed) in a foreign chimaeric DNA sequence of this invention in a transformed plant cell. Preferably, each fertility-restorer DNA, male-sterility DNA, pollen-lethality DNA, and marker DNA of this invention is heterologous to the plant cell being transformed.

By "heterologous" with regard to a DNA, such as a fertility-restorer DNA, a male-sterility DNA, a pollen-lethality DNA, a marker DNA, a fertility-restorer promoter, a sterility promoter, a pollen-specific promoter or a marker promoter or any other DNA sequence in a gene or a genotype of this invention is meant that such a DNA is not naturally found in the nuclear genome of cells of a plant with the same genotype as the plant being transformed. Examples of heterologous DNAs include chloroplast and mitochondrial DNAs obtained from a plant with the same genotype as the plant being transformed, but preferred examples are chloroplast, mitochondrial, and nuclear DNAs from plants having a different genotype than the plant being transformed, DNAs from animal and bacterial genomes, and chromosomal and plasmidial DNAs from fungal, bacterial and viral genomes.

By "chimaeric" with regard to a foreign DNA sequence of this invention is meant that at least one of its coding sequences : 1) is not naturally found under the control of the promoter present in the

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foreign DNA sequence; and/or 2) is not naturally found in the same genetic locus as at least one of its associated marker DNAs. Examples of foreign chimaeric DNA sequences of this invention comprise: a pollen-lethality DNA of bacterial origin under the control of a pollen-specific promoter of plant origin; and a pollen-lethality DNA of plant origin under the control of a pollen-specific promoter of plant origin and in the same genetic locus as a marker DNA of bacterial origin.

By "endogenous" with respect to a gene or genotype of this invention is meant that it is not foreign.

The foreign genes and genotypes of this invention, such as the male-sterility gene and genotype, the fertility-restorer gene and the pollen-lethality gene, can be described like any other genotype: capital letters denote the presence of the foreign genes and genotypes (the dominant allele) while small letters denote their absence (the recessive allele). Hence, in this description of the invention, "S" and "s" will denote the respective presence and absence of the male-sterility gene, "R" and "r" will denote the respective presence and absence of the fertility-restorer gene, and "P" and "p" will denote the respective presence and absence of the maintainer gene.

For an endogeneous male-sterility genotype of this invention, "m" will denote the recessive allele, and "M" will denote the dominant allele. Thus, the recessive allele m in homozygous condition (m/m) at a male-sterility locus would result in male-sterility, and the dominant allele M, when present at a male-sterility locus either in homozygous or heterozygous condition, results in male-fertility.

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The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus), can be transformed using a vector that is a disarmed Ti-plasmid containing the male-sterility gene and/or the fertility-restorer gene and/or the pollen-lethality gene and/or the maintainer gene and/or the marker gene(s) of this invention and carried by Agrobacterium. This transformation can be carried out using the procedures described, for example, in EP 0,116,718 and EP 0,270,822. Preferred Ti-plasmid vectors contain a foreign DNA sequence of this invention between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0, 233,247), pollen mediated transformation (as described, for example, in EP 0,270,356, PCT publication WO 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants, such as the major cereals including corn, rice, wheat, barley and rye, can be transformed as described in PCT application PCT/EP 91/02198. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the methods described for certain lines of corn by Fromm et al (1990) Bio/Technology 8:833, Gordon-Kamm et al (1990) Bio/Technology 2:603 and Gould et al (1991) Plant Physiol. 95:426. In case the plant to be transformed is rice, recently developed

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methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al (1989) Nature 338:274, Datta et al (1990) Bio/Technology 8:736 and Hayashimoto et al (1990) Plant Physiol. 93:857.

The so-transformed cell can be regenerated into a mature plant, and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the male-sterility gene, the fertility-restorer gene, the pollen-lethality gene, the marker genes and/or the maintainer gene of this invention in other varieties of the same or related plant species. Seeds obtained from such plants contain the gene(s) of this invention as a stable genomic insert.

The maintainer plant of this invention is of the same species as a male-sterile plant line and can be used for the maintenance of the male-sterile line, i.e. to maintain a homogeneous population of male-sterile plants and a stock of pure seed of the female parent. The maintainer plant of this invention is itself a plant in which male-fertility has been restored and the genome of which contains both a male-sterility genotype and, in the maintainer locus, a fertility-restorer gene of this invention.

If a plant line with a homozygous male-sterility genotype ($A^{m/m}$ or $A^{s/s}$) is available, a maintainer plant for the male-sterile line can be directly obtained by transforming a male-sterile plant of the line with the maintainer gene of this invention and then selecting those transgenic plants which are male-fertility restored plants and in which the maintainer gene is stably integrated in the nuclear genome so that the genetic locus of the male-

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sterility genotype and of the maintainer gene are unlinked and segregate independently.

If the male-sterility genotype is foreign to the plant line, alternative strategies can be followed. For example, the maintainer plant of the present invention can be obtained by: transforming a plant cell of the plant line (A) with the maintainer gene of this invention (P); and then regenerating, from the so-transformed plant cell, a transgenic plant containing, stably integrated in its genome, the maintainer gene. Such a transgenic plant ($A^{P/P}$) can then be crossed as a female parent with a plant $A^{S/s,R/r}$ of the same line, which contains at separate loci in its genome a male-sterility gene (S) and a corresponding fertility-restorer gene (R), both in heterozygous condition, but which lacks the maintainer gene. Thus, the cross is in fact: $A^{S/s,R/r,P/P}$ (male) x $A^{S/s,r/r,P/P}$ (female), and the offspring with the genotype $A^{S/s,r/r,P/P}$ (or hereinafter " $A^{S/s,P/P}$ " for convenience) are selected and selfed. One eighth of the offspring that have the desired genotype ($A^{S/S,P/P}$) for a maintainer plant of this invention can then be selected. Another eighth of the offspring with the genotype ($A^{S/S,P/P}$) can be used as male-sterile plants to be maintained.

Isolation of plants with desired genotypes can be achieved by means of conventional testcrosses (see, e.g., Fehr (1987) supra), preferably supplemented by detection of the presence of specific genes at the DNA level, e.g., by means of amplification of DNA fragments by the polymerase chain reaction, by Southern blot analysis and/or by phenotypic analysis for the presence and expression of first or second marker genes of this invention.

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The cross of a male-sterile plant containing a male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$) with a maintainer plant of this invention ($A^{S/S,P/P}$ or $A^{m/m,P/P}$, respectively) results in a population of seeds that all contain the male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$, respectively) because the maintainer gene is not transmitted through the pollen. This property can be used to advantage in maintaining the basic seed and in the multiplication of basic seed for the final production of parent seed.

The maintainer plants of this invention ($A^{S/S,P/P}$ or $A^{m/m,P/P}$) can themselves be maintained by selfing. The offspring of such selfing will consist of 50% male-fertile maintainer plants ($A^{S/S,P/P}$ or $A^{m/m,P/P}$, respectively) and 50% male-sterile plants containing the male-sterility genotype in homozygous condition ($A^{S/S}$ or $A^{m/m}$, respectively). If desired, the male-sterile plants can be removed either manually on the basis of the male-sterile phenotype or, if the maintainer gene comprises a suitable first marker gene, preferably a first marker gene whose expression confers herbicide resistance to the plant, by using the phenotypic expression of the first marker gene (e.g., by applying herbicide to the offspring so that male-sterile plants that lack the herbicide-resistance gene are killed while maintainer plants with the herbicide-resistance gene survive).

Thus, the maintainer plant of this invention can be easily used to maintain a homogeneous population of male-sterile plants. In this regard, basic seed of a female parent of a given plant species can be crossed with an appropriate male parent to produce hybrid seed. Also, the maintainer plant of this invention can be used economically to multiply the

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basic seed of a female parent of a given plant species, so as to obtain sufficient quantities of female parent seed that can be crossed with an appropriate male parent to produce desired quantities of hybrid seed.

A male-sterile line, that is maintained and multiplied by the use of the maintainer plants of this invention, can be used for the production of hybrid seed. In principle, the male-sterile line ($A^{s/s}$) can be crossed directly with another male parent line ($B^{s/s}$) to produce hybrid seed ($AB^{s/s}$). However, as all hybrid plants are male-sterile, no reproduction and no seed set will occur. This is not a problem if the seed is not the harvested product (e.g., with lettuce), but where seed is the harvested product (e.g., with corn and oilseed rape), male-fertility in the hybrid plants should be at least partially restored. This can be accomplished by crossing the male-sterile line with a male-fertile parent line (e.g., $B^{R/R}$) that is also a restorer line, i.e. that also contains a fertility-restorer gene (R). The hybrids produced ($AB^{s/s,R/r}$) will be fully male-fertile. Alternatively the male-sterile-line ($A^{s/s}$) can first be crossed with the male-fertile line ($A^{s/s}$) just prior to hybrid seed productions. This has the advantage of giving a further multiplication of the female parent line. The offspring ($A^{s/s}$) can then be crossed with a suitable male-fertile parent line ($B^{s/s,r/r}$) to produce hybrid seed that is 50% male-fertile. If hybrid seed with 100% male fertility is desired, the offspring can be crossed with a suitable restorer male parent line ($B^{s/s,R/R}$).

In the case of a male-sterile line in which male-sterility is due to an endogeneous male-sterility genotype (A^m/m) at a male-sterility locus,

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hybrid seed can easily be produced by crossing the male-sterile line ($A^{M/m}$) with a line that is homozygous with respect to the endogenous dominant (male-fertility) allele at that male-sterility locus ($B^{M/M}$). All hybrid offspring of this cross will have the genotype $AB^{M/m}$ and will be fertile.

The maintainer plants of this invention can also be used as pollinator (i.e., male-fertile) plants in a cross with wild-type plants ($A^{S/s, P/p}$) of the same inbred line. The progeny of this cross will all be male-sterile and heterozygous for the male-sterility genotype ($A^{S/s, P/p}$). The progeny can therefore be used directly for hybrid seed production by crossing with a pollinator plant line B ($B^{S/s, P/p}$). This scheme only requires a male-sterilization of the wild-type plants, for example by manually removing the anthers (e.g., in corn) or by using a male gametocide.

Of course, by using the maintainer plants of this invention to maintain a homogeneous population of plants that are homozygous with respect to a male-sterility allele (whether dominant or recessive) that is encoded in the nuclear genome, the maintainer plants acquire many of the characteristics of plants of a cytoplasmic male-sterile line. However, such plants do not have one of the major disadvantages of cytoplasmic male-sterile plants, namely the cytoplasmic uniformity of the various male-sterile lines which, in corn, has led to serious problems (see Craig (1977) In "Corn and Corn Improvement", G.F. Sprague, ed., American Society of Agronomy, Inc., Publisher, p. 671).

Thus, the maintainer gene of this invention, when introduced into a particular line of a plant species, can always be introduced into any other line by backcrossing, but since the maintainer gene can

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only be transmitted through an egg, it will always be associated with the cytoplasm of the line in which it was initially introduced. However, since a maintainer plant line is only used for maintenance of a male-sterile line and not as a female parent for hybrid seed production, the hybrid seed will always contain the cytoplasm of the female parent, as desired.

The following Examples illustrate this invention. Unless otherwise indicated, all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al (1989) "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, N.Y. USA. All polymerase chain reactions ("PCR") were performed under conventional conditions, using the Vent™ polymerase (Cat. No. 254L - Biolabs New England, Beverley, MA 01915, U.S.A.) isolated from Thermococcus litoralis (Neuner et al (1990) Arch. Microbiol. 153:205-207). Oligonucleotides were designed by the methods described by Kramer and Fritz (1968) Methods in Enzymology 154:350 and synthesized by the phosphoramidite method (Beaucage and Caruthers (1981) Tetrahedron Letters 22:1859) on an applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands).

The following bacterial strains and plasmids, used in the Examples, are available from the Deutsche Sammlung für Mikroorganismen und Zellkulturen ("DSM"), Mascheroder Weg 1B, Braunschweig, Germany:

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<u>Bacterial strain</u>	<u>plasmid</u>	<u>DSM No</u>	<u>Date of Deposit</u>
E. coli WK6	pMa5-8	DSM 4567	May 3, 1988
E. coli WK6	pMc5-8	DSM 4566	May 3, 1988

In the Examples, reference will be made to the following Figure and Sequence Listing:

Figure

Figure 1: Ten-step procedure to obtain corn (e.g. H99) maintainer plants of the invention

Sequence Listing

SEQ ID no. 1: genomic DNA comprising the promoter of the Zm13 gene from Zea mays

SEQ ID no. 2: sequence of plasmid "pVE144"

SEQ ID no. 3: sequence of plasmid "pVE108"

SEQ ID no. 4: sequence of oligonucleotide "MDB80"

SEQ ID no. 5: sequence of oligonucleotide "MDB81"

SEQ ID no. 6: sequence of oligonucleotide "MDB82"

SEQ ID No. 7: genomic DNA comprising the anther specific promoter "PT72" from rice

SEQ ID No. 8: genomic DNA comprising the anther specific promoter "PT42" from rice

SEQ ID No. 9: genomic DNA comprising the anther specific promoter "PE1" from rice

SEQ ID No. 10: genomic DNA comprising the anther specific promoter "PCA55" from corn

SEQ ID No. 11: Oligonucleotide Zm13OLI2

SEQ ID No. 12: Oligonucleotide Zm13OLI1

SEQ ID No. 13: Oligonucleotide Zm13OLI5

SEQ ID No. 14: Oligonucleotide BX0L2

SEQ ID No. 15: Oligonucleotide TA29SBX0L2

SEQ ID No. 16: Oligonucleotide PTA290L5

SEQ ID No. 17: EcoRI-HindIII fragment of pTS218 carrying the maintainer gene.

Examples

Example 1 : Isolation of the pollen-specific promoter of the Zm13 gene from maize.

A pollen-specific cDNA from Zea mays inbred line W-22, designated as "Zmcl3", has been isolated and characterized by Hanson et al (1989) The Plant Cell 1:173. The corresponding genomic clone, designated as "Zmg13", containing substantial portions of the 5' flanking region has been isolated and characterized by Hamilton et al (1989) Sex. Plant Reprod. 2:208 (see also Hamilton et al (1992) Plant Mol. Biol. 18:211). The complete sequence of Zmg13 is shown in SEQ ID no. 1, and its promoter region will hereinafter be referred to as the "Zm13 promoter".

A corresponding promoter region from corn inbred line H99 was isolated as follows. Genomic DNA of inbred line H99 was prepared as described by Dellaporta et al (1983) Plant Mol. Biol. Reports 1:19-21. Using the genome as a substrate, a 1471 bp fragment was amplified by PCR using the oligonucleotides MDB80 and MDB82, the sequences of which are shown in SEQ ID no. 4 and SEQ ID no. 6, respectively. MDB80 corresponds to nucleotides 8 to 28 of Zmg13, while MDB82 is complementary to nucleotides 1458 to 1478 of Zmg13. Then, the purified amplified 1471 bp fragment was used as a substrate for the amplification by PCR of a 1422 bp fragment, using the oligonucleotides MDB80 and MDB81. MDB81 is complementary to nucleotides 1409 to 1429 of Zmg13, and its sequence is shown in SEQ ID no. 5. By using MDB81, a NcoI site is created in the amplified 1422 bp fragment at the ATG translation initiation codon.

The 1422 bp fragment is then ligated in an SmaI site of pGEM2* (Promega Corporation, Madison,

*Trade-mark

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Wisconsin 53711, U.S.A.), yielding plasmid pMDB13, and the fragment is sequenced (Maxam and Gilbert (1980) Meth. Enzymol. 65:499). The pollen-specific promoter of the Zm13 gene of corn inbred line H99 is obtained from pMDB13 as a EcoRV-NcoI fragment.

The Zm13 promoter is also cloned as follows. Genomic DNA of Zea mays line H99 is prepared as described above. Using the genomic DNA as a substrate, the following two fragments are amplified by means of PCR: 1) a 875 bp fragment is amplified using the oligonucleotides MDB80 (SEQ ID No. 4) and ZM13OLI2 (which is complementary to nucleotides 859 to 882 of Zmg13 and which sequence is given in SEQ ID No. 11); and 2) a 661 bp fragment is amplified using the oligonucleotides Zm13OLI1 (which corresponds to nucleotides 767 to 791 of Zmg13 and which sequence is given in SEQ ID No. 12) and Zm13OLI5 (which is partially complementary to nucleotides 1397 to 1423 of Zmg13 and which sequence is given in SEQ ID No. 13). The 875 bp fragment, corresponding to the upstream region of the Zm13 promoter, is cloned into the SmaI site of pGEM2, yielding plasmid pTS204. The 661 bp fragment, corresponding to the downstream region of the Zm13 promoter, is digested with NcoI and cloned into plasmid pJB66 (Botterman and Zabeau (1987) DNA 6:583) digested with EcoRV and NcoI, yielding plasmid pTS203. Both fragments partly overlap and share a BstXI site in the region of overlap. Ligation of the 567 bp EcoRV-BstXI fragment of pTS204 and the 638 bp BstXI-NcoI fragment of pTS203 results in a 1205 bp fragment corresponding to the Zm13 promoter. This 1205 bp fragment, as cloned from line H99, is sequenced, and its sequence is found to be identical to the corresponding fragment of Zmg13 from line W-22 as given in SEQ ID No.1

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except at position 276 (G in W-22 is T in H99), 410 (G in W-22 is A in H99), and 1205-1206 (GC in W-22 is GGC in H99, thus corresponding to a 1 nucleotide insertion), numberings being as in SEQ ID No. 1.

Example 2 : Construction of plant transformation vectors comprising a maintainer gene that contain DNA encoding Barstar under the control of the TA29 promoter and DNA encoding Barnase under the control of the Zml3 promoter.

The 1205 bp EcoRV-NcoI fragment of pMDB13 is ligated to the large EcoRI-SmaI fragment of plasmid pVE144 and to the 739 bp EcoRI-NcoI fragment of pVE108, yielding plasmid pGSJVR1. Plasmid pVE144, the sequence of which is shown in SEQ ID no. 2, is a plasmid derived from plasmid pUC18 (Yanisch-Perron et al (1985) Gene 33:103) and containing DNA encoding neomycin phosphotransferase (neo) under the control of the 35S3 promoter (EP 0,359,617) from Cauliflower Mosaic Virus isolate CabB-JI (Hull and Howell (1978) Virology 86:482) and DNA encoding the Barstar (Hartley (1988) J.Mol.Biol. 202:913) under the control of the tapetum-specific promoter of the TA29 gene of Nicotiana tabacum (EP 0,344,029; Seurinck et al (1990) Nucleic Acids Res. 18:3403). Plasmid pVE108, the sequence of which is shown in SEQ ID no. 3, is a plasmid derived from pUC18 and containing DNA encoding phosphinothricin acetyl transferase (bar) (EP 0,242,236) under the control of the 35S3 promoter and DNA encoding Barnase (Hartley (1980) supra) under the control of the TA29 promoter. The resulting plasmid, pGSJVR1 (which is subsequently renamed "pTS210"), is a pUC18-derived plasmid that contains a maintainer gene of this invention comprising: DNA encoding Barnase as the pollen-lethality DNA, the Zml3 promoter as the pollen-specific promoter, DNA

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encoding Barstar as the fertility-restorer DNA, the TA29 promoter as the restorer promoter, neo as the first marker DNA and the 35S3 promoter as the first marker promoter.

pTS210 is also obtained as follows. The 0.9 kb BstXI-SacI fragment of pTS204 is ligated to the large BstXI-SacI fragment of pTS203, yielding plasmid pTS206. The 1.47 BglII-NcoI kb fragment of pTS206 is then ligated to the large NcoI-BglII fragment of pVE108, yielding plasmid pTS207. Finally, the 1.9 kb EcoRV-Eco-RI fragment of pTS207 is ligated to the large Eco-RI-SmaI fragment of pVE144, yielding plasmid pTS210.

A plasmid pTS218, which differs from pTS210 by carrying the bar gene as a selectable marker gene, is also obtained as follows:

- a 255 bp DNA fragment, designated as bxx and carrying the translation initiation site of the PTA29-barstar gene, is obtained by PCR using pVE144 as a template and oligonucleotides BXOL2 (SEQ ID No. 14) and TA29SBXOL2 (SEQ ID No. 15) as primers.
- a 492 bp DNA fragment is prepared by PCR using pVE108 and bxx as a template and oligonucleotides PTA29OL5 (SEQ ID No. 16) and BXOL2 as primers. This 492 bp fragment is digested with AsnI and BspEI, and a 274 bp fragment is purified on gel and ligated to the 6.28 kb fragment of pVE144 which was digested with BspEI and partially digested with AsnI. The resulting plasmid is designated as pVEK144 and carries the PTA29-barstar-3'nos chimeric gene

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with an optimized translational initiation context.

- pVEK144 is digested with MunI and HindIII, and the 3.7 kbp fragment is isolated and ligated to the 1.7 kbp MunI-HindIII fragment of pVE108, yielding plasmid pVEB144 which carries the PTA29-barstar-3'nos and the P35S-bar-3'nos chimeric genes.
- theEcoRI-HindIII fragment of pVEB144, containing the two chimeric genes, is ligated to the large EcoRI-HindIII fragment of pUCNew2, yielding plasmid pVEC144. pUCNew2 is derived from pUC19 as described in WO 92/13956.
- finally, the large EcoRI-SmaI fragment of pVEC144 is ligated to the 1.9 bp EcoRV-EcoRI fragment of pTS207, yielding plasmid pTS218.

Plasmid pTS218 carries three chimeric genes, i.e., PTA29-barstar-3'nos (with optimized translational initiation context), P35S-bar-3'nos, and PZM13-barnase-3'nos. The EcoRI-HindIII fragment of pTS218 carrying these three chimeric genes is presented in the sequence listing as SEQ ID No. 17.

All steps of vector construction involving fragments of the barnase DNA, such as pVE108, pVE144, and pTS210, are carried out in E. coli strain MC1061 containing the cointegrate plasmid R702::pMc5BS which is obtained as follows. Plasmid pMc5BS, containing the barstar gene (encoding an inhibitor of barnase) under the control of the tac promoter (De Boer et al (1983) Proc. Natl. Acad. Sci. USA 80:21), is constructed by: cloning the EcoRI-HindIII fragment of plasmid pMT416 (Hartley (1988) supra) into the EcoRI and HindIII sites of plasmid pMc5-8 (DSM 4566); and then deleting the sequence starting with the initiation codon of the phoA signal sequence and ending with the last nucleotide before the

translation initiation codon of the barstar-coding region by means of a looping-out mutagenesis procedure as generally described by Sollazo et al (1985) Gene 37:199.

Plasmid R702 is from Proteus mirabilis and can replicate in E.coli (Villarroel et al (1983) Mol. Gen. Genet. 189:390). Plasmid R702::pMc5BS is obtained by cointegration through illegitimate recombination between pMc5BS and R702, mediated by transposable elements present on R702 (Leemans (1982) "Technieken voor het gebruik van Ti-plasmieden van Agrobacterium tumefaciens als vectoren voor de genetic engineering van planten", Ph.D. Thesis Vrije Universiteit Brussel, Brussels, Belgium) and checked for induced expression of Barstar.

The use of E.coli (R702::pMc5BS) allows the construction, maintenance, amplification, and purification of plasmids containing the barnase DNA, such as pGSJVR1, without any lethal effect on the host due to accidental expression of the barnase DNA. However, because the Zml3 promoter is not expressed in E. coli, all steps of vector construction involving this promoter are also carried out in E. coli strain MC1061.

Example 3 : Transformation of corn with the maintainer gene of Example 2.

Zygotic immature embryos of about 0.5 to 1 mm are isolated from developing seeds of corn inbred line H99. The freshly isolated embryos are enzymatically treated for 1-2 minutes with an enzyme solution II (0.3% macerozyme (Kinki Yakult, Nishinomiya, Japan) in CPW salts (Powell & Chapman (1985) "Plant Cell Culture, A Practical Approach", R.A. Dixon ed., Chapter 3) with 10% mannitol and 5 mM 2-[N-Morpholino] ethane sulfonic acid (MES), pH 5.6).

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After 1-2 minutes incubation in this enzyme solution, the embryos are carefully washed with N6aph solution (macro- and micro-elements of N6 medium (Chu et al (1975) Sci. Sin. Peking 18:659) supplemented with 6mM asparagine, 12 mM proline, 1 mg/l thiamine-HCl, 0.5 mg/l nicotinic acid, 100 mg/l casein hydrolysate, 100 mg/l inositol, 30 g/l sucrose and 54 g/l mannitol). After washing, the embryos are incubated in the maize electroporation buffer, EPM-KCl (80 mM KCl, 5 mM CaCl_2 , 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 0.425 M mannitol, pH 7.2). Approximately 100 embryos in 200 μl EPM-KCl are loaded in each electroporation cuvette. About 20 μg of a plasmid DNA, pPGSJVR1 (of Example 2) linearized with EcoRI, is added per cuvette.

After 1 hour DNA incubation with the explants, the cuvettes are transferred to an ice bath. After 10 minutes incubation on ice, the electroporation is carried out: one pulse with a field strength of 375 V/cm is discharged from a 900 μF capacitor. The electroporation apparatus is as described by Dekeyser et al (1990) The Plant Cell 2:591. Immediately after electroporation, fresh liquid N6aph substrate is added to the explants in the cuvette, after which the explants are incubated for a further 10 minute period on ice.

Afterwards, the embryos are transferred to Mahl VII substrate (macro- and micro-elements and vitamins of N6 medium supplemented with 100 mg/l casein hydrolysate, 6 mM proline, 0.5 g/l MES, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2% sucrose solidified with 0.75 g/l MgCl_2 and 1.6 g/l Phytigel* (Sigma Chemical Company, St Louis, Mo. U.S.A.), pH 5.8) and supplemented with 0.2M mannitol. After 3 days, the embryos are transferred to the same substrate supplemented with 200 mg/l kanamycin. After

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approximately 14 days, the embryos are transferred to Mahl VII substrate without mannitol, supplemented with kanamycin. The embryos are further subcultured on this selective substrate for approximately 2 months with subculturing intervals of about 3 weeks. The induced embryogenic tissue is carefully isolated and transferred to MS medium (Murashige and Skoog (1962) *Physiol. Plant* 15:473) supplemented with 5 mg/l 6-benzylaminopurine for line H99. The embryogenic tissue is maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones and 6% sucrose for line H99. Developing shoots are transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. These plantlets are transferred to soil and cultivated in the greenhouse.

In an analogous way, corn embryos are transformed with a fragment of pTS218 DNA which contains the maintainer gene and the chimeric P35S-bar-3'nos and which is obtained by digestion of the plasmid with EcoRI, XhoI and PstI and by purifying the longest fragment. Transformation and plant regeneration is as described in Example 5.

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Example 4 : Analysis of the transgenic corn plants of Example 3.

Plants from Example 3 transformed with pGSJVR1 are analysed for the presence of the maintainer gene by means of PCR. DNA is prepared according to the protocol described by Dellaporta et al (1983) Plant Mol. Biol. Reporter 1:19, adapted for application to tissue amounts of about 10 to 20 mg. For each plant, such an amount of tissue is macerated in extraction buffer in a microfuge tube. Representative fragments of the maintainer gene are amplified using appropriate oligonucleotide probes.

Activity of the expression product of the first marker gene (i.e., neomycin phosphotransferase II (NPTII)) is assayed in plants as follows. Crude extracts are prepared by grinding plant tissue in extraction buffer (McDonnell et al (1987) Plant Molecular Biol. Reporter 5:380). The extracts are then subjected to non-denaturing polyacrylamide gel electrophoresis according to the procedure described by Reiss et al (1984) Gene 30:211. NPTII activity is then assayed by in situ phosphorylation of kanamycin using [γ -³²P]ATP as a substrate (McDonnell et al (1987) supra).

The plants that are found to be positive on both the PCR and NPTII assay are further analyzed by means

of Southern hybridization. Genomic DNA is prepared from plant tissue according to the protocol described by Dellaporta et al (1983) supra, supplemented by a treatment with RNase to remove remaining RNA. A non-transformed H99 plant is used as a control. Samples of the DNA are digested with appropriate restriction enzymes and subjected to horizontal agarose electrophoresis. Southern transfer to Hybond* N+ (Amersham International PLC, Amersham, United Kingdom) membranes by means of the "alkali blotting of DNA" protocol and the subsequent hybridization are performed as recommended by the manufacturer (Amersham Hybond-N+ leaflet). Suitable radioactive probes are prepared with the multi-prime DNA labelling kit (Amersham) according to the protocol supplied by the manufacturer which is derived from published procedures (Feinberg and Vogelstein (1983) Anal. Biochem. 132:6). The banding patterns show that at least the maintainer gene is integrated into the plant genomic DNA.

The PCR assays show that the maintainer gene is present. The NPTII assays show that the first marker DNA is expressed. The mature transformed plants can then be analyzed phenotypically to see whether the barstar DNA is expressed in tapetum cells and the barnase gene is expressed in pollen cells. Expression of barstar is determined by northern blotting of anther mRNA and by making testcrosses to determine the restoration in the progeny. Expression of the pollen-lethality gene is determined by cytological examination of the anther. In this regard, viable and nonviable mature pollen is determined by analyzing the staining of isolated pollen upon incubation for 30 minutes at 24°C in the following reaction mixture:
100 mM phosphate buffer pH 7.8, 100 mM

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Sodiumsuccinate and 1 mM NitroBlue Tetrazolium, followed by visual inspection of formazan precipitation in viable pollen. Alternative techniques for the differentiation between viable and nonviable mature pollen are those described for example by Alexander (1969) Stain Technology 44:117, and by Heslop-Harrison and Heslop-Harrison (1970) Stain Technology 45:115. The viability of microspores is determined by embedding flower buds in plastic at different developmental stages and subjecting the buds to histochemical staining with the succinate dehydrogenase assay, both as described by De Block and Debrouwer (1992) The Plant Journal 2:261.

Ultimately, the progeny of the plant transformed with the pollen-lethality gene is determined. None of the offspring obtained from a cross using this plant as a male parent have this gene, while 50% of the offspring obtained from a cross using this plant as a female parent possess the gene.

Plants from Example 3, transformed with pTS218 DNA, are analyzed in the same way, except that the expression product of the first marker gene, i.e., phosphinothricine acetyltransferase, is assayed by means of a PAT assay as described in Example 5.

Example 5 : Production of male-sterile corn plants.

Zygotic embryos of corn inbred line H99 were isolated, enzymatically treated, washed, and loaded in electroporation buffer as described in Example 3. Approximately 100 embryos in 200 μ l EPM-KCl were loaded in each electroporation cuvette. About 20 μ g of a plasmid DNA, pVE108 linearized with HindIII, was added per cuvette. pVE108 is a 5620 bp plasmid which contains: a chimaeric gene comprising the bar DNA (EP 242236), encoding phosphinothricin acetyl transferase (PAT) and conferring resistance to an herbicidal glutamine synthetase inhibitor such as phosphinothricin (PPT), under the control of the 35S3 promoter; and another chimaeric gene comprising the DNA coding for barnase (Hartley (1988) supra) under the control of the tapetum-specific promoter of the

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TA29 gene (EP 344029) of N. tabacum. The complete sequence of plasmid pVE108 is given in SEQ ID no. 4. All vector constructions involving DNA fragments comprising the barnase gene were carried out in E. coli strain MC1061 containing the plasmid R702::pMc5BS of Example 3. After a 1 hour DNA incubation with the explants, the cuvettes were transferred to an ice bath. After 10 minutes incubation on ice, the electroporation was carried out as described in Example 3. Immediately after electroporation, fresh liquid N6aph substrate was added to the explants in the cuvette, after which the explants were incubated for a further 10 minute period on ice.

Afterwards, the embryos from one electroporation experiment were transferred to Mh1 VII substrate supplemented with 0.2 M mannitol and 2 mg/l PPT. After approximately 14 days, the embryos were transferred to Mh1 VII substrate (Mh1 VII substrate of Example 3 but without proline and casein hydrolysate) supplemented with 2 mg/l PPT but without mannitol. After approximately 4 weeks, the embryos were subcultured for another month on Mh1 VII substrate supplemented with 10 mg/l PPT. The induced embryogenic tissue was carefully isolated and transferred to MS medium supplemented with 5 mg/l 6-benzylaminopurine. The embryogenic tissue was maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones and sucrose. Developing shoots were transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. These plantlets survived an in vitro spraying with doses of BASTA^R (Hoechst AG, Frankfurt am Main, Germany) corresponding to 2 l/ha. These plantlets were then

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transferred to soil and cultivated in the greenhouse, and two of the transformed plantlets, designated RZM35-1 and RZM35-18, were further characterized.

The embryos from a second electroporation experiment were transferred to Mh1 VII substrate supplemented with 2 mg/l PPT and 0.2 M mannitol. After about 14 days, the embryos were transferred to Mh1 VII substrate supplemented with 2 mg/l PPT but without mannitol. After approximately another three weeks, the embryos were transferred to Mh1 VII substrate supplemented with 10 mg/l PPT but without mannitol. After another three weeks, the induced embryogenic tissue was carefully isolated and transferred to MS medium supplemented with 2 mg/l PPT and 5 mg/l 6-benzylaminopurine. The embryogenic tissue was maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones, sucrose or PPT. Developing shoots were transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. The resulting plantlets were transferred to soil and cultivated in the greenhouse, and three of the transformed plantlets, designated RZM34-1, RZM34-12, and RZM34-14, were further characterized.

RZM34-1, RZM34-12, RZM34-14, RZM35-1, and RZM35-18 were grown in the greenhouse. Activity of the expression product of the bar gene in leaves of the plants was assayed as follows in a "PAT assay". 100 mg of leaf tissue from each plant, together with 50 mg of acid-treated sea sand (Merck, Darmstadt, Germany) and 5 mg polyvinylpolypyrrolidone (PVPP), were ground in an Eppendorf tube with a glass rod in 50 μ l of extraction buffer (25 mM Tris-HCL pH 7.5, 1 mM Na₂-EDTA (ethylenediaminetetraacetic acid disodium salt), 0.15 mg/ml phenylmethylsulfonylfluoride

(PMSF), 0.3 mg/ml dithiothreitol (DTT), and 0.3 mg/ml bovine serum albumin). The extract was centrifuged in a microfuge for 5 minutes at 16000 rpm. The supernatant was recovered and diluted ten times with TE 25/1 (25 mM Tris-HCL pH 7.5, 1 mM Na₂-EDTA. To twelve μ l of the diluted extract was then added: 1 μ l of 1 mM PPT in TE 25/1, 1 μ l of 2 mM AcetylCoenzyme A in TE 25/1, and 2 μ l of [14C]AcetylCoenzym A (60 mCi/mmol, 0.02 mCi/ml, [NEN Research Products, Dupont, Wilmington, Delaware, USA). The reaction mixture was incubated for 30 minutes at 37°C and spotted on a aluminium sheet silicagel* 60 t.l.c. plate with concentrating zone (Merck). Ascending chromatography was carried out in a 3 to 2 mixture of 1-propanol and NH₄OH (25% NH₃). 14C was visualized by overnight autoradiography (XAR-5 Kodak film). The tolerance to the herbicide BASTA[®] was tested by brushing a small area near the top of one leaf per plant with a 1% solution of the herbicide and observing the damage symptoms at and near the brushed sites. While RZM34-1, RZM35-1 and RZM35-18 showed no damage symptoms at all, RZM34-12 and RZM34-14 displayed slight browning and drying-out of the brushed site. RZM34-1, RZM34-12, RZM34-14, RZM35-1 and RZM35-18 were also shown to be male-sterile but otherwise phenotypically completely normal; female fertility, for instance, was normal. The spikelets of the male flowers were of about normal length but were very thin and appeared to be empty, and they never opened. A detailed analysis showed that the anthers were reduced to almost microscopic structures. This phenotype indicates not only that at least one copy of the barnase gene was expressed but also that it was selectively expressed in some or all of the tissues of the anthers.

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Southern analysis showed RZM35-1 and RZM35-18 to have an identical integration pattern, with only one copy of plasmid pVE108 being present in the genome of each plant. A small part of the plasmid DNA sequence adjacent to the HindIII site (used for linearization prior to electroporation) seemed to be absent in the integrated copy. Southern analysis of RZM34-1, RZM34-12 and RZM34-14 showed that each of these plants probably has two or three copies of part or all of pVE108 integrated into its genome. The copies are most likely not inserted in a concatemer configuration.

Transformants RZM35-1 and RZM34-1 were pollinated with pollen from an untransformed H99 plant, and progeny plantlets were recovered. From the 35 plantlets recovered from RZM35-1, 16 (46%) scored positive in a PAT assay, while 19 (54%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation of one active copy of the chimaeric bar gene ($X^2 = 0.26$).

From the 34 plantlets recovered from RZM34-1, 19 (56%) scored positive in a PAT assay, while 15 (44%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation, assuming that the transformed female parent had one active copy, or alternatively multiple active, but closely linked copies, of the chimaeric bar gene ($X^2 = 0.47$).

Example 6 : Production of restorer corn plants.

Zygotic embryos of corn inbred line H99 were isolated, enzymatically treated, washed and loaded in electroporation buffer as described in Example 5. Approximately 100 embryos in 200 μ l EPM-KCl were loaded in each electroporation cuvette. About 20 μ g

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of a plasmid DNA, pVE144 linearized with HindIII, was added per cuvette. pVE144 is a 6555 bp plasmid which was described in Example 2.

The embryos were electroporated, and the transformed cells were selected, grown into callus, and regenerated as described in Example 3. Transgenic plants were analyzed for the presence of the fertility-restorer gene and the marker gene by means of Southern hybridization and PCR. The expression of the fertility-restorer gene is assayed by means of Northern blotting, and the expression of the marker gene is determined by NPTII assay as described in Example 3.

Example 7 : Production of maintainer corn plants and a male-sterile corn line and maintenance of the male-sterile corn line

Maintainer plants of this invention of corn line H99 are obtained as outlined in Figure 1. A plant of corn inbred line H99 with the male-sterility genotype $H99^{S/s, r/r, P/p}$, transformed with the male-sterility gene of Example 5, is crossed with plants with the genotype $H99^{S/s, R/r, P/p}$, transformed with the fertility-restorer gene of Example 6. The progeny that have the genotype $H99^{S/s, R/r, P/p}$ are identified by PCR analysis for the presence of the S and R genes. These plants are selfed, yielding progeny with nine different genotypes. Two of these genotypes ($H99^{S/S, r/r}$ and $H99^{S/s, r/r}$) will develop into male-sterile plants, while all the other genotypes will develop into male-fertile plants. When these male-fertile plants are selfed, progeny analysis allows the identification of their genotype. Thus: a) the progeny of selfings of $H99^{S/S, R/R}$, $H99^{S/s, R/R}$, $H99^{s/s, R/R}$, $H99^{S/s, R/r}$ and $H99^{s/s, r/r}$ would all develop into male-fertile plants; b) selfings of $H99^{S/s, R/r}$ plants would

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produce progeny, of which 13 out of 16 would be male-fertile, and since the male-sterility gene is linked to the herbicide resistance gene, bar, 4 out of the 13 male-fertile plants would be sensitive to the herbicide BASTA^R ; and c) selfings of H99^{S/S,R/r} plants would produce progeny, of which 12 out 16 would be fertile (4 out of 16 would have the genotype H99^{S/S,R/R} and 8 out of 16 would have the genotype H99^{S/S,R/r}), all of which would be resistant to the herbicide, and the male-sterile progeny of which (4 out of 16) would all be homozygous for the male-sterility gene (H99^{S/S,r/r}).

The homozygous male-sterile progeny (H99^{S/S,r/r}) of selfing (c) are then crossed with their male-fertile siblings, and only when the cross is with plants with the genotype H99^{S/S,R/r} are the resulting plants 50% male-sterile (all with the genotype H99^{S/S,r/r}) and 50% male-fertile (all with the genotype H99^{S/S,R/r}). Indeed, the alternative cross between H99^{S/S,r/r} and H99^{S/S,R/R} would result in 100% male-fertile progeny plants.

Maintainer plants are selected by crossing the plant with the genotype H99^{S/s,R/r,P/p} with a plant that is heterozygous for the maintainer gene of Example 2, i.e., (H99^{S/s,r/r,P/p}), using the latter plant as the female parent. The offspring with the genotype H99^{S/s,r/r,P/p} are selected by means of testcrosses supplemented with PCR analysis of the progeny (which can be easily identified by PCR and Southern blotting for the presence of the S and P genes and the absence of the R gene). The selected fertile offspring are then selfed. One out of eight offspring have the desired genotype for a maintainer plant of this invention (H99^{S/S,P/p}) and can be further selected by means of testcrosses and PCR analysis of the

progeny. Indeed, only plants with this genotype will produce 50% male-sterile offspring (all $H99^{S/S,P/P}$) and 50% male-fertile offspring (all $H99^{S/s,P/p}$), thus growing at once both the desired homozygous male-sterile line and the maintainer line of this invention. Testcrosses also include the pollination of wild type H99 plants with pollen of the progeny plants obtained from the selfing of $H99^{S/s,P/p}$ plants.

Homozygous male-sterile plants with the genotype $H99^{S/S,r/r,P/p}$ are then pollinated by maintainer plants ($H99^{S/S,r/r,P/p}$) of this invention. All progeny have the genotype $H99^{S/S,r/r,P/p}$, so that the male-sterile line is maintained, as desired.

Example 8 : Introduction of the male-sterility gene and the maintainer gene in inbred corn lines through classical breeding.

The male-sterility gene of Example 5 and the maintainer gene of Example 2 are transferred from corn inbred line H99 to another corn inbred line (A) by repeated backcrossings as follows. The maintainer plant $H99^{S/S,P/P}$ is crossed as a female parent with an untransformed plant of line A ($A^{s/s,P/p}$). The offspring with the genotype $A-H99^{S/s,P/p}$ are selected by screening, using PCR, for the presence of both the maintainer gene (P) and the male-sterility gene (S). These plants are then crossed again as female parents with $A^{s/s,P/p}$ plants, and the offspring that are heterozygous for both the P and S genes are again selected by PCR. This process of backcrossing is repeated until finally plants with the genotype $A^{S/s,P/p}$ are obtained. These plants are then selfed, and the progeny are analyzed in the same way as

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described in Example 7. In this way, male-sterile plants with the genotype $A^{S/S,P/P}$ and maintainer plants of this invention with the genotype $A^{S/S,P/P}$ are obtained.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Maintenance of male-sterile plants

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

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(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/970,849
- (B) FILING DATE: 03-NOV-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2661 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Zea mays
- (B) STRAIN: inbred line W-22

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Hamilton et al.,
- (C) JOURNAL: Sex Plant Reprod.
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GTAGACCAGA	ATAATACGAA	GGTTGGTGAA	GCGCCGAAGG	TGTAAGCAGG	AAAGCTTCGG	300
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GCCAGCCCGC	GCAAGGACTG	CGACGAGGTC	CAGGCGCTCA	GGGACCGCGC	CGGCGTCCTG	1800
CTCACCAGGA	ACGTTGGCAT	CTCCGACAGC	CTGCGCCCCG	CCAACCCGCT	AGGCTACTTC	1860
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CATAACATGT GGGCCGGCTT GGCCCAGGCA CAAGCCCATC TACGCATGGC CTACGGTCCG      2160
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TACGATACAA CCCACAATTA CTTATGTGTG ATGGGCCGGC CAAAAAGCC TAAGATGTCTG      2280
TAGTGTGCTA GACCGACTCA TATATATAAA ACATTAAAC ATATTGTCTG GGACCATAAT      2340
TAGGGGTACC CTTAAGGCTC CTAATTCTCA GCTGGTAACC CTCATCAGCG TAAAGCTGCA      2400
AAGGCCTGAT GGGTGCGATT AAGTCAGGGA TCAGTCCATT CGAGGGACTC GATCAGGCCT      2460
CGCCCGAGCC TAGCCTCGGA CAAGGGCAGC CGACCCCGGA GGATCTCCGT CTCGCCCGAG      2520
GCCCTCCTCC AGCGGCGAAC ATATTTCCGG CTCGCCCGAG GCCCTGTCTT CGCCAAGAAG      2580
CAACCCTGAC CAAATCGCCG CACCGACCGA CCAAATCGCA GGAGCATTTA ATGCAAAGGT      2640
GGCCTGACAC ATTTATCCTG A                                              2661

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6555 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: plasmid pVE144 (replicable in E.coli)

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..396
- (D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (397..751)
- (D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the
polyadenylation site derived from Agrobacterium
T-DNA nopaline synthase gene"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (752..1024)
- (D) OTHER INFORMATION: /label= barstar

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/note= "coding region of the barstar gene of
Bacillus amyloliquefaciens"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1025..1607)
- (D) OTHER INFORMATION: /label= TA29
/note= "promoter derived from the TA29 gene of
Nicotiana tabacum"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1608..2440
- (D) OTHER INFORMATION: /label= 35S3
/note= "35S3 promoter sequence derived from
cauliflower mosaic virus isolate CabbB-JI"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 2441..3256
- (D) OTHER INFORMATION: /label= neo
/note= "coding region of the neomycine
phosphotransferase gene of Tn5"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 3257..4315
- (D) OTHER INFORMATION: /label= 3'ocs
/note= "3' regulatory sequence containing the
polyadenylation site derived from Agrobacterium
T-DNA octopine synthase gene"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 4316..6555
- (D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC	180
ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC	240
ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT	300
TACGCCAGCT GGC GAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT	360
TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT CGAGCTCGGT ACCCGGGGAT	420
CTTCCCGATC TAGTAACATA GATGACACCG CGCGCGATAA TTTATCCTAG TTTGCGCGCT	480
ATATTTTGTT TTCTATCGCG TATTAAATGT ATAATTGCGG GACTCTAATC ATAAAAACCC	540
ATCTCATAAA TAACGTCATG CATTACATGT TAATTATTAC ATGCTTAACG TAATTCAACA	600
GAAATTATAT GATAATCATC GCAAGACCGG CAACAGGATT CAATCTTAAG AAACCTTTATT	660

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GCCAAATGTT	TGAACGATCT	GCTTCGGATC	CTCTAGACCA	AGCTAGCTTG	CGGGTTTGTG	720
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CTTCCGCTTT	CGCTTCACGG	AAAACCTGAA	GCACACTCTC	GGCGCCATTT	TCAGTCAGCT	840
GCTTGCTTTG	TTCAAACCTGC	CTCCATTCCA	AAACGAGCGG	GTACTCCACC	CATCCGGTCA	900
GACAATCCCA	TAAAGCGTCC	AGGTTTTTCAC	CGTAGTATTC	CGGAAGGGCA	AGCTCCTTTT	960
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TCATCGGTAG	CTAATTTCTT	TAAGTAAAAA	CTTTGATTTG	AGTGATGATG	TTGTACTGTT	1080
ACACTTGCAC	CACAAGGGCA	TATATAGAGC	ACAAGACATA	CACAACAAC	TGCAAAACTA	1140
ACTTTTGTTG	GAGCATTTTCG	AGGAAAATGG	GGAGTAGCAG	GCTAATCTGA	GGGTAACATT	1200
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GTTTATTCTA	GTCCAGCCAC	CCACCTTATG	CAAGTCTGCT	TTTAGCTTGA	TTCAAAAAC	1380
GATTTAATTT	ACATTGCTAA	ATGTGCATAC	TTCGAGCCTA	TGTCGCTTTA	ATTCGAGTAG	1440
GATGTATATA	TTAGTACATA	AAAAATCATG	TTTGAATCAT	CTTTCATAAA	GTGACAAGTC	1500
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AAGTGGATTG	ATGTGACATC	TCCACTGACG	TAAGGGATGA	CGCACAATCC	CACTATCCTT	2340
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ACCAGTCTCT	CTCTATAAAT	CTATCTCTCT	CTCTATAACC	ATGGATCCGG	CCAAGCTAGC	2460
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CACGCTCACC	GGCTCCAGAT	TTATCAGCAA	TAAACCAGCC	AGCCGGAAGG	GCCGAGCGCA	5700
GAAGTGGTCC	TGCAACTTTA	TCCGCCTCCA	TCCAGTCTAT	TAATTGTTGC	CGGGAAGCTA	5760
GAGTAAGTAG	TTCGCCAGTT	AATAGTTTGC	GCAACGTTGT	TGCCATTGCT	ACAGGCATCG	5820
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CTCTTACTGT	CATGCCATCC	GTAAGATGCT	TTTCTGTGAC	TGGTGAGTAC	TCAACCAAGT	6060
CATTCTGAGA	ATAGTGATG	CGGCGACCGA	GTTGCTCTTG	CCCGGCGTCA	ATACGGGATA	6120
ATACCGCGCC	ACATAGCAGA	ACTTTAAAAG	TGCTCATCAT	TGGAAAACGT	TCTTCGGGGC	6180
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CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA 6300
 GGCAAAATGC CGCAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT 6360
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5620 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: plasmid pVE108 (replicable in E.coli)

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..395
- (D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (396..802)
- (D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the polyadenylation site derived from the nopaline synthase gene from Agrobacterium T-DNA"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (803..1138)
- (D) OTHER INFORMATION: /label= barnase
/note= "coding region of the barnase gene of Bacillus amyloliquefaciens"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: complement (1139..1683)
- (D) OTHER INFORMATION: /label= TA29
/note= "sequence derived from tapetum specific promoter of Nicotiana tabacum"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1684..2516
- (D) OTHER INFORMATION: /label= 35S3

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/note= "35S3" promoter sequence derived from
cauliflower mosaic virus isolate CabbB-JI"

(ix) FEATURE:

- (A) NAME/KEY: -
(B) LOCATION: 2517..3068
(D) OTHER INFORMATION: /label= bar
/note= "coding sequence of phosphinotricin
acetyltransferase gene"

(ix) FEATURE:

- (A) NAME/KEY: -
(B) LOCATION: 3069..3356
(D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the
polyadenylation site derived from Agrobacterium
T-DNA nopaline synthase gene"

(ix) FEATURE:

- (A) NAME/KEY: -
(B) LOCATION: 3357..5620
(D) OTHER INFORMATION: /label= pUC18
/note= "pUC18 derived sequence"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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TATAGTTAAT ATCCGCTTCA CGCCATGTTC GTCCGCTTTT GCCCGGGAGT TTGCCTTCCC	960
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TATGATATGT	CTGAAGATAA	TCCGCAACCC	CGTCAAACGT	GTTGATAACC	GGTACCATGG	1140
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CACCACAAGG	GCATATATAG	AGCACAAAGAC	ATACACAACA	ACTTGCAAAA	CTAACTTTTG	1260
TTGGAGCATT	TCGAGGAAAA	TGGGGAGTAG	CAGGCTAATC	TGAGGGTAAC	ATTAAGGTTT	1320
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CAATCCCCT	ATCCTTCGCA	AGACCCTTCC	TCTATATAAG	GAAGTTCATT	TCATTTGGAG	2460
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TGGGGTGCCT	AATGAGTGAG	CTAACTCACA	TTAATTGCGT	TGCGCTCACT	GCCCGCTTTC	3540
CAGTCGGGAA	ACCTGTCGTG	CCAGCTGCAT	TAATGAATCG	GCCAACGCGC	GGGGAGAGGC	3600
GGTTTGCGTA	TTGGGCGCTC	TTCCGCTTCC	TCGCTCACTG	ACTCGCTGCG	CTCGGTCGTT	3660
CGGCTGCGGC	GAGCGGTATC	AGCTCACTCA	AAGGCGGTAA	TACGGTTATC	CACAGAATCA	3720
GGGGATAACG	CAGGAAAGAA	CATGTGAGCA	AAAGGCCAGC	AAAAGGCCAG	GAACCGTAAA	3780
AAGGCCGCGT	TGCTGGCGTT	TTTCCATAGG	CTCCGCCCCC	CTGACGAGCA	TCACAAAAT	3840
CGACGCTCAA	GTCAGAGGTG	GCGAAACCCG	ACAGGACTAT	AAAGATACCA	GGCGTTTCCC	3900
CCTGGAAGCT	CCCTCGTGCG	CTCTCCTGTT	CCGACCCTGC	CGCTTACCGG	ATACCTGTCC	3960
GCCTTTCTCC	CTTCGGGAAG	CGTGGCGCTT	TCTCAATGCT	CACGCTGTAG	GTATCTCAGT	4020
TCGGTGTAGG	TCGTTCGCTC	CAAGCTGGGC	TGTGTGCACG	AACCCCCCGT	TCAGCCCGAC	4080
CGCTGCGCCT	TATCCGGTAA	CTATCGTCTT	GAGTCCAACC	CGGTAAGACA	CGACTTATCG	4140
CCACTGGCAG	CAGCCACTGG	TAACAGGATT	AGCAGAGCGA	GGTATGTAGG	CGGTGCTACA	4200
GAGTTCTTGA	AGTGGTGGCC	TAACACGGC	TACACTAGAA	GGACAGTATT	TGGTATCTGC	4260
GCTCTGCTGA	AGCCAGTTAC	CTTCGGAAAA	AGAGTTGGTA	GCTCTTGATC	CGGCAAACAA	4320
ACCACCGCTG	GTAGCGGTGG	TTTTTTTGTT	TGCAAGCAGC	AGATTACGCG	CAGAAAAAAA	4380
GGATCTCAAG	AAGATCCTTT	GATCTTTTCT	ACGGGGTCTG	ACGCTCAGTG	GAACGAAAAC	4440
TCACGTTAAG	GGATTTTGGT	CATGAGATTA	TCAAAAAGGA	TCTTCACCTA	GATCCTTTTA	4500
AATTAAAAAT	GAAGTTTTAA	ATCAATCTAA	AGTATATATG	AGTAAACTTG	GTCTGACAGT	4560
TACCAATGCT	TAATCAGTGA	GGCACCTATC	TCAGCGATCT	GTCTATTTTCG	TTCATCCATA	4620
GTTGCCTGAC	TCCCGTCGT	GTAGATAACT	ACGATACGGG	AGGGCTTACC	ATCTGGCCCC	4680
AGTGCTGCAA	TGATACCGCG	AGACCCACGC	TCACCGGCTC	CAGATTTATC	AGCAATAAAC	4740
CAGCCAGCCG	GAAGGGCCGA	GCGCAGAAGT	GGTCCTGCAA	CTTTATCCGC	CTCCATCCAG	4800

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TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC	4860
GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC	4920
AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG	4980
GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC	5040
ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT	5100
GTGACTGGTG AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC	5160
TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC	5220
ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC	5280
AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC	5340
GTTTCTGGGT GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA	5400
CGGAAATGTT GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT	5460
TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT	5520
CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA	5580
TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC	5620

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: oligonucleotide MDB80

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /label= MDB80
/note= "oligonucleotide designated as MDB80"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCGCTTGTC A GTGAATGTTG C

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide MDB81

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..21

(D) OTHER INFORMATION: /label= MDB81

/note= "oligonucleotide designated as MDB81"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCGAGGCCAT GGTGCGCC G

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide MDB82

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..21

(D) OTHER INFORMATION: /label= MDB82

/note= "oligonucleotide designated as MDB82"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGCATAGGC ATAGGATGAC G

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3627 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Oryza sativa*

(B) STRAIN: Akihikari

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..2845

(D) OTHER INFORMATION: /label= PT72

/note= "sequence comprising anther specific promoter PT72"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 2733..2739

(D) OTHER INFORMATION: /label= TATA

/note= "TATA Box"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 2765

(D) OTHER INFORMATION: /note= "transcription initiation determined by primer extension"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 2846..2848

(D) OTHER INFORMATION: /label= ATG

/note= "ATG start of translation of rice T72 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACAATACAT CAAGTAAATC AAACATTACA AATCAGAACC TGTCTAAGAA TCCATCTTAA	60
TTCAGAAAAA AACTCAGATT AGATGTTTCAT GCTTCCACCA GAAGCAGGAA TGTGCAACCT	120
ACACTTCCTG TAATTTCCAT ACTACAATGT CCCCCTGAC CACTGTGCCT GATGCTCTAT	180
TAGAATACCA CATCCTCCAT GGCTCCATGT AAATGCATAT AAATTTGACT CTTTAAATTA	240
GTAAC TACAA TTTAAAATTT ATCGAACATT GTTCAAATTT ATAAACAGTT TCCCCAAATT	300
TAGATGCTCC CAAATGTACA CAGCTACTAG TAAAGCACCA TCCAGTTTCA CCTGAACAGG	360
ACTGACATAA ATGTGTGAAA AGGGGACGTC ATTCCCCCAA ATACAACTGA ACAATCCTCC	420
ATCAGAACAT TCATTTGATT GACATTACTC GGAGAGATAC AGCTCGCAGG CACACGAGAT	480
TCTTCTGCCT TTCCAATTGC CACGAACCCA CATGTCACAC GACCAACCAA AAAGAGAGAA	540
TTTTTCTTTG CACAAACAAA AAGTGAGATT TTTTTTTCGC CACAAAGGTG CGAACTTTCT	600
TCTCTCTCCC ACTTTCCAAT CAAGAAACGA AGCACTCAAA CCAAGAACAA ACCAAGGAAG	660
GAGAGATCGC TCCCTCTCCC AGAGCAAACG AAAGGAGAGA ACTCAGATGG ATGCGAACTA	720
CTACCTTGCC TCTTTCCCCG GAGAAGCAGC GAAGGAGAAG AGCGCGATGC CGCCGCCGCC	780
GCCGCCTCCG GCAACCTCCG GCTCCGGCGA GTCCGCCTCC TCCTCCTCTC TCACCTCTCT	840

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CTTCCCAACC	GTGTGGTGTT	CGAGAAGCTT	TTATGCGAGC	GACGTGCAGT	GGAAGCGGTT	900
GCTCCCAAGT	CAAAC TGATG	GAGACCACCT	ACTATCTTCC	TCTTGTTTTT	TTCTGCTTTT	960
CTTTTCTTTA	TCTTTTTTCT	TTCATTTTAT	TTTGAGCGAT	GAAC TTGAGA	ACAGTTTGGT	1020
TGTGGGT TAA	ATTAAACGGT	GCAGAATTGC	AAAGCTACGT	CCTTTTCGTC	TGATTAAGGT	1080
GGTATCAGAA	TCCTAATCTG	TTAGCTCAGC	ATTTGTTTTT	GTGTGTTTAA	TTGGCCATGA	1140
CATCAGATGG	TTCAGACCGG	TGGCAGGTCT	TCATCGGAGA	GGAGAATGAG	AGCAATGCAA	1200
GTTGCAAACA	ACAAACAGGT	CCTTCCAAAC	GGGTTGGTTT	CATTCCACAG	AACAGGATAG	1260
CAACCAGAGC	ACAAACCGTT	CAACAATATA	TATATATATA	TATATATATA	TATATATATA	1320
TATATATATA	TATATATATG	ATTTAAAATT	ATATTACTAT	TTTTAGGATA	CGGAACTCTT	1380
AACACATGAA	AATCTAAACA	TTTTCAACCA	ATCAGAACTA	CTAGAAAGAT	AATCTAACTA	1440
CTTCAAAATT	TAAAATTTGA	CAAATAAAAT	AACTAGTTTT	TTCTAAAGCT	ATCTTCACTG	1500
GACAACTTAT	GAATATTTAT	ATTTATGAAG	CGAGTACTCT	CCTAGTACAT	ATTACATATA	1560
TATTCTTCTT	CTCATGAAAA	ATTAAC TTCT	CGCTATAAAT	CCGAACATAT	ATTATGCGTA	1620
GCAAGTTGTT	TTTTTTAACG	GGTGGAGTAA	TATTAGAGTA	TTTAAATTCC	TTCAAATTGC	1680
CATCCCTCTG	GGACTTTGCT	GCTGTTGTTC	TTCCACGGTT	GCTGTCAGTG	TCACCCAGAT	1740
TTGCATCCTT	TCCAGCTCGT	AGCTACTGTT	CTGCATGTAT	TGGACTTGGA	TTAAGATCAA	1800
ATGCAGTTGC	TATTGTA ACT	GCACAATAGC	AACTGCACAC	AATCATGTCC	ATTCGTTTTT	1860
AGATCCAACG	GCTCTAGATG	ACTGCTACAG	TACATGCATA	ATAGTACATC	TCTGCTACAG	1920
TGTTTTTTGCT	GCAGTACCAC	TTCATATCCT	GGCCTTCCGT	TCTAGATCAT	GTGATGTACA	1980
TGTTTTTTT TG	AAACAACCCG	CACAAGACAT	TGATAGAGTA	GGAAATGTGA	TGTACATGTT	2040
AACGGCTTAA	GTTACAGTTA	CAATAACAAC	TGCACAGGAT	CTTGATCCAT	TGGACTTGTA	2100
TAATATCTCA	TCTCGTCGTT	CCATTATCGT	GGTAACAGTT	GGCAACTTGG	CATCCAGTGC	2160
TGGAAACTAT	GCCGTGTGTA	CATCAGGATC	GTCCTTTTTG	TTCAGTTCCA	AGATAGAACA	2220
AGTCCAAAAG	ATGGCCGTAG	TTTTTTTAGT	CACAGTGGAA	GCTGACATAG	CCGTGGAATA	2280
AGTTCTGCAC	AAAAGTTGCC	ATTCGAGATC	AACTACTGGT	AGTAGTAGTC	ATCTTCTACC	2340
ACTGCGAATA	TTCGAAGGGA	CACAAAAAGA	TCAACGAGTA	AATTAGTTCA	CCGGAAGACG	2400
ACACATTATC	ACCACAAAAA	GACTAAAAAC	AAAAAGAAAT	TGCCAGGCCA	AAAAAGGCAA	2460
AAAAGAAAAA	AAAAGATGGC	ACGAGGCCCA	GGGCTACGGC	CCATCTTGTC	GCCGGCCCAA	2520
CCGCGCGCGC	GAAACGCTCT	CGTCGGCTCT	CGGCTCGCCG	CGACGCGATG	GAGAGTTCGC	2580
GCCGCGGCGC	GCGCGCGCGT	TCGGTGGCTC	ACACGCTTGC	GCCCTCGTCC	TCCCGGCCGG	2640
CGCGGGCGCC	GACCGCGCGT	CCGCCGCATG	CGCGCGGCGT	AGGTGAGCAA	CGCGGGCCTC	2700

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GCCGCGCGCG CTCCCCTCCT TCGATCCCCT CCTATAAATC GAGCTCGCGT CGCGTATCGC 2760
CACCACCACC ACGACACACA CGCACGCACC GTGCAGGCAT CGACGACGAG CGAGAGCCCC 2820
TCGGCGGCAG AAGACACTCA CGGCGATGGC GGTGACGAGG ACGGCGCTGC TGGTGGTGTT 2880
GGTAGCGGGG GCGATGACGA TGACGATGCG CGGGGCGGAG GCGCAGCAGC CGAGCTGCGC 2940
GGCGCAGCTC ACGCAGCTGG CGCCGTGCGC GCGAGTCGGC GTGGCGCCGG CGCCGGGGCA 3000
GCCGCTGCCG GCGCCCCCGG CGGAGTGCTG CTCGGCGCTG GGCGCCGTGT CGCACGACTG 3060
CGCCTGCGGC ACGCTCGACA TCATCAACAG CCTCCCCGCC AAGTGCGGCC TCCCGCGCGT 3120
CACCTGCCGT AAGAAAACGA ATAAAATCGA TTTGCTATCT ATCGATGATT GTGTTTTTGT 3180
AGACTAAACT AAACCCCTAT TAATAATCAA CTAACCGATG AACTGATCGT TGCAGAGTGA 3240
TGGAGATGGT GTGCCAAGGT AATTGCGTTT GCTCGTGCGA GGATGAGAAG AGAAGATTGA 3300
ATAAGATGTT TGATGGCAAC AAGTCATCAG GCGATCCGAT CCCTGCAGCT ATGAATGGGA 3360
GTATACGTAG TAGTGGTCTC GTTAGCATCT GTGTGTCGCA TATGCACGCC GTGCGTGCCG 3420
TGTCTGTCCT GCTTGCTCTG CTGATCGTTC AATGAACGAC AAATTAATCT AACTCTGGAG 3480
TGACAAGTCG TTCGAGATAT ACTAATACTA CCATGTGCAG GGTCTTTCAA CCAAGGTTCA 3540
TGTTTTCCAC GAAAGCCGAT TGAAACGAAA CCGCGAAATT TTGATGCGAG ATGAAAGCAG 3600
ATTCCGAGTG AAATTTTAAA TGGTTTTT 3627

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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2370 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Oryza sativa*
- (B) STRAIN: Akihikari

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..1808
- (D) OTHER INFORMATION: /label= PT42
/note= "sequence comprising anther specific promoter PT42"

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1748..1755
- (D) OTHER INFORMATION: /label= TATA

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/note= "TATA Box"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1780

(D) OTHER INFORMATION: /note= "transcription initiation site determined by primer extension"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1809

(D) OTHER INFORMATION: /label= ATG

/note= "ATG start of translation of rice T42 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGCCATCACT GTCGGGTGCT GCGCCATGGA CATCACCGTC TCCTTCCTGC GCCGCCGTGC	60
CCGGTGAGCT CCAAGGCCGA AGCCTTCTTC CCCTCAGCC ACTACCTCTC TCTTCCCCAA	120
TTCCGGCCAA CGCCGTCCGT TGCCACAGCG CCACCTCCAC GCCATCCCAG AGCCCCGTGC	180
CGTGCCACCG GGTTCGCCTC CATCTCCTCT TGCCAACGCC GACGCTCGTC GCGGCAGCCA	240
TGCGCTGTCA CCGATGAACA CCGCCGCGCC ACAGCCATGG CAGAGCACGG CCAGGGAGCC	300
ATGGCTGCTC TGCTCCTCC TCCTTCTCTC ACATCTGGTT GCAGCCGGAC CTAGTCGGCT	360
TATACAAATG GCCCATGGGC AAAATTGTCT TTTATGAAAG TTTCTCTCAC CGTTTCAGTC	420
GGAAATAATA AAATAATGGG AGGATTGTCC GCCAGCAAAT TACCATATTT TTTCGGTGTC	480
CAAGAGCAAA TACACGATCT TCGGGTGTTT CACAGCAAAG ACCACAATTT CTAAGTGTCC	540
TGTAACAAAT TTGCCAATA AAAATTTAAA ACCAAAGGAG AAGACTGTAC ATGAAGAAAA	600
ACAAAGAGAA TGAAATTACA TAAGCTCAGG GGTATAAAG TTGATTTATT TTTAGGATGA	660
AGGAAGTGTG TGAAAACAAT GGCCAATTGG GTGTCGGAAA ATATAACGTG CTTGCTAAAA	720
TGTCGTCCCC ATATCCTGTA GCTGATTATA GATAGACCCT GATGGTCAAG ATGCCCTGTA	780
CTGGATCGTG TTTCCATGCT TCATCTCCGC TTCTCTCAAG TACTCCCCGA ACTCACATAT	840
CTGGTGGGCT GGATCCACAG TAAGAAACAG TCAAACAACA CTCACTTCAT AGATAACCAA	900
TTGTTTAATT ATTCTTAGTC CCTTATCTTA TACTCCTAGT AAGTGCTTAA AACTTGCTA	960
TAAATATCAA ATTTATCGTA CAATTACAAT ATAATTATAA CGTATACCAT GTAATTTTAA	1020
AAACTATTTT TAGATAAAAA AAATATGGTG ATGAGCAGCC GCAGCAGCGG ACGCCGAACC	1080
ACCTGCCGAA CATCACCAAG ATAGCGAGTC CTAAAAATTT TTAGTGTTTCG TTTGCTGGGT	1140
TGGTAACTAA TTAATAAAAA AGAGCGACTC ATTAGCTCAT AAATAATTAC GTATTAGCTA	1200
ATTTTTTTAA AAAATAAATT AATATAACTT ATAAAGCAGC TTTTGTATAA TTTTTTTTTT	1260
AAAAAAGTGT TGTTTAGCAG TTTTGGAAG TGTGCCGAGG GAAAACGATG AGATGGGTG	1320
GGGAAGGAGG GGGAAGAAGT GAAGAACACA GCAAATATAG GCAGCATCGT CCCGTACAGA	1380

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TCAGGCTGCA ACCACGCCCC GCGGAGATAG TTAACGCGGC CCACGTTGTG CTATAGCCCCG	1440
TCACTCTCGC GGGCCTCTCC AACCTCCAGT TTTTTTTCTA GCCCATCAGC TGATACGGGG	1500
CCTTCCCCCC ATGCAGGAGG ATGGCCCCGCC ACGCGGTGTT TTGGGCGGTT CTCGCCGCGC	1560
GCGCCCGTGC CGATCCGGGA CTCATCCCAC GTGCCGCCTC GCCACCGCCG CCGCCGCCGC	1620
TGCTGCTCCG GCTGCCGGCT GGACCTTCAC GCTCACGCGC TCTCCCCTGC CCAACCACCA	1680
CGCAAACAAA CACGAAGTTC GCGCCGTCGA CCGGCTCCCC TCCTCCCCCG CGCGCATCGG	1740
ATCCCCCTAC ATAAACCCTC TCGCTCGCCA TCGCCATGGC AGCAACTCCC CTCCTCCACT	1800
AGACCACCAT GCACAGATCG ATGGCCTCTC AGGCGGTGGC GCCCTCCTC CTCATCCTCA	1860
TGCTCGCGGC GCGGCGGGG GCGCGTCGG CCGCGGTGCA GTGCGGGCAG GTGATGCAGC	1920
TGATGGCGCC GTGCATGCCG TACCTCGCCG GCGCCCCCGG GATGACGCCC TACGGCATCT	1980
GCTGCGACAG CCTCGGCGTG CTCAACCGGA TGGCCCCGGC CCCC GCCGAC CGCGTCGCCG	2040
TCTGCAACTG CGTCAAGGAC GCCGCCGCCG GCTTCCCCGC CGTCGACTTC TCCCGCGCCT	2100
CCGCCCTCCC CGCCGCCTGC GGCTCTCCA TCAGCTTCAC CATCGCCCC AACATGGACT	2160
GCAACCAGTA AGTTCATTCA TTCTTTCTTA ACTCCAATTC AATTTATCCA TCACCTCGAC	2220
TTAAGCCTGA TTAACTTAA CTTGTTCTTT GCATGCTTGC ACTATTGCAG GGTACAGAG	2280
GAAGTGAAG TCTGAGAGCG TGAGGAATCG AGTTCATGTT GCATTTATCA TCAATCATCA	2340
TCGACTAGAT CAATAAATCG AGCAAAGCTT	2370

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2407 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Oryza sativa*
- (B) STRAIN: Akihikari

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..2263
- (D) OTHER INFORMATION: /label= PE1
/note= "sequence comprising anther specific promoter PE1"

(ix) FEATURE:

- (A) NAME/KEY: -

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(B) LOCATION: 2181..2187
 (D) OTHER INFORMATION: /label= TATA
 /note= "TATA Box"

(ix) FEATURE:

(A) NAME/KEY: -
 (B) LOCATION: 2211
 (D) OTHER INFORMATION: /note= "transcription initiation
 site determined by primer extension"

(ix) FEATURE:

(A) NAME/KEY: -
 (B) LOCATION: 2264..2266
 (D) OTHER INFORMATION: /label= ATG
 /note= "ATG start of translation of E1 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGATAGTGAC ATACTCACAT GCTTTGTCAA TTCAAGTATC AGTTCCTTTC ATATTGATTT	60
CTTAGTTGAT GAAAGTATAC ATATTTCTTG CCATCAATTC TTTTAGTAGG TACATTTGGA	120
CACTAGTGGT CAGGGTTGAA CTCTTAACTG GAGTCTCATC TGATTTGCTT ATCTGAGACT	180
GGGTTTGTGC AAATCCTGTC ATGAGGCAAG GTGGACTGTC AGTCCATGAC ACTTTGCTAC	240
TTCTATTAAG TTCTCGAAAT CTTTTCAGT GTATGTCCGT TCTCTTTC AA ATGAATTATT	300
TATATGTTCT GACAGCCTCG CGGTGTACAT TTCATTTAAC TTTTGTCTTC ACAGGGCCTC	360
TTGGTATTTT GTTGAGCAGA TTGGAATCAA CCTTCTTGTA GAACTTCTTG ATGTCGTCGC	420
TACCCTTTGC AACTAGATGG TCAACTTCTG TCTTATATCT TTGGTACAAC ACTGGCAAAG	480
TGTGCGCGCA CAAGAATCCT GTGAAGTAAG AAATACAAAC TTGTCATTGT GAAAGTTTAG	540
CTTTATATGA TCTTGACTCT AAATTGTTTC TCCTCAGATC CTTCTGTGTG ATTGTTTTAT	600
TAAAATTTAA TATTTATCTG GAATACCTAC CAATATATAG TAGACTTGTC AAGCTGCAAG	660
AACTTCCAAT CGCCGACAAT ACCAATAGAG ATCCAACCAC CTTAATATCA TAAACAATCT	720
GATTGTTAGT CCAGAACTAT ATTGAGTAGT GAACAACAAT AGCACATTAA CATTATGAGG	780
ATTATTGGCT AACTCTGCAA TTCAATATTC TGATGCGTCT AATCTGGTCA ATTTTAGCGC	840
TCCAGAAAGA ATTGCACAAT CCTTGGACAA TGTTGGCACT GGAAGTGTG CATGTTTTTA	900
CATCTCTTAT TAACGTAGCA AAGGAGTAGA TTATTATGTA CCAGGAGAAA TCTCTTCAGA	960
TCCTTTCCAC ATGCAATGTC GTAAAGAACA GATACAGTGT ACGTTAGTTT GTAATGGACG	1020
GTCAATGCCA TTTCTCTGAA GGCATGTTCA GAGATGATGA TTTCTGGGAT CCTTGGAGGG	1080
GCCCTGAAAT TCGGAAACAG TTAGTTGAGT TTAGTACCT AATGTCTTGC GTTATACTAC	1140
GTGAAATGCC ATTTCTGTAA GCTGAGTTTT CTACCATCTC CACAGGAAAT AAAGCTAATA	1200
CCTGTCCAAG AGTGGTGCGG CATTGACCA AATGAAGATC ACAAGCATGG CAAGAATGGC	1260
AATCTGGCAA AGGAGCGGAA TTATATTGTA TTCTACTACA TCGAACAGGA ACCATATCAA	1320

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TGTTGCCCCA GCAAGGACCC CCGCAGATAA GTTCCTGTTC TTCCACAGCA GAATATCCGC	1380
AACTGCATAG CTCCCAACAA TGAAATCCAA AACCACATCG GCTCAGAGAG AAGTTATGAT	1440
AAAAGGCACT AATTCTGAAT AATTCCTAG AAAGCGAATA ATAATAGCAC ACCTTGACCT	1500
CCACCAAGAA GCTTGTGGAT CGACTTGTGC CCATGAAATG GCATTCTGAC ATTCTGGTCA	1560
CTGTCAGAAT CTCTCGGAAA ATGAGGAGGC ATAGCTTCGT GTGTGTATGT GTGTGGGATA	1620
TTACGCTGCT AAAACTTTGT GTTCTGATC GATCTGGTTA GAGAGCATCG TCTTTATAAG	1680
CACTTAAAAA TGGTAGTATA ATCTCTCAAG GAGCCTATAC TGCCAAGGAA AGGATAGCTT	1740
GGCCTGTGGG GATTGAGCCG TTGAAGGGAA CAAACGAATA CAGTTACCTT ACCAGATGTT	1800
TGCCACGACA TGGGCAACGT CATTGCTAGA CCAAGAAGGC AAGAAGCAAA GTTTAGCTGT	1860
CAAAAAGAT ATGCTAGAGG CTTTCCAGAA TATGTTCTAT CTCAGCCAGA CCAATGGGGG	1920
CAAAATTTAC TACTATTTGC CATACTTAA CCACGTAAAA GTCCTACACT CAACCTAACT	1980
GTTGAACGGT CCGTGTCTGG CCAACGGTGA GAATGCACCT AATGGACGGG ACAACACTTC	2040
TTTCACCGTG CTACTGCTAC ATCCTGTAGA CGGTGGACGC GTGAGGTGCT TTCGCCATGA	2100
CCGTCCTTGG TTGTTGCAGT CACTTGCGCA CGCTTGCACC GTGACTCACC TGCCACATTG	2160
CCCCCGCCGT CGCCGGCGCC TACAAAAGCC ACACACGCAC GCCGGCCACG ATAACCCATC	2220
CTAGCATCCC GGTGTCCAGC AAGAGATCCA TCAAGCCGTC GCGATGACGA CGAGGCCTTC	2280
TGTTTTTTCC ACCGTTGTCG CGGCGATCGC CATCGCCGCG CTGCTGAGCA GCCTCCTCCT	2340
CCTGCAGGCT ACCCCGGCCG CGGCCAGCGC GAGGGCCTCG AAGAAGGCTT CGTGCGACCT	2400
GATGCAG	2407

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2784 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Zea mays

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..1179
- (D) OTHER INFORMATION: /label= PCA55
/note= "region comprising the anther specific promoter and the leader sequence, PCA55"

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(ix) FEATURE:

- (A) NAME/KEY: -
 (B) LOCATION: 1072
 (D) OTHER INFORMATION: /label= TATA
 /note= "TATA Box"

(ix) FEATURE:

- (A) NAME/KEY: -
 (B) LOCATION: 1180..1596
 (D) OTHER INFORMATION: /note= "presumed coding sequence of
 corn CA55 gene"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGTATGCAT CAATAGAGCC GGAAGATGGT CTGGAGTAAG GACCTGGCAG TGTGATACGG	60
GAACCTTGACA TCTGAATAGA TATTCTCCCT TGTCCCTCTG GTAAAAAAA CTGTTGTCAC	120
ATTTGCCTTC GCTGTGACTT GGATGTATCA TGTATATCTT TGACCATTGA TATCTTGGTT	180
AATCAGACGG TGCATTACAA TCATGGCCTC ATTCATATAG GGTTTAGGGT TACCACGATT	240
GGTTTGCATA AGTAGTACCC CTCCGTTTCA AATTATGTCG TATTTTGATT TTTTAGATAC	300
ACTTTTATA TAATTTTATA TTTTAAATTA GGTGTTTTAT ATAATACGTA TCTAAGTGTA	360
TAATAAAATA TATGTATCTA AAAGCTGTAA TTTAGTATAA ATTAGAATGG TGTATATCTT	420
CAATGTATGA CAAATAATTT GAAATGGAGG AGGGTATGAA AAGCCAAAAC CTCCTAGAAT	480
ATGGAATGGA GGGAATACAT ACAAATTCTT TGCTTCAGTT AAAAGAAACG AGAAAAGGAG	540
GGGAATGGGG AATCGTACTT CAGTTTTTAC GAGTTTTTCAT CAAACATGTA TGCACGTCTT	600
CCCTTG GTTG ATGCATCTTT TTGGCAAATC TTCGTTTAAT TGCGGCTTCT TTTTATAACC	660
GTTCGAAGGT TTTCGTCGTC AATGCTGAAA CTCCACTTTC ACCACCTTCG GTTGCATCTG	720
CTTGCTTTCA ATTCACCTCT AATTAGTCCA AGTGTTTCAT TGGACGAAGG TCCAAGTCCT	780
TCAGATCATC TCAATTTTCT TTGATCTGAA ACAACAATTT AAAACTGATT TTGTTACCTT	840
GACCTGTCGA AGACCTTCGA ACGAACGGTA CTGTAAAAAT ACTGTACCTC AGATTTGTGA	900
TTTCAATTCTG ATTCGGGTCT CCTGGCTGGA TGAAACCAAT GCGAGAGAAG AAGAAAAAAT	960
GTTGCATTAC GCTCACTCGA TCGGTTACGA GCACGTAGTT GGCGCCTGTC ACCCAACCAA	1020
ACCAGTAGTT GAGGCACGCC CTGTTTGCTC ACGATCACGA ACGTACAGCA CTATAAAACA	1080
CGCAGGGACT GGAAAGCGAG ATTTACAGC TCAAAGCAGC CAAAACGCAG AAGCTGCACT	1140
GCATATACAG AAGATACATC GAGCTAACTA GCTGCAGCGA TGTCTCGCTC CTGCTGCGTC	1200
GCCGTGTCGG TGCTTCTCGC TGTCGCCGCG ACAGCCAGCG CCACCGCGCC GGCATGGCTG	1260
CACGAGGAGC AGCACCTCGA GGAGGCCATG GCCACGGGCC CGCTGGTCGC AGAGGGTGCG	1320
AGGGTGGCGC CCTCCGCGTC CACCTGGGCT GCCGACAAGG CGTCGCCGCG GAGGCCGAGC	1380
GGCGGCATGG CCACGCAGGG CGACGACCAG AGCTCGTCGG GCGGCAGTGG CAGCAGCGGT	1440

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GAGCACGGCA	AGGCGGAGGG	CGAGAAGCAG	GGCAAGAGCT	GCCTCACCAA	GGAGGAGTGC	1500
CACAAGAAGA	AGATGATCTG	TGGCAAGGGC	TGCACGCTCT	CGGCGCACAG	CAAGTGCGCC	1560
GCCAAGTGCA	CCAAGTCCTG	TGTCCCCACC	TGCTAGGAGC	CGAGGCCGGA	GCTTGCCGGC	1620
GGCGAGACCT	CGATCGATCG	AGTGCTTCAC	TTCACCTTCTT	TGTTATAGTT	CTTGTGTGTT	1680
GCCGTTGCGT	TGCGTTGCGT	AGACGAAGGG	AATAAGGAAG	GGTAATTGGA	TTACCTGTTC	1740
CAGATCTCTG	TGTAAGCGTG	TTGTCGTGAC	AAGTCTTTTG	ATCCAGAGCG	AGGGATGGAT	1800
AGATCGCGCT	CGCAGTTTTA	ATTGCAATGC	TAGTTCAATA	TGTGTGCATC	ATGTTGGCAA	1860
CTACATAGTC	CAGATTCAAA	CCGAGATCGC	TGTTTAGCAT	GCCAGCACAA	TAATAACGGT	1920
ACAATCATAT	TATATTTTAT	ACAAATGCAC	AATTTATCTC	TAGAGATGTC	AATGGGAAAT	1980
TCCTCATCGG	GTTATATCAT	CTCAGACTCA	TCCCCATCAT	ATTTGATTCA	TCCTCATACT	2040
CATCCTCATA	TCTATCATGA	GTGCAAAACT	CATTTCATAC	CCATCTCTAT	TTTGGTTTAG	2100
GGTCTCCATC	CCTAATTAAG	GGATAACTAG	TACTAACAAC	TAGCACAAAC	TATCTAGATT	2160
TCAGATATCA	CCACATTGAC	AAACAATCAT	CCATGAACTA	TGATCCATTC	ATCCATCCAT	2220
CAAAAATAA	ATCGGTATTT	CGAGAACGAT	AGAAGAAATG	AAGTCGGCTC	ACCTTTCTTG	2280
GTCACCATTT	GAGTTTGTTG	GTGCCTGAGA	ATCCATGGTC	GTCATCGTCG	TCCTAGGGAT	2340
CGGCGGTGCT	CCTCGTTGTT	GGTAAAGTCG	CCAGTGTGTA	GTGCTAGCGC	AACTGTCCAG	2400
GCGTGCAACG	GTTGGCCGGC	TGGAAAGGGC	ATAGCGTATG	GCTGGTTATT	TTTAGGGTTT	2460
TGTTTTTTTA	CTAATCTGCT	AGTTGCCTTG	CCATGTTGTC	TTATTGGGCT	AGGATCTAGG	2520
GCTTGTTACG	CTGCTGTGTT	GGGCTTGGTG	TCCGGTTCAG	CCTCAACTCA	TTCATACAAA	2580
TCAGATTCAT	ACAAAACAGG	TATACACGTA	TGAAATATCC	ATGGATAATC	AGGTTCGAAT	2640
TATTGTCCCC	TAAACCCATA	CACGTTTACC	CAATGGATGG	ATATTTTGTC	TCATATCCAT	2700
ACACATGAGA	CGATTTTTGT	CCCATACCTG	TGCTCTAATA	GGAGAATTTC	TCTCGGGATA	2760
GCGAGTATCG	GATCCTCTAG	AGTC				2784

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide Zm13Oli2

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..24

(D) OTHER INFORMATION: /label= Zm13Oli2

/note= "oligonucleotide designated as Zm13Oli2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTGGATTGAA CGGGACTGAG TTGG

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(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide Zm13Oli1

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..25

(D) OTHER INFORMATION: /label= Zm13Oli1

/note= "oligonucleotide designated as Zm13Oli1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAGTCTCCAA GACTTTGGTT ATTCC

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(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Oligonucleotide Zm13Oli5

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION: 1..31

(D) OTHER INFORMATION: /label= Zm13Oli5

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/note= "oligonucleotide designated as Zm130li5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGATCCATGG TTGCCGCCGG GTGAATGTAC G

31

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: oligonucleotide BXOL2

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..24
- (D) OTHER INFORMATION: /label= BXOL2
/note= "oligonucleotide designated as BXOL2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGGAAAACC TGAAGCACAC TCTC

24

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: oligonucleotide TA29SBXOL2

(ix) FEATURE:

- (A) NAME/KEY: -
- (B) LOCATION: 1..49
- (D) OTHER INFORMATION: /label= TA29SBXOL2
/note= "oligonucleotide designated as TA29SBXOL2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GTTTTTACTT AAAGAAATTA GCTACCATGA AAAAAGCAGT CATTAACGG

49

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(i)
i) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide PTA29OL5

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 1..27
(D) OTHER INFORMATION: /label= PTA29OL5
/note= "oligonucleotide designated as PTA29OL5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGGCCATAAC TGAAATCAGG GTGAGAC

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(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4808 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: EcoRI-HindIII fragment of plasmid pTS218

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (18..401)
(D) OTHER INFORMATION: /label= 3'nos
/note= "3' regulatory sequence containing the
polyadenylation site derived from Agrobacterium
T-DNA nopaline synthase gene"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (402..737)
(D) OTHER INFORMATION: /label= barnase
/note= "coding region of the barnase gene of
Bacillus amyloliquefaciens"

(ix) FEATURE:

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- (A) NAME/KEY: -
 - (B) LOCATION: complement (738..1944)
 - (D) OTHER INFORMATION: /label= PZM13
/note= "promoter region of the Zm13 gene of Zea mays"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: complement (1945..2281)
 - (D) OTHER INFORMATION: /label= 3'nos
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: complement (2282..2554)
 - (D) OTHER INFORMATION: /label= barstar
/note= "coding region of the barstar gene of Bacillus amyloliquefaciens"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: complement (2555..3099)
 - (D) OTHER INFORMATION: /label= PTA29
/note= "promoter region of the TA29 gene of Nicotiana tabacum"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: 3100..3932
 - (D) OTHER INFORMATION: /label= 35S3
/note= "'35S3" promoter sequence derived from cauliflower mosaic virus isolate CabbB-JI"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: 3933..4484
 - (D) OTHER INFORMATION: /label= bar
/note= "coding region of the phosphinothricin acetyltransferase gene"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: 4485..4763
 - (D) OTHER INFORMATION: /label= 3'nos
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: 2333..2356
 - (D) OTHER INFORMATION: /label= BXOL2
/note= "region corresponding to oligonucleotide BXOL2"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: complement (2538..2586)
 - (D) OTHER INFORMATION: /label= TA29SBXOL2
/note= "region complementary to oligonucleotide TA29SBXOL2"
- (ix) FEATURE:
- (A) NAME/KEY: -
 - (B) LOCATION: complement (2800..2823)
 - (D) OTHER INFORMATION: /label= PTA29OL5

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/note= "region complementary to part of
oligonucleotide PTA290L5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAATTCGAGC TCGGTACCCG GGGATCTTCC CGATCTAGTA ACATAGATGA CACCGCGCGC	60
GATAATTTAT CCTAGTTTGC GCGCTATATT TTGTTTTCTA TCGCGTATTA AATGTATAAT	120
TGCGGGACTC TAATCATAAA AACCCATCTC ATAAATAACG TCATGCATTA CATGTTAATT	180
ATTACATGCT TAACGTAATT CAACAGAAAT TATATGATAA TCATCGCAAG ACCGGCAACA	240
GGATTCAATC TTAAGAAACT TTATTGCCAA ATGTTTGAAC GATCTGCTTC GGATCCTCTA	300
GAGNNNNCCG GAAAGTGAAA TTGACCGATC AGAGTTTGAA GAAAAATTTA TTACACACTT	360
TATGTAAAGC TGAAAAAAC GGCCTCCGCA GGAAGCCGTT TTTTTCGTTA TCTGATTTTT	420
GTAAAGGTCT GATAATGGTC CGTTGTTTTG TAAATCAGCC AGTCGCTTGA GTAAAGAATC	480
CGGTCTGAAT TTCTGAAGCC TGATGTATAG TTAATATCCG CTTACGCCA TGTTCGTCCG	540
CTTTTGCCCG GGAGTTTGCC TTCCCTGTTT GAGAAGATGT CTCCGCCGAT GCTTTTCCCC	600
GGAGCGACGT CTGCAAGGTT CCCTTTTGAT GCCACCCAGC CGAGGGCTTG TGCTTCTGAT	660
TTTGTAATGT AATTATCAGG TAGCTTATGA TATGTCTGAA GATAATCCGC AACCCCGTCA	720
AACGTGTTGA TAACCGGTAC CATGGTTGCC GCCGGGTGAA TGTACGTGTT TTCCCCTCCC	780
CCCTTGTTGA TGTCGGAGGA AAAGGGCGGG ACCTTTCCTT ATTATTTGTG TGAGGGAGGG	840
AGGGTGAGAC GAGGGTGGCA AATCTGGCCT GGTTCGTAA CGCAGCCTGT GGTGTCCTTA	900
AATAATCTGC GAGACCCGAA GATTTGTCTG GCTGCCCGTA GAAATAGTGG CCGACGGCCA	960
GAAAGCCGTT TTGACCTAGT TTTGTGGCAT GGGGAGGTGA AAAATATGTC GAATTTGGTT	1020
GAGTTTTTCA GCGGTCCACA AAAGTTGGAT TTGCGACTTC TAATCTTCTA TGCTGCTATG	1080
TAAACTAAA TAAGACGCGC TCAACAAAAC GGTATCAGTT AAATGTATCC ATAAGTGAAT	1140
AGTATATAGA ATCACTAAAT GGCAGCTTCT CATATTTAGC ATAGTACTTG GACAAATGTC	1200
ACGCAAGATG TATTATAAAA ACACATCGGT GTTGAGTGTT GATACAATAA AATCTCAATA	1260
GTTGTTTTAA TCTCAATCCA TGTGGATTGA ACGGGACTGA GTTGGTTTTA ATCCCTAGTA	1320
AGTCAAAATT ATTTCTAATT TTATCTAAAT CTCATCTAAT CCACGTGGAA TAGGAATAAC	1380
CAAACTGTTG GAGACTTGTT CTCAAGTGCT ATGAGTTAAG AACAAGGCAA CATAGAAAAT	1440
ATTAATCGTT AAGGTCCTTC GTCCTTCGAA GCATTATTTT CCTTAGGATA TAATGGTTTA	1500
CGGACGAAGG TTATGAAGGA CGTACCTTCA TAAATTCATT AAACAATGAC GAAGGATGAA	1560
ATATAAAGAA TATAAAAGAC AACATGAACA ATTATATATT ATTATTAGGT ACAAACAGAA	1620
ATATCGTTGA ATTACAAGTG TACCTTCAAT AGGAATGAGA TGACAGTACA AGCGTGACGC	1680
AAAAGCGAA TGCCAAGTCA GCGTGAACAG TACGGGAATA CTGTTCACCT ATTTATAGGC	1740

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ACGGGACGTA	GCCTGTGCAA	AATTACATTA	ATGCCCTTTA	CACTTAATAA	TAAACCTATA	1800
GTAATCTGTT	GAGGTCTAAA	TAGCCTTTTC	ATCTTTAAGT	CGGTTTCAAC	TGCTGCTGTC	1860
TTGCCGAAGC	TTTCCTGCTT	ACACCTTAGG	CGCTTCACCA	ACCTTCGTAT	TATTCTGGTC	1920
TACTGTGATG	CCTGACTTGA	GTCCGAAGAT	GGGGATCTTC	CCGATCTAGT	AACATAGATG	1980
ACACCGCGCG	CGATAATTTA	TCCTAGTTTG	CGCGCTATAT	TTTGTTTTCT	ATCGCGTATT	2040
AAATGTATAA	TTGCGGGACT	CTAATCATAA	AAACCCATCT	CATAAATAAC	GTCATGCATT	2100
ACATGTTAAT	TATTACATGC	TTAACGTAAT	TCAACAGAAA	TTATATGATA	ATCATCGCAA	2160
GACCGGCAAC	AGGATTCAAT	CTTAAGAAAC	TTTATTGCCA	AATGTTTGAA	CGATCTGCTT	2220
CGGATCCTCT	AGACCAAGCT	AGCTTGCGGG	TTTGTGTTTC	CATATTGTTC	ATCTCCCATT	2280
GATCGTATTA	AGAAAGTATG	ATGGTGATGT	CGCAGCCTTC	CGCTTTCGCT	TCACGGAAAA	2340
CCTGAAGCAC	ACTCTCGGCG	CCATTTTCAG	TCAGCTGCTT	GCTTTGTTCa	AACTGCCTCC	2400
ATTCCAAAAC	GAGCGGGTAC	TCCACCCATC	CGGTCAGACA	ATCCCATAAA	GCGTCCAGGT	2460
TTTCACCGTA	GTATTCCGGA	AGGGCAAGCT	CCTTTTTCAA	TGTCTGGTGG	AGGTCGCTGA	2520
TACTTCTGAT	TTGTTCCCCG	TTAATGACTG	CTTTTTTCAT	GGTAGCTAAT	TTCTTTAAGT	2580
AAAAACTTTG	ATTTGAGTGA	TGATGTTGTA	CTGTTACACT	TGCACCACAA	GGGCATATAT	2640
AGAGCACAAG	ACATACACAA	CAACTTGCAA	AACTAACTTT	TGTTGGAGCA	TTTCGAGGAA	2700
AATGGGGAGT	AGCAGGCTAA	TCTGAGGGTA	ACATTAAGGT	TTCATGTATT	AATTTGTTGC	2760
AAACATGGAC	TTAGTGTGAG	GAAAAAGTAC	CAAAATTTTG	TCTCACCTTG	ATTTCAAGTTA	2820
TGGAAATTAC	ATTATGAAGC	TGTGCTAGAG	AAGATGTTTA	TTCTAGTCCA	GCCACCCACC	2880
TTATGCAAGT	CTGCTTTTAG	CTTGATTCAA	AACTGATTT	AATTTACATT	GCTAAATGTG	2940
CATACTTCGA	GCCTATGTCG	CTTTAATTCG	AGTAGGATGT	ATATATTAGT	ACATAAAAAA	3000
TCATGTTTGA	ATCATCTTTC	ATAAAGTGAC	AAGTCAATTG	TCCCTTCTTG	TTTGGCACTA	3060
TATTCAATCT	GTTAATGCAA	ATTATCCAGT	TATACTTAGC	TAGATCCTAC	GCAGCAGGTC	3120
TCATCAAGAC	GATCTACCCG	AGTAACAATC	TCCAGGAGAT	CAAATACCTT	CCCAAGAAGG	3180
TTAAAGATGC	AGTCAAAAGA	TTCAGGACTA	ATTGCATCAA	GAACACAGAG	AAAGACATAT	3240
TTCTCAAGAT	CAGAAGTACT	ATTCCAGTAT	GGACGATTCA	AGGCTTGCTT	CATAAACCAA	3300
GGCAAGTAAT	AGAGATTGGA	GTCTCTAAAA	AGGTAGTTCC	TACTGAATCT	AAGGCCATGC	3360
ATGGAGTCTA	AGATTCAAAT	CGAGGATCTA	ACAGAACTCG	CCGTGAAGAC	TGGCGAACAG	3420
TTCATACAGA	GTCTTTTACG	ACTCAATGAC	AAGAAGAAAA	TCTTCGTCAA	CATGGTGGAG	3480
CACGACACTC	TGGTCTACTC	CAAAAATGTC	AAAGATACAG	TCTCAGAAGA	CCAAAGGGCT	3540
ATTGAGACTT	TTCAACAAAG	GATAATTTCG	GGAAACCTCC	TCGGATTCCA	TTGCCCAGCT	3600

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ATCTGTCACT TCATCGAAAG GACAGTAGAA AAGGAAGGTG GCTCCTACAA ATGCCATCAT	3660
TGCGATAAAG GAAAGGCTAT CATTCAAGAT GCCTCTGCCG ACAGTGGTCC CAAAGATGGA	3720
CCCCCACCCA CGAGGAGCAT CGTGGAAAAA GAAGACGTTC CAACCACGTC TTCAAAGCAA	3780
GTGGATTGAT GTGACATCTC CACTGACGTA AGGGATGACG CACAATCCCA CTATCCTTCG	3840
CAAGACCCTT CCTCTATATA AGGAAGTTCA TTTCATTTGG AGAGGACACG CTGAAATCAC	3900
CAGTCTCTCT CTATAAATCT ATCTCTCTCT CTATAACCAT GGACCCAGAA CGACGCCCGG	3960
CCGACATCCG CCGTGCCACC GAGGCGGACA TGCCGGCGGT CTGCACCATC GTCAACCACT	4020
ACATCGAGAC AAGCACGGTC AACTTCCGTA CCGAGCCGCA GGAACCGCAG GAGTGGACGG	4080
ACGACCTCGT CCGTCTGCGG GAGCGCTATC CCTGGCTCGT CGCCGAGGTG GACGGCGAGG	4140
TCGCCGGCAT CGCCTACGCG GGCCCCTGGA AGGCACGCAA CGCCTACGAC TGGACGGCCG	4200
AGTCGACCGT GTACGTCTCC CCCC GCCACC AGCGGACGGG ACTGGGCTCC ACGCTCTACA	4260
CCCACCTGCT GAAGTCCCTG GAGGCACAGG GCTTCAAGAG CGTGGTCGCT GTCATCGGGC	4320
TGCCCAACGA CCCGAGCGTG CGCATGCACG AGGCGCTCGG ATATGCCCCC CGCGGCATGC	4380
TGCGGGCGGC CGGCTTCAAG CACGGGA ACT GGCATGACGT GGGTTTCTGG CAGCTGGACT	4440
TCAGCCTGCC GGTACCGCCC CGTCCGGTCC TGCCCGTCAC CGAGATCTGA TCTCACGCGT	4500
CTAGGATCCG AAGCAGATCG TTCAAACATT TGGCAATAAA GTTTCTTAAG ATTGAATCCT	4560
GTTGCCGGTC TTGCGATGAT TATCATATAA TTTCTGTTGA ATTACGTTAA GCATGTAATA	4620
ATTAACATGT AATGCATGAC GTTATTTATG AGATGGGTTT TTATGATTAG AGTCCCGCAA	4680
TTATACATTT AATACGCGAT AGAAAACAAA ATATAGCGCG CAAACTAGGA TAAATTATCG	4740
CGCGCGGTGT CATCTATGTT ACTAGATCGG GAAGATCCTC TAGAGTCGAC CTGCAGGCAT	4800
GCAAGCTT	4808

FIGURE 1

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1. Action : Transform corn embryos (e.g. H99) with male-sterility gene S, linked to herbicide resistance gene bar (Example 5)
Result : transformed plants with genotype S/s
2. Action : Transform corn embryos (e.g. H99) with fertility-restorer gene R (Example 6)
Result : transformed plants with genotype R/r
3. Action : Transform corn embryos (e.g; H99) with maintainer gene P (Example 3)
Result : transformed plants with genotype P/p
4. Action : Cross S/s,r/r x s/s,R/r.
Select offspring for presence of both S and R genes by means of PCR.
Result : plants with genotype S/s,R/r
5. Action : Self selected plants of 4 (optional)
Result : Progeny plants with 9 different genotypes

gamete ♀ ♂ ↓ →	S,R	S,r	s,R	s,r
S,R	S/S,R/R	S/S,R/r	S/s,R/R	S/s,R/r
S,r	S/S,R/r	S/S,r/r**	S/s,R/r	S/s,r/r**
s,R	S/s,R/R	S/s,R/r	s/s,R/R	s/s,R/r
s,r	S/s,R/r	S/s,r/r**	s/s,R/r	s/s,r/r

** male-sterile plants

6. Action : self male-fertile progeny plants of 5 (Optional)
Result :
 - 6.1. Self of S/S,R/R : 100 % male-fertile plants
Self of S/s,R/R : 100 % male-fertile plants
Self of s/s,R/R : 100 % male-fertile plants
Self of s/s,R/r : 100 % male-fertile plants
Self of s/s,r/r : 100 % male-fertile plants
 - 6.2 Self of S/s,R/r : Same progeny as in 5
13/16 male-fertile plants
with 4/13 herbicide
sensitive

FIGURE 1 (continued 1)

6.3 Self of S/S,R/r : Progeny as follows :

gamete ♀ ♂ ↓	S,R	S,r
S,R	S/S,R/R	S/S,R/r
S,r	S/S,R/r	S/S,r/r''

'' male-sterile plants

Thus : 3/4 male-fertile plants, 0% herbicide sensitive
All male-sterile plants are of genotype S/S,r/r

7. Action : Cross

♀ : P/p (from 3) x ♂ : S/s,R/r (from 4)

this equals in fact

♀ : s/s,r/r,P/p x ♂ : S/s,R/r,p/p

Result : Progeny with the following genotypes

gamete ♀ ♂ ↓	S,R,p	S,r,p	s,R,p	s,r,p
s,r,P	S/s,R/r,P/p	S/s,r/r,P/p	s/s,R/r,P/p	s/s,r/r,P/p
s,r,p	S/s,R/r,p/p	S/s,r/r,p/p	s/s,R/r,p/p	s/s,r/r,p/p

8. Action : From offspring of 7, select plants with genotype S/s,r/r,P/p by screening, by means of PCR and/or Southern blotting, for presence of S and P gene and absence of R gene.

Result : plants with genotype S/s,P/p

FIGURE 1 (continued 2)

9. Action : Self plants with genotype S/s,P/p (from 8)
Result : progeny with the following genotypes

gamete ♀ ♂→ ↓	S,P	S,p	s,P	s,p
S,P	S/S,P/P	S/S,P/p	S/s,P/P	S/s,P/p
S,p	S/S,P/p	S/S,p/p ^{**}	S/s,P/p	S/s,p/p ^{**}
s,P	S/s,P/P	S/s,P/p	s/s,P/P	s/s,P/p
s,p	S/s,P/p	S/s,p/p ^{**}	s/s,P/p	s/s,p/p

^{**} male-sterile plants

Shaded genotypes cannot develop because male gametes (pollen) are killed off by expression of the maintainer gene P.

10. Action : self male fertile plants of 9.

Result

10.1. Self of s/s,P/p : 100 % male-fertile plants

Self of s/s,p/p : 100 % male-fertile plants

10.2 Self of S/s,P/p : Same progeny as in 9
5/8 male-fertile plants with
2/5 herbicide sensitive

10.3 Self of S/S,P/p : Progeny as follows :

gamete ♀ ♂→ ↓	S,P	S,p
S,P	S/S,P/P	S/S,P/p
S,p	S/S,P/p	S/S,p/p ^{**}

^{**} male-sterile plants

Shaded genotypes cannot develop because male gametes (pollen) are killed off by expression of the maintainer gene P.

FINAL RESULT

- 1/2 male-fertile plants, 0% herbicide sensitive. All these plants are maintainer plants
- 1/2 male sterile plants. All homozygous for the male-sterility gene S.

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CLAIMS:

1. A process for maintaining a male-sterile line of a plant species, said process comprising the steps of:

1) crossing:

5 a) a male-sterile line to be maintained comprising male-sterile parent plants which comprise a male-sterility gene, at a first genetic locus; wherein said male-sterility gene is homozygous at said first genetic locus; with

10 b) a maintainer line comprising male-fertile parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising:

15 (i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

(ii) a pollen-lethality gene comprising, under the control of a first promoter that directs expression
20 selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants, significantly disrupts the metabolism, functioning
25 or development of said microspore or pollen cell;

wherein said foreign DNA is heterozygous at said second genetic locus; and

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2) harvesting seeds from said male-sterile parent plants, wherein said seeds grow into a new generation of male-sterile parent plants.

2. A process for maintaining a male-sterile line of a plant species, said process comprising the steps of:

1) obtaining a maintainer line comprising male-fertile parent plants which comprise a male-sterility gene at a first genetic locus, wherein said male-sterility gene is homozygous at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising:

a) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

b) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants, significantly disrupts the metabolism, functioning or development of said microspore or pollen cell;

wherein said foreign DNA is heterozygous at said second genetic locus; and

2) crossing said maintainer line with a male-sterile line to be maintained comprising male-sterile parent plants which comprise said homozygous male-sterility gene at said first genetic locus; and

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3) harvesting seeds from said male-sterile parent plants, wherein said seeds grow into a new generation of male-sterile parent plants.

3. A process for obtaining seed of male-sterile parent plants which comprise a homozygous male-sterility gene at a first genetic locus, said process comprising the steps of:

1) crossing said male-sterile line with a maintainer line comprising male-fertile parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising:

a) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

b) a pollen-lethality gene comprising, under the control of a first promoter that directs expression selectively in a microspore or a pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants, significantly disrupts the metabolism, functioning or development of said microspore or pollen cell; and

2) harvesting seeds from said male-sterile parent plants.

4. The process of any one of claims 1 to 3, in which said first genetic locus in said male-sterile parent plants and said male-fertile parent plants is an endogenous locus comprising a recessive allele in homozygous condition.

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5. The process of claim 4, in which said restorer gene is dominant at said endogenous locus.

6. The process of any one of claims 1 to 3, in which said first genetic locus in said male-sterile parent plants and said male-fertile parent plants is a foreign genetic locus and comprises a foreign male-sterility gene, which comprises a sterility DNA under the control of a sterility promoter directing expression selectively in specific stamen cells, and in which said restorer gene at said second genetic locus encodes a protein or polypeptide, which upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene.

7. The process of claim 6, in which said sterility promoter directs expression in tapetum cells.

8. The process of claim 7, in which said sterility promoter is a TA29 promoter.

9. The process of any one of claims 1 to 8, in which said first DNA encodes a protein or polypeptide.

10. The process of any one of claims 1 to 9, in which said first DNA encodes a ribonuclease.

11. The process of claim 10, in which said first DNA encodes a barnase or an RNase T1.

12. The process of any of claims 1 to 8, in which said first DNA encodes a DNase, a protease, an enzyme which catalyzes the synthesis of phytohormones, a glucanase, a lipase, a lipid peroxidase, or a plant cell wall inhibitor.

13. The process of any of claims 1 to 8, in which said first DNA encodes an antisense RNA or a ribozyme targeted

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against an RNA that is naturally transcribed in the microspores or pollen cells of a plant.

14. The process of any one of claims 1 to 13, in which said first promoter is a promoter of a *zml3* gene from maize.

5 15. The process of any one of claims 1 to 14, in which said male-fertile parent plants and said male-sterile parent plants are maize, oilseed rape, wheat or rice plants.

16. A cell of a male-fertile parent plant for use in maintaining male-sterile parent plants which comprise a
10 homozygous male-sterility gene at a first genetic locus, wherein said male-fertile parent plant comprises said homozygous male-sterility gene at said first genetic locus and further comprises, at a second genetic locus which segregates independently from said first genetic locus, a
15 foreign DNA comprising:

(i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

(ii) a pollen-lethality gene comprising, under the
20 control of a first promoter that directs expression selectively in a microspore and/or pollen cell of said male-fertile parent plant, a first DNA encoding a first RNA, protein or polypeptide which when produced in said microspore or pollen cell of said male-fertile parent plant
25 significantly disrupts the metabolism, functioning or development of said microspore or pollen cell;

wherein said foreign DNA is heterozygous at said second genetic locus, and wherein said male-fertile parent plant can be crossed with said male-sterile parent plants to

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produce, on said male-sterile parent plants, seeds which grow into a new generation of male-sterile parent plants.

17. The cell of claim 16, in which said first genetic locus comprising said sterility gene is an endogenous locus
5 comprising a recessive allele in homozygous condition.

18. The cell of claim 17, in which said restorer gene is the dominant allele at said endogenous locus.

19. The cell of claim 16, in which said first genetic locus is a foreign genetic locus and comprises a foreign
10 male-sterility gene which comprises a sterility DNA under the control of a sterility promoter directing expression selectively in specific stamen cells, and in which said restorer gene at said second genetic locus encodes a protein or polypeptide, which upon expression, inhibits or prevents
15 the phenotypic expression of said male-sterility gene.

20. The cell of claim 19, wherein said sterility promoter is a TA29 promoter.

21. The cell of any of claims 16 to 20, in which said first DNA encodes a protein or polypeptide.

20 22. The cell of any one of claims 16 to 21, in which said first DNA encodes a ribonuclease.

23. The cell of claim 22, in which said first DNA encodes a barnase or an RNase T1.

24. The cell of any one of claims 16 to 21, in which
25 said first DNA encodes a DNase, a protease, an enzyme which catalyzes the synthesis of phytohormones, a glucanase, a lipase, a lipid peroxidase, or a plant cell wall inhibitor.

25. The cell of any one of claims 16 to 20, in which said first DNA encodes an antisense RNA or a ribozyme

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targeted against an RNA that is naturally transcribed in the microspores or pollen cells of a plant.

26. The cell of any one of claims 16 to 25, in which said first promoter is a promoter of a *zm13* gene from maize.

5 27. The cell of any one of claims 16 to 26 which is a cell of a maize plant, an oilseed rape plant, a wheat plant or a rice plant.

28. Use of a male-fertile parent plant for maintaining male-sterile parent plants which comprise a homozygous male-sterility gene at a first genetic locus, wherein said male-fertile parent plant comprises said homozygous male-sterility gene at said first genetic locus and further comprises, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA
10 comprising:
15

(i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

(ii) a pollen-lethality gene comprising, under the
20 control of a first promoter that directs expression selectively in a microspore or pollen cell of said male-fertile parent plant, a first DNA encoding a first RNA, protein or polypeptide which when produced in said microspore or pollen cell of said male-fertile parent plant
25 significantly disrupts the metabolism, functioning or development of said microspore or pollen cell;

wherein said foreign DNA is heterozygous at said second genetic locus, and wherein said male-fertile parent plant can be crossed with said male-sterile parent plants to

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produce, on said male-sterile parent plants, seeds which grow into a new generation of male-sterile parent plants.

29. The use of claim 28, in which said first genetic locus comprising said sterility gene is an endogenous locus
5 comprising a recessive allele in homozygous condition.

30. The use of claim 29, in which said restorer gene is the dominant allele at said endogenous locus.

31. The use of claim 28, in which said first genetic locus is a foreign genetic locus and comprises a foreign
10 male-sterility gene which comprises a sterility DNA under the control of a sterility promoter directing expression selectively in specific stamen cells, and in which said restorer gene at said second genetic locus encodes a protein or polypeptide, which upon expression, inhibits or prevents
15 the phenotypic expression of said male-sterility gene.

32. The use of claim 31, wherein said sterility promoter is a TA29 promoter.

33. The use of any one of claims 28 to 32, in which said first DNA encodes a protein or polypeptide.

20 34. The use of any one of claims 28 to 33, in which said first DNA encodes a ribonuclease.

35. The use of claim 34, in which said first DNA encodes a barnase or an RNase T1.

36. The use of any one of claims 28 to 33, in which
25 said first DNA encodes a DNase, a protease, an enzyme which catalyzes the synthesis of phytohormones, a glucanase, a lipase, a lipid peroxidase, or a plant cell wall inhibitor.

37. The use of any one of claims 28 to 32, in which said first DNA encodes an antisense RNA or a ribozyme

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targeted against an RNA that is naturally transcribed in the microspores or pollen cells of a plant.

38. The use of any one of claims 28 to 37, in which said first promoter is a promoter of a *zm13* gene from maize.

5 39. The use of any one of claims 28 to 38, in which said male-fertile parent plant and said male-sterile parent plants are maize, oilseed rape, wheat or rice plants.

40. Use of a kit for maintaining a male-sterile line of a plant species, said kit comprising:

10 a) a male-sterile line comprising male-sterile parent plants which comprise a male-sterility gene at a first genetic locus, wherein said male-sterility gene is homozygous at said first genetic locus; and,

b) a maintainer line comprising male-fertile
15 parent plants which comprise said homozygous male-sterility gene at said first genetic locus, and which further comprise, at a second genetic locus which segregates independently from said first genetic locus, a foreign DNA comprising:

20 (i) a restorer gene which, upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene; and

(ii) a pollen-lethality gene comprising, under the control of a first promoter that directs expression
25 selectively in a microspore and/or pollen cell of said male-fertile parent plants, a first DNA encoding a first RNA, protein or polypeptide which, when produced in said microspore or pollen cell of said male-fertile parent plants significantly disrupts the metabolism, functioning or
30 development of said microspore or pollen cell;

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wherein said foreign DNA is heterozygous at said second genetic locus; and

wherein said male-sterile and male-fertile parent plants can be crossed to produce, on said male-sterile plants, seed which grow into a new generation of male-sterile parent plants.

41. The use of claim 40, in which said first genetic locus comprising said sterility gene is an endogenous locus comprising a recessive allele in homozygous condition.

10 42. The use of claim 41, in which said restorer gene is the dominant allele at said endogenous locus.

43. The use of claim 40, wherein said first genetic locus is a foreign genetic locus and comprises a foreign male-sterility gene, which comprises a sterility DNA under the control of a sterility promoter directing expression selectively in specific stamen cells, and in which said restorer gene at said second genetic locus encodes a protein or polypeptide, which upon expression, inhibits or prevents the phenotypic expression of said male-sterility gene.

20 44. The use of claim 43, in which said sterility promoter directs expression in tapetum cells.

45. The use of claim 44, in which said sterility promoter is a TA29 promoter.

46. The use of any one of claims 40 to 45, in which said first DNA encodes a protein or polypeptide.

47. The use of any one of claims 40 to 46, in which said first DNA encodes a ribonuclease.

48. The use of claim 47, in which said first DNA encodes a barnase or an RNase T1.

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49. The use of any one of claims 40 to 46, in which said first DNA encodes a DNase, a protease, an enzyme which catalyzes the synthesis of phytohormones, a glucanase, a lipase, a lipid peroxidase, or a plant cell wall inhibitor.

5 50. The use of any one of claims 40 to 45, in which said first DNA encodes an antisense RNA or a ribozyme targeted against an RNA that is naturally transcribed in the microspores or pollen cells of a plant.

51. The use of any one of claims 40 to 50, in which
10 said first promoter is a promoter of a zml3 gene from maize.

52. The use of any one of claims 40 to 51 in which said plant species is selected from maize, oilseed rape, wheat and rice.

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