Title: HYBRID WINTER OILSEED RAPE AND METHODS FOR PRODUCING SAME

Abstract: This invention relates to transgenic winter oilseed rape (WOSR) plants, plant material and seeds, characterized by harboring a specific transformation event. It pertains to winter oilseed rape plants, more particularly to a pair of winter oilseed rape plants, which is particularly suited for the production of hybrid seed. More specifically, one plant is characterized by being male-sterile, due to the presence in its genome of a male sterility gene, while the other is characterized by carrying a fertility-restorer gene, capable of preventing the activity of the male-sterility gene. The invention further provides a method for producing hybrid seed, a process for producing a transgenic WOSR plant oil or plant, and a method to identify a transgenic plant, cell or tissue. A kit for identifying the transgenic plants comparing the elite event of the present invention is also described. The WOSR plants of the invention combine the ability to form hybrid seeds with optimal overall agronomic performance, genetic stability and adaptability to different genetic backgrounds.
Title of the invention

HYBRID WINTER OILSEED RAPE AND METHODS FOR PRODUCING SAME

Field of the invention

This invention pertains to winter oilseed rape (WOSR) plants, more particularly a pair of winter oilseed rape plants, which is particularly suited for the production of hybrid seed. More specifically, the one plant is characterized by being male-sterile, due to the presence in its genome of a male-sterility gene, while the other is characterized by carrying a fertility-restorer gene, capable of preventing the activity of the male-sterility gene. The pair WOSR plants of the invention combine the ability to form hybrid seed with optimal overall agronomic performance, genetic stability and adaptability to different genetic backgrounds.

All documents cited herein are hereby incorporated herein by reference.

Background of the invention

The phenotypic expression of a transgene in a plant is determined both by the structure of the gene itself and by its location in the plant genome. At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. The agronomically or industrially successful introduction of a commercially interesting trait in a plant by genetic manipulation can be a lengthy procedure dependent on different factors. The actual transformation and regeneration of genetically transformed plants are only the first in a series of selection steps which include extensive genetic characterization, breeding, and evaluation in field trials.
Oilseed rape (OSR) (Brassica napus, AACC, 2n=38) is a natural hybrid resulting from the interspecies hybridisation between Cole (Brassica Oleracea, CC, 2n = 18) and Turnip (Brassica campestris, AA, 2n = 20). Winter oilseed rape is sown during the last 10 days of August and the first ten days of September and harvested the following July, needing a temperate period for vernalization. The faster growing spring rapes are sown during late March and early April being harvested mid August to September. The main types of OSR grown at present are low and high erucic acid varieties. Double low (00) varieties contain low (typically less than 1%) levels of erucic acids (which humans find hard to digest), and low levels of glucosinolates (which makes the meal by-product indigestible for animals). Current uses for "00" varieties include oil for human consumption and high protein meal for animal feed. Industrial uses include feedstocks for pharmaceuticals and hydraulic oils. High erucic acid rape (HEAR) varieties are grown specifically for their erucic acid content—typically 50-60% of oil. The principal end use of HEAR is to produce erucamide, a "slip agent" used in polyethane manufacture. A small portion is used to produce behenyl alcohol, which is added to a waxy crude mineral oil to improve its flow.

Oilseed rape plants are bisexual and typically 60-70% self pollinated. The production of hybrids and introduction of genetic variation as a basis for selection was traditionally dependent on the adaptation of natural occurring phenomena such as self-incompatibility and cytoplasmic male sterility. Artificial pollination control methods such as manual emasculation or the use of gametocides are not widely applied in OSR breeding due to their limited practicability and high cost respectively.

Transgenic methods have been developed for the production of male or female-sterile plants, which provide interesting alternatives to the traditional techniques.

EP 0,344,029 describes a system for obtaining nuclear male sterility whereby plants are transformed with a male-sterility gene, which comprises, for example a DNA encoding a barnase under the control of a tapetum specific promoter, PTA29, which when incorporated into a plant ensures selective destruction of tapetum cells. Transformation of tobacco and oilseed rape plants with such a chimaeric gene resulted in plants in which pollen formation was completely prevented (Mariani et al. 1990, Nature 347: 737-741).
To restore fertility in the progeny of a male-sterile plant, a system was developed whereby the male-sterile plant is crossed with a transgenic plant carrying a fertility-restorer gene, which when expressed is capable of inhibiting or preventing the activity of the male-sterility gene (US 5,689,041; US 5,792,929). Such a fertility-restorer gene is placed under the control of a promoter directing expression at least in the cells in which the male-sterility gene is expressed. Mariani et al. (1992, Nature 357:384-387) demonstrated that the sterility encoded by the pTA29:barnase gene can be restored by the chimeric pTA29:barstar gene in oilseed rape.

Cytochemical and histochemical analysis of anther development of Brassica napus plants comprising the chimeric pTA29:barnase gene alone or with pTA29:barstar is described by De Block and De Brouwer (1993, Planta 189:218-225).

Successful transformation of Brassica species has been obtained by a number of methods including Agrobacterium infection (as described for example in EP 0,116,718 and EP 0,270,882), microprojectile bombardment (as described for example by Chen et al., 1994, Theor. Appl. Genet. 88:187-192) and direct DNA uptake (as described for example by De Block et al. 1989, Plant Physiol. 914:694-701; Poulsen 1996, Plant Breeding 115:209-225).

However, the foregoing documents fail to teach or suggest the present invention.

**Summary of the invention**

The present invention relates to a pair of WOSR plants, particularly suited for producing hybrid seed. More particularly, the present invention relates to a first transgenic WOSR plant, or seed, cells or tissues thereof, comprising, integrated into its genome, an expression cassette which comprises a male-sterility gene, and a second transgenic WOSR plant, or seed, cells or tissues thereof, comprising, integrated into its genome, an expression cassette which comprises a fertility restorer gene, and the hybrid seed obtained by the crossing of the first and second plant, which comprises the male-sterility gene and/or the fertility restorer gene integrated into its genome.
In one embodiment of the invention, the first WOSR plant or seed, cells or tissues thereof, comprises the expression cassette of pTHW107. In the preferred embodiment of the invention the first WOSR plant or seed, cells or tissues thereof comprise event MS-BN1.

In another embodiment of the invention, the second WOSR plant or seed, cells or tissues thereof, comprises the expression cassette of pTHW1118. In the preferred embodiment of the invention the WOSR plant or seed, cells or tissues thereof comprise event RF-BN1. In a particularly preferred embodiment of the invention the first WOSR plant comprises event MS-BN1 and the second WOSR plant comprises event RF-BN1 and the hybrid seed obtained therefrom comprises even MS-BN1 and RF-BN1 or RF-BN1 alone.

The invention relates to transgenic WOSR seed, or a plant that can be grown from such seed, the genomic DNA of which is characterized by one or both of the following characteristics:

a) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably at least four, most preferably five of the sets of restriction fragments selected from the group of:

i) one set of two EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp;

ii) one set of two EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp;

iii) one set of two HpaI fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp;

iv) one set of three AflIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, and one with a length of between 2140 and 2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp;

v) one set of two NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp;
wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107 described herein; and/or

b) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably four of the sets of restriction fragments selected from the group of:

i) one set of three BamHI fragments, wherein one has a length between 805 and 1099 bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, preferably of about 1849 bp, one has a length between 2450 and 2838 bp, preferably of about 2607 bp, and one has a length between 5077 and 14057 bp, preferably of about 6500 bp;

ii) one set of four EcoRI fragments, one with a length of between 805 and 1159 bp, preferably of about 1094 bp, one with a length between 1986 and 2450 bp, preferably of about 2149 bp, and two with a length of between 5077 and 14057 bp, preferably one of about 7000 bp, and one with a length of about 10 kb;

iii) one set of two EcoRV fragments wherein both have a length of between 5077 and 14057 bp, preferably one has a length of about 5.4 kbp and the other has a length of about 8 kbp;

iv) one set of three HindIII fragments, wherein one has a length of between 1700 and 2140 bp, preferably of about 1969 bp, and two have a length between 2450 and 2838 bp, preferably one has a length of about 2565 bp, and one has a length of about 2635 bp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar sequence obtainable by HpaI digestion of the plasmid pTHW118 described herein.

The present invention relates to a the seed of a WOSR plant, or a plant which can be grown from such seed, or cells, or tissues thereof, the genomic DNA of which is characterized by one or both of the following characteristics:

a) the genomic DNA is capable of yielding at least two, preferably at least three, for instance at least four, more preferably five of the sets of restriction fragments selected from the group described under a) above comprising the sets of restriction fragments
described under a) i), ii), iii), iv), and v) above, whereby the selection can include any combination of i), ii), iii), iv), and v) described under a) above; and/or
b) the genomic DNA is capable of yielding at least two, preferably at least three, most preferably four of the sets of restriction fragments selected from the group described under b) above comprising the sets of restriction fragments described under b) i), ii), iii) and iv) above, whereby the selection can include any combination of i), ii), iii) and iv) described under b) above.

The invention further relates to WOSR seed, or plants grown from such seed, the genomic DNA of which is characterized by one or both of the following characteristics:
c) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, preferably of about 280 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively and/or
d) the genomic DNA can be used to amplify a DNA fragment of between 195 and 235 bp, preferably of about 215 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

The invention further relates to WOSR seed, or plants grown from such seed, the genomic DNA of which is characterized by the characteristics described under a) and c) above and/or the characteristics described under b) and d) above.

The present invention relates to the seed of a WOSR plant, or a plant which can be grown from such seed, the genomic DNA of which is characterized by one or both of the following characteristics:
a) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably at least four, most preferably five of the sets of restriction fragments selected from the group of:
i) One set of two EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp.
ii) one set of two EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp.

iii) one set of two Hpal fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp.

iv) one set of three AflIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, one with a length of between 2140 and 2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp.

v) one set of two NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp; wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107 described herein; and/or,

c) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, preferably of about 280 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.

The present invention relates to a the seed of a WOSR plant, preferably a male-sterile plant, or a plant which can be grown from such seed, or cells, or tissues thereof, the genomic DNA of which is characterized in that it is capable of yielding at least two, preferably at least three, more preferably five of the sets of restriction fragments selected from the group described above comprising the sets of restriction fragments described under i), ii), iii), iv), and v) above, whereby the selection can include any combination of i), ii), iii), iv), and v) described above.

The present invention further relates to the seed of a WOSR plant, or a plant grown from such seed, the genomic DNA of which is characterized by one or both of the following characteristics:
b) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably four of the restriction fragments or sets of restriction fragments selected from the group of:

i) one set of three BamHI fragments, wherein one has a length of between 805 and 1099 bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, preferably of about 1849 bp, one has a length between 2450 and 2838 bp, preferably of about 2607 bp, and one has a length between 5077 and 14057 bp, preferably of about 6500 bp;

ii) one set of four EcoRI fragments, one with a length of between 805 and 1159 bp, preferably of about 1094 bp, one with a length between 1986 and 2450 bp, preferably of about 2149 bp, and two with a length of between 5077 and 14057 bp, preferably one of about 7000 bp, and one with a length of about 10 kbp;

iii) one set of two EcoRV fragments wherein both have a length of between 5077 and 14057 bp, preferably one has a length of about 5.4 kbp and the other has a length of about 8 kbp;

iv) one set of three HindIII fragments, wherein one has a length of between 1700 and 2140 bp, preferably of about 1969 bp, and two have a length between 2450 and 2838 bp, preferably one has a length of about 2565 bp, and one has a length of about 2635 bp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar sequence obtainable by HpaI digestion of the plasmid pTHW118 described herein;

and/or

d) the genomic DNA can be used to amplify a DNA fragment of between 195 and 235 bp, preferably of about 215 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

The present invention relates to the seed of a WOSR plant, preferably a fertility restorer plant, or a plant which can be grown from such seed, or cells, or tissues thereof, the genomic DNA of which is capable of yielding at least two, preferably at least three, most preferably four of the sets of restriction fragments selected from the group described above
comprising the sets of restriction fragments described under b) i), ii), iii) and iv) above, whereby the selection can include any combination of i), ii), iii) and iv) described above.

The present invention relates to transgenic WOSR plants, cells, tissues or seeds which are preferably characterized by both of the characteristics described under b) and/or d) above, respectively.

The invention further relates to transgenic, preferably hybrid fertility restored WOSR plants, cells, tissues or seeds obtained from the crossing of the male-sterile plant with the fertility restorer plant of the invention characterized by the respective characteristics described above, whereby the fertility restored plants, cells tissues or seeds are characterized by both the molecular characteristics of the male-sterile and those of the fertility restorer WOSR plant described above. The invention further relates to transgenic, preferably hybrid WOSR plants, cells, tissues or seeds obtained from the crossing of the male-sterile plant with the fertility restorer plant of the invention characterized by the molecular characteristics described above, whereby the hybrid plants, cells tissues or seeds are characterized by the molecular characteristics of the fertility restorer WOSR plant described above.

The invention also relates to the seed deposited at the ATCC under accession number PTA-730, a plant which is grown from this seed, and cells or tissues from a plant grown from this seed. The invention further relates to plants obtainable by propagation of, and/or breeding with a WOSR plant grown from the seed deposited at the ATCC under accession number PTA-730.

The invention further relates to a process for producing hybrid WOSR seed, which comprises, crossing the male-sterile WOSR plant of the present invention with the fertility-restorer WOSR plant of the invention.

The invention further relates to a WOSR plant, plant cell, plant tissue or seed, which comprises a recombinant DNA comprising at least one transgene, integrated into a part of the chromosomal DNA characterized by the sequence of SEQ ID No 22 and/or a
recombinant DNA comprising at least one transgene, integrated into a part of the chromosomal DNA characterized by the sequence of SEQ ID No 34.

The invention further provides a process for producing a transgenic cell of a WOSR plant or a plant obtained therefrom, which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of an WOSR cell characterized by the sequence of SEQ ID No 22 and, optionally, regenerating a WOSR plant from the transformed WOSR cell.

The invention further provides a process for producing a transgenic cell of a WOSR plant or a plant obtained therefrom, which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of an WOSR cell characterized by the sequence of SEQ ID No 34 and, optionally, regenerating a WOSR plant from the transformed WOSR cell.

The invention further relates to a method for identifying a transgenic plant, or cells or tissues thereof, comprising the elite event MS-BN1 of the invention, which method comprises establishing one or both of the following characteristics of the genomic DNA of the transgenic plant, or its cells or tissues:

a) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably at least four, most preferably five of the sets of restriction fragments selected from the group of:

i) one set of two EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp;

ii) one set of two EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp;

iii) one set of two Hpal fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp;

iv) one set of three AflIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, one with a length of between 2140 and
2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp;

v) one set of two NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107 described herein; and/or

c) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, preferably of about 280 bp, according to the PCR Identification Protocol described herein with two primers identifying the elite event having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.

The invention further relates to a method for identifying a transgenic plant, or cells or tissues thereof, comprising the elite event RF-BN1 of the invention, which method comprises establishing one or both of the following characteristics of the genomic DNA of the transgenic plant, or its cells or tissues:

b) the genomic DNA is capable of yielding at least two, preferably at least three, more preferably four of the restriction fragments or sets of restriction fragments selected from the group of:

i) one set of three BamHI fragments, wherein one has a length of between 805 and 1099 bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, preferably of about 1849 bp, one has a length between 2450 and 2838 bp, preferably of about 2607 bp, and one has a length between 5077 and 14057 bp, preferably of about 6500 bp;

ii) one set of four EcoRI fragments, one with a length of between 805 and 1159 bp, preferably of about 1094 bp, one with a length between 1986 and 2450 bp, preferably of about 2149 bp, and two with a length of between 5077 and 14057 bp, preferably one of about 7000 bp, and one with a length of about 10 kbp;

iii) one set of two EcoRV fragments wherein both have a length of between 5077 and 14057 bp, preferably one has a length of about 5.4 kbp and the other has a length of about 8 kbp;
iv) one set of three HindIII fragments, wherein one has a length of between 1700 and 2140 bp, preferably of about 1969 bp, and two have a length between 2450 and 2838 bp, preferably one has a length of about 2565 bp, and one has a length of about 2635 bp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar sequence obtainable by HpaI digestion of the plasmid pTHW118 described herein, and/or
d) the genomic DNA can be used to amplify a DNA fragment of between 195 and 235 bp, preferably of about 215 bp, using the PCR identification protocol described herein with two primers identifying the elite event having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

The invention also relates to a kit for identifying the plants comprising elite event MS-BN1 of the present invention, said kit comprising the PCR probes having the nucleotide sequence of SEQ ID No. 12 and SEQ ID No. 19.

The invention further relates to a kit for identifying the plants comprising elite event RF-BN1 of the present invention, said kit comprising the PCR probes having the nucleotide sequence of SEQ ID No. 23 and SEQ ID No. 41.

The invention also relates to a kit for identifying elite event MS-BN1 and/or RF-BN1 in biological samples, which kit comprises at least one specific primer or probe having a sequence which corresponds (or is complementary to) a sequence having between 80% and 100% sequence identity with a specific region of MS-BN1 and/or at least one specific primer or probe having a sequence which corresponds (or is complementary to) a sequence having between 80% and 100% sequence identity with a specific region of RF-BN1. Preferably the sequence of the probe corresponds to a specific region comprising part of the 5’ or 3’ flanking region of MS-BN1 and/or RF-BN1. Most preferably the specific probe has (or is complementary to) a sequence having between 80% and 100% sequence identity to the plant DNA sequence within SEQ ID No. 36 or SEQ ID No. 38 for MS-BN1 or to the plant DNA sequence within SEQ ID No. 39 or SEQ ID No. 40 for RF-BN1.
Preferably the kit of the invention comprises, in addition to a primer which specifically recognizes the 5' or 3' flanking region of MS-BN1 and/or RF-BN1, a second primer which specifically recognizes a sequence within the foreign DNA of MS-BN1 and/or RF-BN1, for use in a PCR identification protocol. Preferably, the kit of the invention comprises two (or more) specific primers, one of which recognizes a sequence within the 3' flanking region of MS-BN1 and/or RF-BN1, most preferably a sequence within the plant DNA region of SEQ ID No. 36 or SEQ ID No. 38 for MS-BN1 or to the plant DNA sequence of SEQ ID No. 39 or SEQ ID No. 40 for RF-BN1, and another which recognizes a sequence within the foreign DNA of MS-BN1 and/or RF-BN1 respectively. Especially preferably, the primer recognizing the plant DNA sequence within 5'flanking region of MS-BN1 comprises the nucleotide sequence of SEQ ID No. 19. Particularly, the primer recognizing the plant DNA sequence within 5'flanking region of MS-BN1 comprises the nucleotide sequence of SEQ ID No. 19 and the primer recognizing the foreign DNA of MS-BN1 comprises the nucleotide sequence of SEQ ID No. 12 described herein. Especially preferably, the primer recognizing the plant DNA sequence within 5'flanking region of RF-BN1 comprises the nucleotide sequence of SEQ ID No. 41. Particularly, the primer recognizing the plant DNA sequence within 5'flanking region of MS-BN1 comprises the nucleotide sequence of SEQ ID No. 41 and the primer recognizing the foreign DNA of RF-BN1 comprises the nucleotide sequence of SEQ ID No. 23 described herein.

The methods and kits encompassed by the present invention can be used for different purposes such as, but not limited to the following: to identify MS-BN1 and/or RF-BN1 in plants, plant material or in products such as, but not limited to food or feed products (fresh or processed) comprising or derived from plant material; additionally or alternatively, the methods and kits of the present invention can be used to identify transgenic plant material for purposes of segregation between transgenic and non-transgenic material; additionally or alternatively, the methods and kits of the present invention can be used to determine the quality (i.e. percentage pure material) of plant material comprising MS-BN1 and/or RF-BN1.
It will be understood that particular embodiments of the invention are described by the dependent claims cited herein.

**Brief description of the drawings**

The following detailed description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

**Fig. 1. Plasmid map of pVE113**

**Fig. 2. Restriction map obtained after digestion of MS-BN1 genomic DNA**

Loading sequence of the gel analyzed by Southern blot: lane 1, MS-BN1 DNA digested with EcoRI, lane 2, MS-BN1 DNA digested with EcoRV, lane 3, MS-BN1 DNA digested with HpaI, lane 4, MS-BN1 DNA digested with AflIII, lane 5, MS-BN1 DNA digested with NdeI, lane 6, non-transgenic WOSR DNA digested with BamHI, lane 7, non-transgenic WOSR digested with BamHI + control plasmid pTHW107 DNA digested with BamHI.

**Fig. 3. Restriction map obtained after digestion of RF-BN1 genomic DNA**

Loading sequence of the gel analyzed by Southern blot: lane 1, RF-MS1 DNA digested with BamHI, lane 2, RF-BN1 DNA digested with EcoRI, lane 3, RF-BN1 DNA digested with EcoRV, lane 4, RF-BN1 DNA digested with HindIII, lane 5, non-transgenic WOSR DNA digested with BamHI, lane 6, non-transgenic WOSR digested with BamHI+control plasmid pTHW118 DNA digested with BamHI.

**Fig. 4. PCR analysis of different lines using the MS-BN1 PCR identification protocol.**

Loading sequence of the gel: lane 1, DNA sample from an OSR plant comprising the transgenic event MS-BN1, lane 2, DNA sample from an OSR plant comprising another transgenic event, lane 3, DNA from wild-type OSR, lane 4, negative control (water), lane 5, molecular weight marker (100bp ladder).
Fig. 5. PCR analysis of different lines using the RF-BN1 PCR identification protocol.
Loading sequence of the gel: lane 1, DNA sample from an OSR plant comprising the transgenic event RF-BN1, lane 2, DNA sample from an OSR plant comprising another transgenic event, lane 3, DNA from wild-type OSR, lane 4, negative control (water), lane 5, molecular weight marker (100bp ladder).

Detailed description

The term “gene” as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5’ untranslated region (the 5’UTR), which together form the promoter region, a coding region (which may or may not code for a protein), and an untranslated 3’ region (3’UTR) comprising a polyadenylation site. Typically in plant cells, the 5’UTR, the coding region and the 3’UTR are transcribed into a RNA which, in the case of a protein encoding gene, is translated into the protein. A gene may include additional DNA fragments such as, for example, introns. As used herein, a genetic locus is the position of a given gene in the genome of a plant.

The term “chimeric” when referring to a gene or DNA sequence is used to indicate that the gene or DNA sequence comprises at least two functionally relevant DNA fragments (such as promoter, 5’UTR, coding region, 3’UTR, intron) that are not naturally associated with each other and originate, for example, from different sources. “Foreign” referring to a gene or a DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species, or is not naturally found in that genetic locus in that plant species. The term “foreign DNA” will be used herein to refer to a DNA sequence as it has incorporated into the genome of a plant as a result of transformation. The “transforming DNA” as used herein refers to a recombinant DNA molecule used for transformation. The transforming DNA usually comprises at least one “gene of interest” (e.g. a chimeric gene) which is capable of conferring one or more specific characteristics to the transformed plant. The term “recombinant DNA molecule” is used to exemplify and thus can include an isolated nucleic acid molecule which can be DNA and which can be obtained through recombinant or other procedures.
As used herein the term “transgene” refers to a gene of interest as incorporated in the genome of a plant. A “transgenic plant” refers to a plant comprising at least one transgene in the genome of all of its cells.

The foreign DNA present in the plants of the present invention will preferably comprise two genes of interest, more specifically, either a male-sterility gene and a herbicide resistance gene or a fertility restorer gene and a herbicide resistance gene. A “male-sterility gene” as used herein refers to a gene which upon expression in the plant renders the plant incapable of producing fertile, viable pollen. An example of a male sterility gene is a gene comprising a DNA sequence encoding barnase, under the control of a promoter directing expression in tapetum cells. More specifically, according to the present invention the male-sterility gene is “TA29-barnase” as described herein.

A “fertility restorer gene” as used herein refers to a gene which upon expression in a plant comprising a male-sterility gene, is capable of preventing phenotypic expression of the male-sterility gene, restoring fertility in the plant. More specifically the fertility restorer gene will comprise a DNA encoding a protein or polypeptide capable of preventing phenotypic expression of the male-sterility gene, under the control of a promoter directing expression in at least the cells in which the male-sterility DNA is expressed. More specifically, according to the present invention, the fertility restorer gene is “TA29-barstar” as described herein.

The incorporation of a recombinant DNA molecule in the plant genome typically results from transformation of a cell or tissue (or from another genetic manipulation). The particular site of incorporation is either due to chance or is at a predetermined location (if a process of targeted integration is used).

The foreign DNA can be characterized by the location and the configuration at the site of incorporation of the recombinant DNA molecule in the plant genome. The site in the plant genome where a recombinant DNA has been inserted is also referred to as the “insertion site” or “target site”. Insertion of the transgene into the plant genome can be associated with a deletion of plant DNA, referred to as “target site deletion”. A “flanking region” or
“flanking sequence” as used herein refers to a sequence of at least 20 bp, preferably at least 50 bp, and up to 5000 bp of the plant genome which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the foreign DNA. Transformation procedures leading to random integration of the foreign DNA will result in transformants with different flanking regions, which are characteristic and unique for each transformant. When the transgene is introduced into a plant through traditional crossing, its insertion site in the plant genome, or its flanking regions will generally not be changed. An “insertion region” as used herein refers to the region corresponding to the region of at least 40 bp, preferably at least 100 bp, and up to more than 10000 bp, encompassed by the upstream and the downstream flanking regions of a transgene in the (untransformed) plant genome and including the insertion site (and possible target site deletion). Taking into consideration minor differences due to mutations within a species, an insertion region will retain at least 85%, preferably 90%, more preferably 95%, and most preferably 100% sequence identity with the sequence comprising the upstream and downstream flanking regions of the foreign DNA in a given plant of that species.

Expression of a gene of interest refers to the fact that the gene confers on the plant one or more phenotypic traits (e.g. herbicide tolerance) that were intended to be conferred by the introduction of the recombinant DNA molecule – the transforming DNA - used during transformation (on the basis of the structure and function of part or all of the gene(s) of interest).

An “event” is defined as a (artificial) genetic locus that, as a result of genetic manipulation, carries a foreign DNA comprising at least one copy of the gene(s) of interest. The typical allelic states of an event are the presence or absence of the foreign DNA. As used herein an “MS” event and an “RF” event will refer to events carrying the “TA29-barnase” and “TA29-barstar” transgenes respectively. An event is characterized phenotypically by the expression of one or more transgene. At the genetic level, an event is part of the genetic makeup of a plant. At the molecular level, an event is characterized by the restriction map (e.g. as determined by Southern blotting) and/or by the upstream
and/or downstream flanking sequences of the transgene, and/or the molecular configuration of the transgene. Usually transformation of a plant with a transforming DNA comprising at least one gene of interest leads to a multitude of events, each of which is unique.

An “elite event”, as used herein, is an event which is selected from a group of events, obtained by transformation with the same transforming DNA or by back-crossing with plants obtained by such transformation, based on the expression and stability of the transgene and its compatibility with optimal agronomic characteristics of the plant comprising it. Thus the criteria for elite event selection are one or more, preferably two or more, advantageously all of the following:

a) That the presence of the transgene does not compromise other desired characteristics of the plant, such as those relating to agronomic performance or commercial value;

b) That the event is characterized by a well defined molecular configuration which is stably inherited and for which appropriate diagnostic tools for identity control can be developed;

c) That the gene(s) of interest in the transgene show(s) a correct, appropriate and stable spatial and temporal phenotypic expression, both in heterozygous (or hemizygous) and homozygous condition of the event, at a commercially acceptable level in a range of environmental conditions in which the plants carrying the event are likely to be exposed in normal agronomic use;

It is preferred that the foreign DNA is associated with a position in the plant genome that allows introgression into desired commercial genetic backgrounds.

The status of an event as an elite event is confirmed by introgression of the elite event in different relevant genetic backgrounds and observing compliance with one, two or all of the criteria e.g. a), b) and c) above.

Additionally, for the transgenes encoding male sterility and fertility restoration described herein, selection of the elite events will also be determined on the compatibility between these events, more specifically that the progeny resulting from a cross between a plant
carrying a male-sterility event and a plant carrying a fertility restorer event, in which both events are present have the following characteristics:

a) adequate phenotypic expression of the fertility restored phenotype, i.e. male fertility; and

b) phenotypic expression at a commercially acceptable level in a range of environmental conditions in which plants carrying the two events are likely to be exposed in normal agronomic use;

An "elite event" thus refers to a genetic locus comprising a transgene, which answers to the above-described criteria. A plant, plant material or progeny such as seeds can comprise one or more elite events in its genome.

The "diagnostic tools" developed to identify an elite event or the plant or plant material comprising an elite event, are based on the specific genomic characteristics of the elite event, such as, a specific restriction map of the genomic region comprising the foreign DNA and/or the sequence of the flanking region(s) of the transgene. A "restriction map" as used herein refers to a set of Southern blot patterns obtained after cleaving plant genomic DNA with a particular restriction enzyme, or set of restriction enzymes and hybridization with a probe sharing sequence similarity with the transgene under standard stringency conditions. Standard stringency conditions as used herein refers to the conditions for hybridization described herein or to the conventional hybridizing conditions as described by Sambrook et al. (1989) (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, NY) which for instance can comprise the following steps: 1) immobilizing plant genomic DNA fragments on a filter, 2) prehybridizing the filter for 1 to 2 hours at 42°C in 50% formamide, 5 X SSPE, 2 X Denhardt's reagent and 0.1% SDS, or for 1 to 2 hours at 68°C in 6 X SSC, 2 X Denhardt's reagent and 0.1% SDS, 3) adding the hybridization probe which has been labeled, 4) incubating for 16 to 24 hours, 5) washing the filter for 20 min. at room temperature in 1 X SSC, 0.1% SDS, 6) washing the filter three times for 20 min. each at 68°C in 0.2 X SSC, 0.1% SDS, and 7) exposing the filter for 24 to 48 hours to X-ray film at -70°C with an intensifying screen.
Due to the (endogenous) restriction sites present in a plant genome prior to incorporation of the foreign DNA, insertion of a foreign DNA will alter the specific restriction map of that genome. Thus, a particular transformant or progeny derived thereof can be identified by one or more specific restriction patterns. The conditions for determining the restriction map of an event are laid out in a “restriction map identification protocol”. Alternatively, once one or both of the flanking regions of the transgene have been sequenced, PCR-probes can be developed which specifically recognize this (these) sequence(s) in a “PCR identification protocol”. Plants or plant material comprising an elite event can be identified by testing according to the PCR identification protocol using these specific primers.

As used in herein, a “biological sample” is a sample of a plant, plant material or products comprising plant material. The term “plant” is intended to encompass WOSR (Brassica napus) plant tissues, at any stage of maturity, as well as any cells, tissues, or organs taken from or derived from any such plant, including without limitation, any seeds, leaves, stems, flowers, roots, single cells, gametes, cell cultures, tissue cultures or protoplasts. “Plant material”, as used herein refers to material which is obtained or derived from a plant. Products comprising plant material relate to food, feed or other products which are produced using plant material or can be contaminated by plant material. It is understood that, in the context of the present invention, such biological samples are preferably tested for the presence of nucleic acids specific for MS-BN1 and/or RF-BN1, implying the presence of nucleic acids in the samples. Thus the methods referred to herein for identifying elite event MS-BN1 and/or RF-BN1 in biological samples, preferably relate to the identification in biological samples of nucleic acids which comprise the elite event.

A “kit” as used herein refers to a set of reagents for the purpose of performing the method of the invention, more particularly, the identification of the elite event MS-BN1 and/or RF-BN1 in biological samples. More particularly, a preferred embodiment of the kit of the invention comprises at least one or two specific primers, as described above. Optionally, the kit can further comprise any other reagent described herein in the PCR identification protocol. Alternatively, according to another embodiment of this invention, the kit can comprise a specific probe, as described above, which specifically hybridizes with nucleic
acid of biological samples to identify the presence of MS-BN1 and/or RF-BN1 therein. Optionally, the kit can further comprise any other reagent (such as but not limited to hybridizing buffer, label) for identification of MS-BN1 and/or RF-BN1 in biological samples, using the specific probe.

The kit of the invention can be used, and its components can be specifically adjusted, for purposes of quality control (e.g., purity of seed lots), detection of the elite event in plant material or material comprising or derived from plant material, such as but not limited to food or feed products.

The present invention relates to the development of a set of elite events in WOSR, MS-BN1 and RF-BN1, to the plants comprising these events, the progeny obtained from the crossing of these plants and to the plant cells, or plant material derived from these events. Plants comprising elite event MS-BN1 were obtained through transformation with pTHW107 as described in example 1. Plants comprising elite event RF-BN1 were obtained through transformation with pTHW118, also described in example 1.

The recombinant DNA molecule used for generation of elite event MS-BN1 comprises a DNA sequence encoding a barnase molecule, under the control of a promoter directing expression selectively in tapetum cells (termed “TA29-barnase”). The TA29 promoter has a “tapetum selective” expression pattern in OSR (De Block and Debrouwer, Planta 189:218-225, 1993). The expression of the TA29-barnase gene in WOSR plants results in destruction of the tapetum rendering the plants male-sterile (Mariani et al, 1990, above). The recombinant DNA molecule used for generation of elite event RF-BN1 comprises a DNA sequence encoding a barstar molecule, wherein the under the control of a tapetum specific promoter (termed “PTA29-barstar”). The expression of the TA29-barstar gene in WOSR plants will, in the presence of a “TA29-barnase” gene in the plant prevent the activity of barnase in the tapetum cells of the plant, preventing the destruction of the tapetum and thus restoring fertility in these plants (Mariani et al. 1992, above).

The recombinant DNAAs used for the generation of elite event MS-BN1 and RF-BN1 both additionally comprise a DNA sequence encoding the enzyme phosphinothricin acetyl
transferase and the 35S promoter of Cauliflower Mosaic Virus, wherein the sequence encoding phosphinothricin acetyl transferase is under the control of the 35S promoter (termed “35S-bar”). The 35S promoter has a “constitutive” expression pattern in OSR, which means that it is significantly expressed in most cell types, during most of the plant life cycle. The expression of the 35S-bar gene in OSR plants confers resistance to herbicidal compounds phosphinothricin or bialaphos or glufosinate, or more generally, glutamine synthetase inhibitors, or salts or optical isomers thereof.

WOSR Plants or plant material comprising MS-BN1 can be identified according to the restriction map identification protocol described for MS-BN1 in Example 5 herein. Briefly, WOSR genomic DNA is digested with a selection (preferably two to five) of the following restriction enzymes: EcoRI, EcoRV, Ndel, Hpal, AflIII, is then transferred to nylon membranes and hybridized with the 3942 bp HindIII fragment of plasmid pTHW107 (or of the T-DNA comprised therein). It is then determined for each restriction enzyme used whether the following fragments can be identified:

- EcoRI: one fragment of between 2140 and 2450 bp, preferably of about 2266 bp, and one fragment of more than 14 kbp;
- EcoRV: one fragment of between 1159 and 1700 bp, preferably of about 14 kbp and one fragment of more than 14 kbp;
- Hpal: one fragment of between 1986 and 2140 bp, preferably of about 1990 bp, and one fragment of between 2140 and 2450 bp, preferably of about 2229 bp;
- AflIII: one fragment of between 514 and 805 bp, preferably of about 522 bp, one fragment of between 2140 and 2450 bp, preferably of about 2250 bp, and one fragment of between 2450 and 2838 bp, preferably of about 2477 bp;
- Ndel: two fragments with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp;

The lengths of the DNA fragments are determined by comparison with a set of DNA fragments of known length, particularly the PstI fragments of phage lambda DNA. A fragment of more than 14 kbp is estimated to have a length between 14 kbp and 40 kbp, when extraction of the DNA occurs according to the method of Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol. 3, p. 19-21).
If the plant material after digestion with at least two, preferably at least three, particularly with at least four, more particularly with all of these restriction enzymes, yields DNA fragments with the same length as those described above, the WOSR plant is determined to harbor elite event MS-BN1.

Plants or plant material comprising MS-BN1 can also be identified according to the PCR identification protocol described for MS-BN1 in Example 5 herein. Briefly, WOSR genomic DNA is amplified by PCR using a primer which specifically recognizes a flanking sequence of MS-BN1, preferably recognizing the 5' or 3' flanking sequence of MS-BN1 described herein, particularly the primer with the sequence of SEQ ID No 19, and a primer which recognizes a sequence in the transgene, particularly the primer with the sequence of SEQ ID No 12. Endogenous WOSR primers are used as controls. If the plant material yields a fragment of between 260 and 300 bp, preferably of about 280 bp, the WOSR plant is determined to harbor elite event MS-BN1.

Plants harboring MS-BN1 are phenotypically characterized by the fact that, in the absence of a restorer gene in their genome, they are male sterile. A male sterile plant is defined as not being able to produce fertile, viable pollen. Plants harboring MS-BN1 can, for example, be obtained from seeds comprising MS-BN1 deposited at the ATCC under accession number PTA-730. Such plants can be further propagated to introduce the elite event of the invention into other cultivars of the same plant species.

WOSR Plants or plant material comprising RF-BN1 can be identified according to the restriction map identification protocol described for RF-BN1 in Example 5 herein. Briefly, WOSR genomic DNA is digested with a selection (preferably two to four) of the following restriction enzymes: BamHI, EcoRI, EcoRV, and HindIII, is then transferred to nylon membranes and hybridized with the 2182 bp Hpal fragment of plasmid pTHW118 (or of the T-DNA comprised therein). It is then determined for each restriction enzyme used whether the following fragments can be identified:
- BamHI: one fragment of between 805 and 1099 bp, preferably of about 814 bp, one fragment of between 1700 and 1986 bp, preferably of about 1849 bp, one fragment of between 2450 and 2838 bp, preferably of about 2607 bp, and one fragment of between 5077 and 14057 bp, preferably of about 6500 bp;

- EcoRI: one fragment of between 805 and 1159 bp, preferably of about 1094 bp, one fragment of between 1986 and 2450 bp, preferably of about 2149 bp, and two fragments of between 5077 and 14057 bp, preferably one of about 7000 bp, and one of about 10 kbp;

- EcoRV: two fragments of between 5077 and 14057 bp, preferably one of about 5.4 kbp and of about 8 kbp;

- HindIII: one fragment of between 1700 and 1986 bp, preferably of about 1969 bp, and two fragments of between 2450 and 2838 bp, preferably one of about 2565 bp, and one of about 2635 bp;

The lengths of the DNA fragments are determined by comparison with a set of DNA fragments of known length, particularly the PstI fragments of phage lambda DNA.

If the plant material after digestion with at least two, preferably at least 3, more particularly with all of these restriction enzymes, yields DNA fragments with the same length as those described above, the WOSR plant is determined to harbor elite event RF-BN1.

Plants or plant material comprising RF-BN1 can also be identified according to the PCR identification protocol described for RF-BN1 in Example 5 herein. Briefly, WOSR genomic DNA is amplified by PCR using a primer which specifically recognizes a flanking sequence of RF-BN1, preferably the 5' or 3' flanking sequence of RF-BN1 described herein, particularly the primer with the sequence of SEQ ID No 41, and a primer which recognizes a sequence in the transgene, particularly the primer with the sequence of SEQ ID No 23. Endogenous WOSR primers are used as controls. If the plant material yields a fragment of between 195 and 235 bp, preferably of about 215 bp, the WOSR plant is determined to harbor elite event RF-BN1.
Plants harboring RF-BN1 are characterized by the fact that barstar is expressed in the cells of the tapetum. The production of barstar in the tapetum cells of the plant has been shown to be neither beneficial nor detrimental to the production of pollen (Mariani et al. 1992, above). Thus, in the absence of a male sterility gene in the genome of the plant, the TA29-barstar gene will not result in an observable phenotype. In the presence of a male-sterility gene in the genome of the plant, the TA29-barstar gene will result in a fertility restored i.e., fertile phenotype. A plant with a fertility restored phenotype is defined as a plant which, despite the presence of a male sterility gene in its genome, is capable of producing fertile, viable pollen.

Plants harboring RF-BN1 can, for example, be obtained from seeds deposited at the ATCC under accession number PTA-730. Such plants can be further propagated and/or used in a conventional breeding scheme to introduce the elite event of the invention into other cultivars of the same plant species.

Plants harboring MS-BN1 and/or RF-BN1 are also characterized by their glufosinate tolerance, which in the context of the present invention includes that plants are tolerant to the herbicide Liberty™. Tolerance to Liberty™ is defined by the criterium that spraying of the plants in the three to four leaf stage (3V to 4V) with at least 200 grams active ingredient/hectare (g.a.i./ha), preferably 400 g.a.i./ha, and possibly up to 1600 g.a.i./ha, does not kill the plants. Plants harboring MS-BN1 and/or RF-BN1 can further be characterized by the presence in their cells of phosphinothricin acetyl transferase as determined by a PAT assay (De Block et al, 1987, supra).

The WOSR plants of this invention can be cultivated in a conventional way. The presence of the 35S-bar gene ensures that they are tolerant to glufosinate. Therefore, weeds in the fields where such WOSR plants are grown can be controlled by application of herbicides comprising glufosinate as an active ingredient (such as Liberty™).

Plants harboring MS-BN1 and/or RF-BN1 are also characterized by having agronomical characteristics which are comparable to commercially available WOSR varieties in the
US. The agronomical characteristics of relevance are: plant height, strength/stiffness of straw, tendency to lodge, winter-hardiness, shatter resistance, drought tolerance, disease resistance (Black leg, Light leafspot, Sclerotinia) and grain production and yield.

It has been observed that the presence of the foreign DNA in the insertion regions of the Brassica napus WOSR plant genome described herein, more particularly at these insertion sites in the Brassica napus WOSR plant genome, confers particularly interesting phenotypic and molecular characteristics to the plants comprising these events. More specifically, the presence of the foreign DNA in these particular regions in the genome of these plants results in stable phenotypic expression of the transgenes without significantly compromising any aspect of desired agronomic performance of the plants, making them particularly suited for the production of hybrid WOSR. Thus, the insertion regions, corresponding to SEQ ID No 22 and SEQ ID No 34, more particularly the insertion site of MS-BN1 and RF-BN1 therein, is shown to be particularly suited for the introduction of a gene(s) of interest. More particularly, the insertion regions of MS-BN1 (SEQ ID No 22) and of RF-BN1 (SEQ ID No 34), or the insertion sites of MS-BN1 and RF-BN1 respectively therein, are particularly suited for the introduction of plasmids comprising a male-sterility gene and a fertility restorer gene respectively ensuring optimal expression of each of these genes or of both genes in a plant without compromising agronomic performance.

A recombinant DNA molecule can be specifically inserted in an insertion region by targeted insertion methods. Such methods are well known to those skilled in the art and comprise, for example, homologous recombination using a recombinase such as, but not limited to either FLP recombinase from Saccharomyces cerevisiae (US Patent 5,527,695), the CRE recombinase from Escherichia coli phage P1 (published PCT application WO 9109957, the recombinase from pSRI of Saccharomyces rouxii (Araki et al. 1985, J Mol Biol 182:191-203), or the lambda phage recombination system such as described in US Patent 4,673,640.

As used herein, "sequence identity" with regard to nucleotide sequences (DNA or RNA), refers to the number of positions with identical nucleotides divided by the number of
nucleotides in the shorter of the two sequences. The alignment of the two nucleotide sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann, 1983) using a window-size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can, e.g., be conveniently performed using the programs of the IntelligeneticsTM Suite (Intelligenetics Inc., CA). Sequences are indicated as “essentially similar” when such sequence have a sequence identity of at least about 75%, particularly at least about 80 %, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially are identical. It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

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

The following examples describe the development and characteristics of WOSR plants harboring the elite events MS-BN1 and RF-BN1.

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

SEQ ID No 1: plasmid pTHW107
SEQ ID No 2: plasmid pTHW118
SEQ ID No 3: primer 248
SEQ ID No 4: primer 249
SEQ ID No 5: primer 247
SEQ ID No 6: primer 250
SEQ ID No 7: primer 251
SEQ ID No 8: primer 254
SEQ ID No 9: primer 258
SEQ ID No 10: primer SP6
SEQ ID No 11: primer T7
SEQ ID No 12: primer 201 (BNA01)
SEQ ID No 13: sequence comprising the 5’ flanking region of MS-BN1
SEQ ID No 14: primer 611
SEQ ID No 15: primer 259
SEQ ID No 16: primer 260
SEQ ID No 17: primer 24
SEQ ID No 18: sequence comprising the 3’ flanking region of MS-BN1
SEQ ID No 19: primer 51 (BNA02)
SEQ ID No 20: primer 48
SEQ ID No 21: sequence comprising the target site deletion of MS-BN1
SEQ ID No 22: insertion region MS-BN1
SEQ ID No 23: primer 193 (BNA03)
SEQ ID No 24: sequence comprising the 5’ flanking region of RF-BN1
SEQ ID No 25: primer 286
SEQ ID No 26: primer 314
SEQ ID No 27: primer 315
SEQ ID No 28: primer 316
SEQ ID No 29: primer 288
SEQ ID No 30: sequence comprising the 3’ flanking region of RF-BN1
SEQ ID No 31: primer 269
SEQ ID No 32: primer 283
SEQ ID No 33: primer 284
SEQ ID No 34: integration region RF-BN1
SEQ ID No 35: primer 57
SEQ ID No 36: sequence comprising the 5’ flanking region of MS-BN1 in WOSR
SEQ ID No 37: primer 68
SEQ ID No 38: sequence comprising the 3’ flanking region of MS-BN1 in WOSR
SEQ ID No 39: sequence comprising the 5’ flanking region of RF-BN1 in WOSR
SEQ ID No 40: sequence comprising the 3’ flanking region of RF-BN1 in WOSR
SEQ ID No 41: primer 268 (BNA04)
SEQ ID No 42: primer BNA05
SEQ ID No 43: primer BNA06
EXAMPLES

Example 1. Transformation of *Brassica napus* with a male-sterility gene and a restorer gene

a) Construction of the chimeric DNA comprising the barnase gene under the control of a tapetum specific promoter (pTHW107).

Plasmid pTHW107 (SEQ ID No. 1) was essentially derived from the intermediate vector pGSV1. PGSV1 is itself derived from pGSC1700 (Cornelissen and Vandewiele, 1989), but comprises an artificial T-region consisting of the left and right border sequences of the TL-DNA form pTiB6S3 and multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats. The pGSV1 vector is provided with a barstar gene on the plasmid mainframe, with regulatory signals for expression in E. coli.

A full description of the DNA comprised between the border repeats of pTHW107 is given in Table 1:

<table>
<thead>
<tr>
<th>nt positions</th>
<th>Orientation</th>
<th>Description and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-97</td>
<td></td>
<td>Synthetic polylinker derived sequences</td>
</tr>
<tr>
<td>310-330</td>
<td></td>
<td>Synthetic polylinker derived sequences</td>
</tr>
<tr>
<td>882-331</td>
<td>Counter clockwise</td>
<td>The coding sequence of the <em>bar</em> gene of <em>Streptomyces hygroscopicus</em> (Thompson <em>et al.</em> (1987) The EMBO Journal 6: 2519-2523). The N-terminal two codons of the wild type <em>bar</em> coding region have been substituted for the codons ATG and GAC respectively.</td>
</tr>
<tr>
<td>2608-883</td>
<td>Counter clockwise</td>
<td>The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from <em>Arabidopsis thaliana</em> (PssuAra) (Krebbers <em>et al.</em> (1988) Plant Molecular Biology 11: 745-759).</td>
</tr>
</tbody>
</table>
b) Construction of the chimeric DNA comprising the barstar gene under the control of a constitutive promoter (pTHW118)

Plasmid pTHW118 (SEQ ID No. 2) was also essentially derived from the intermediate vector pGSV1 (described above). A full description of the DNA comprised between the border repeats of pTHW118 is given in Table 2:

<table>
<thead>
<tr>
<th>nt positions</th>
<th>Orientation</th>
<th>Description and references</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-53</td>
<td></td>
<td>Synthetic polylinker derived sequences</td>
</tr>
<tr>
<td>54-90</td>
<td></td>
<td>Residual sequence from the TL-DNA at the right border repeat.</td>
</tr>
<tr>
<td>91-97</td>
<td></td>
<td>Synthetic polylinker derived sequences.</td>
</tr>
<tr>
<td>310-330</td>
<td>Synthetic polynucleotide derived sequences</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>883-331</td>
<td>Counter clockwise</td>
<td></td>
</tr>
</tbody>
</table>
|         | The coding sequence of the bialaphos resistance gene (bar) of *Streptomyces hygroscopicus* (Thompson *et al.* (1987) The EMBO Journal 6: 2519-2523). The N-terminal two codons of the wild type bar coding region have been substituted for the codons ATG and GAC respectively.  
2608-883 | Counter clockwise                        |
|         | The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PssuAra) (Krebbers *et al.* (1988) Plant Molecular Biology 11: 745-759).  
2609-2658 | Synthetic polynucleotide derived sequences |
| 2919-2659 | Counter clockwise                        |
2920-2940 | Synthetic polynucleotide derived sequences |
| 2941-2980 | 3'untranslated region downstream from the barstar coding sequence from *Bacillus amyloliquefaciens* |
| 3253-2981 | Counter clockwise                        |
4762-3254 | Counter clockwise                        |
|         | The promoter region of the anther-specific gene TA29 from *Nicotiana tabacum*. The promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon (Seurinck *et al.* (1990) Nucleic Acids Research 18: 3403).  
4763-4807 | Synthetic polynucleotide derived sequences |

c) Transformation of *Brassica napus*

For transformation of *Brassica napus* the vector system as described by Deblaere *et al.* (1985, 1987) was used. The vector system consists of an Agrobacterium strain and two plasmid components: 1) a non-oncogenic Ti-plasmid (pGV400) and 2) an intermediate cloning vector based on plasmid pGSV1. The non-oncogenic Ti-plasmid from which the
T-region has been deleted carries the vir genes required for transfer of an artificial T-DNA cloned on the second plasmid to the plant genome. The Agrobacterium strains resulting from the triparental mating between these components can be used for plant transformation.

Selection was done on phosphinothricin (PPT) at all stages except plantlet regeneration, which was done in the absence of PPT to accelerate growth. This resulted in a set of primary transformants (plants of generation \( T_0 \)).

Example 2. Development of events

2.1. Characterization of transgenic events

2.1.1. Southern blot analysis of MS events

Presence of the transgene and the number of gene insertions were checked by standard Southern blot analysis. Total genomic DNA is isolated from 1g of shoot tissue according to Dellaporta (1983, Plant Molecular Biology Reporter, 1, vol.3, p.19-21 or Doyle et al. 1987, Phytochem. Bull. 19:11) and digested with SacI restriction enzyme. SacI has a unique restriction site within the T-DNA fragment, situated between the barnase and bar constructs. Southern analysis was performed with the following two probes:

“barnase” probe: 478 bp PstI-EcoRI fragment of plasmid pVE113
“bar” probe: 546 bp NcoI-BgIII fragment of plasmid pDE110

Plasmid pVE113 and pDE110 are described in Figure 1 and WO 92/09696 respectively. Hybridisation of the MS events with the barnase probe yielded a 12 Kb band, while hybridization with the bar probe yielded a 14 Kb fragment. The relative band intensity provided an indication on whether plants were homozygous or hemizygous for the transgenic locus. Two events were found to have simple insertions.
This was confirmed by the fact that the segregation pattern of the transgene could be explained by Mendelian inheritance of a simple locus.

2.1.2. Southern blot analysis of RF events

Presence of the transgene and the number of gene insertions were checked by standard Southern blot analysis. Total genomic DNA was isolated from 1g of shoot tissue (according to Doyle et al. 1987, Phytochem. Bull. 19:11) and digested with SacI restriction enzyme. SacI has a unique restriction site within the T-DNA fragment, situated between the barnase and bar constructs. Southern analysis was performed with the following two probes:

“barstar” probe: 436 bp HindIII-PstI fragment of plasmid pVE113
“bar” probe: 546 bp NcoI-BgIII fragment of plasmid pDE110

Hybridisation of the RF events with the barstar probe yielded a 3 Kb band, while hybridization with the bar probe yielded a 14 Kb fragment. The relative band intensity provided an indication on whether plants were homozygous or hemizygous for the transgenic locus. Several events were found to have simple insertions. This was confirmed by the fact that the segregation pattern of the transgene could be explained by Mendelian inheritance of a simple locus.

2.1.3. General plant phenotype and agronomic performance

T1 plants of both MS and RF events were evaluated for a number of phenotypic traits including plant height, strength/stiffness of straw, tendency to lodge, shatter resistance, drought tolerance, disease resistance (Black leg, Light leafspot, Sclerotinia) and grain production and yield.

Lines were evaluated to be similar (or improved) in displayed agronomic characteristics compared to the untransformed variety as well as a number of oilseed rape cultivars. In some cases, the plants segregated for somaclonal variation for one or more of the above-
mentioned traits. Unless this resulted in the introduction of a commercially interesting phenotypic trait, these plants were discarded.

2.2. Development of lines carrying the MS or RF trait

The various T₀ hemizygous plantlets ("Ms/-" or "RF/-") were transitioned from tissue culture, transferred to greenhouse soil. Presence of the transgene and copy number was checked by southern blot analysis (described above). The plants were allowed to flower and sterility or fertility of flowers was evaluated respectively. The T₀ plants were crossed with wildtype plants (-/-) to produce T₁ seed (MsT₁ and RF₁). T₁ seeds were planted and grown up in the greenhouse. Plants were evaluated for tolerance to glufosinate ammonium. Ms-T₁ plants were also evaluated for sterility/fertility segregation (in non-sprayed plants), while RF-T₁ plants were checked for fertile flowers.

Ms-T₁ plants comprising the transgene were crossed with a tester plant homozygous for a fertility restorer gene (RF/Rf), for the production of MsRF-F₁ seed. This seed (Ms/-, RF/- and -/-, RF/-) was planted in the greenhouse and sprayed with Liberty™. Remaining F₁ progeny is evaluated for fertility/sterility segregation to test whether the male sterility trait could be adequately restored in Brassica napus (fertility close to 100%).

The best events were selected for further testing. Ms-T₁ plants were crossed with a homozygous fertility restorer and the seed was planted in the field. Plants were evaluated for tolerance to the Liberty™ herbicide (at 800 grams active ingredient per hectare (g.a.i./ha) recommended dosage for farmers is 400 g.a.i./ha), for fertility/sterility segregation and for general phenotypic characteristics. The lines in which fertility was 100% restored and for which no negative penalties on phenotype or agronomic performance (detailed under (d)) was observed as compared to the wild-type isogenic control were selected.

RF-T₁ plants comprising the transgene were crossed with a tester plant comprising the male sterility gene (Ms/-), for the production of F₁ seed. This seed was planted in the
greenhouse, sprayed with Liberty™ and restoration of fertility was evaluated (close to 100%).
Meanwhile Rf-T1 plants are selfed to produce S1. The S1 plants are grown in the
greenhouse, sprayed with Liberty™ and again selfed to produce S2; from the S2, homozygous individuals were selected.

2.3. combination of MS and RF events.

The selected Ms-T1 plants were crossed with the selected Rf-S2 events in the greenhouse for fertility restoration testing. The seed was replanted in the greenhouse, plants were sprayed with Liberty™ and fertility of the flowers was checked.

2.4. testing of MS and RF events in different genetic backgrounds and in different locations

The selected events were introduced into two different genetic backgrounds which are heterotically distinct, to prove that the MS and RF events function well and have no negative penalty on yield or quality in any background tested.

At the same time the selected MS and RF events are tested in four to five different environments to ensure that there is no negative interaction between environment and the MS or RF events.

In a next stage the production of hybrid seed using the selected MS and RF events was tested more extensively in the field. The selected MS event in its original background and in two different and heterotically distinct backgrounds was crossed with the selected RF event in its original background and two different and heterotically distinct backgrounds. The F1 hybrid was evaluated for resistance to Liberty™, for fertility, as well as overall agronomic performance (yield and quality).

2.5. selection of elite events
The above described selection procedure in the development of transgenic MS lines, yielded several elite events which displayed optimal expression of the transgene, i.e. resistance to glufosinate ammonium, a male-sterile phenotype and susceptibility to complete fertility restoration with a homozygous restorer line, more specifically with the selected RF elite event.

The above described selection procedure in the development of transgenic RF lines, yielded several elite events which displayed optimal expression of the transgene, i.e. resistance to the glufosinate ammonium and the ability to restore fertility of the F1 when crossed with a plant carrying a male sterility gene, more specifically with the selected MS elite event.

**Example 3: introduction of candidate elite events selected into WOSR**

Several MS and RF elite events which were developed in *B. napus* as described above were introduced by repeated backcrossing of Drakkar variety plants, into a WOSR cultivar.

Plants were examined and it was established that:

a) the presence of the foreign DNA did not compromise other desired characteristics of the plant, such as those relating to agronomic performance or commercial value;

b) the event was characterized by a well defined molecular configuration which was stably inherited;

c) the gene(s) of interest in the foreign DNA showed a correct, appropriate and stable spatial and temporal phenotypic expression, both in heterozygous (or hemizygous) and homozygous condition of the event, at a commercially acceptable level in a range of environmental conditions in which the plants carrying the event are likely to be exposed in normal agronomic use;

Furthermore, the plants were evaluated for their agronomical characteristics and performance as compared with wild-type WOSR species.

Extensive testing in the field demonstrated that certain candidate elite events of spring oilseed rape, when introduced into WOSR resulted in plants which showed adequate
expression of the genes of interest in the foreign DNA, combined with optimal agronomic performance. These events were selected as MS and RF elite events in WOSR and were named MS-BN1 and RF-BN1 respectively.

5  Example 4. Characterization of elite events MS-BN1 and RF-BN1

Once the MS-BN1 and RF-BN1 events were identified as the elite events in which expression of the respective transgenes as well as overall agronomic performance were optimal, the loci of the transgenes were analyzed in detail on a molecular level. This included detailed Southern blot analysis (using multiple restriction enzymes) and sequencing of the flanking regions of the transgene.

4.1. Southern blot analysis using multiple restriction enzymes

Leaf tissue was harvested from transgenic and control plants. Total genomic DNA was isolated from leaf tissue according to Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol.3, p.19-21). The DNA concentration of each preparation was determined by measuring the optical density in a spectrophotometer at a wavelength of 260 nm.

10  10 μg of genomic DNA was digested with restriction enzyme in a final reaction volume of 40 μl, applying conditions proposed by the manufacturer. The time of digestion and/or amount of restriction enzyme were adjusted to ensure complete digestion of the genomic DNA samples without non-specific degradation. After digestion, 4 μl of loading dye was added to the digested DNA samples, and they were loaded on a 1% agarose gel.

20  The following control DNAs were also loaded on the gel:
- a negative control with genomic DNA prepared from a non-transgenic Brassica plant. This negative control is used to confirm the absence of background hybridization.
- a DNA positive control: With a heterozygous single copy integration of the transgene into the Brassica napus genome, 10 μg of genomic DNA has the same number of molecule equivalents as ± 19 picogram of 1501 bp PvuI-HindIII fragment of pTHW118
DNA (*Brassica napus* diploid genome size: 0.8x10^9 bp). The amount representing one plasmid copy per genome is added to 1 μg of digested non-transgenic *Brassica napus* DNA. This reconstitution sample is used to show that the hybridizations are performed under conditions allowing hybridization of the probe with target sequences.

Phage Lambda DNA (strain Clind 1 ts 857 Sam 7, Life Technologies) digested with PstI was included as size standard.

After electrophoresis, the DNA samples (digested *Brassica* genomic DNA, controls and size standard DNA) were transferred to a Nylon membrane by capillary blotting during 12 to 16 hours.

The DNA templates used for probe preparation for MS-BN1 events were prepared by restriction digestion of PTW107 with HindIII. This released a 3942 bp DNA fragment that encompasses a relevant part of the transforming DNA (part of PSSUARA, 3’nos, barnase, PTA29).

The DNA templates used for probe preparation for RF-BN1 events were prepared by restriction digestion of PTW118 with Hpal. This released a 2182 bp DNA fragment that encompasses a relevant part of the transforming DNA (part of PSSUARA, 3’nos, barstar, PTA29).

After purification, the DNA fragments were labeled according to standard procedures, and used for hybridizing to the membrane.

Hybridization was performed under standard stringency conditions: The labeled probe was denaturated by heating for 5 to 10 minutes in a water bath at 95°C to 100°C and chilling on ice for 5 to 10 minutes and added to the hybridization solution (6 X SSC (20 X SSC is 3.0 M NaCl, 0.3 M Na citrate, pH 7.0), 5 X Denhardt's (100 X Denhardt's = 2% Ficoll, 2% Polyvinyl pyrollidone, 2% Bovine Serum Albumin), 0.5 % SDS and 20 μg/ml denatured carrier DNA (single-stranded fish sperm DNA, with an average length of 120 - 3000 nucleotides). The hybridization was performed overnight at 65°C. The blots were washed three times for 20 to 40 minutes at 65°C, with the wash solution (2 X SSC, 0.1 % SDS).

The autoradiographs were electronically scanned.
4.1.1. MS-BN1

The restriction patterns obtained after digestion of MS-BN1 genomic DNA with different restriction enzymes is presented in Figure 2 and summarized in Table 3.

<table>
<thead>
<tr>
<th>Lane number</th>
<th>DNA loaded</th>
<th>Migration of hybridizing DNA fragments between size marker bands</th>
<th>Estimated length of the hybridizing DNA fragments.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS-BN1 – EcoRI</td>
<td>2140 2450</td>
<td>2266 bp (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14057 -</td>
<td>&gt;14 kbp</td>
</tr>
<tr>
<td>2</td>
<td>MS-BN1 – EcoRV</td>
<td>1159 1700</td>
<td>1.4 kbp (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14057 -</td>
<td>&gt;14 kbp</td>
</tr>
<tr>
<td>4</td>
<td>MS-BN1- HpaI</td>
<td>1986 2140</td>
<td>1990 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2140 2450</td>
<td>2229 bp</td>
</tr>
<tr>
<td>5</td>
<td>MS-BN1 – AflIII</td>
<td>2450 2838</td>
<td>2477 bp (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2140 2450</td>
<td>2250 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>514 805</td>
<td>552 bp (*)</td>
</tr>
<tr>
<td>6</td>
<td>MS-BN1 – NdeI</td>
<td>5077 14057</td>
<td>10 kbp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077 14057</td>
<td>6510 bp</td>
</tr>
<tr>
<td>7</td>
<td>Non-transgenic WOSR</td>
<td>- -</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Control plasmid DNA – BamHI</td>
<td>1700 1986</td>
<td>1966 bp (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2450 2838</td>
<td>2607 bp (*)</td>
</tr>
</tbody>
</table>

(*) the lengths of these fragments are those predicted from the restriction map of the plasmid pTHW107

4.1.2. RF-BN1

The restriction patterns obtained after digestion of RF-BN1 genomic DNA with different restriction enzymes is presented in Figure 3 and summarized in Table 4.
Table 4: Restriction map of RF-BN1

<table>
<thead>
<tr>
<th>Lane number</th>
<th>DNA loaded</th>
<th>Migration of hybridizing DNA fragments between size marker bands</th>
<th>Estimated length of the hybridizing DNA fragments.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Larger than</td>
<td>Smaller than</td>
</tr>
<tr>
<td>1</td>
<td>MS-BN1 – BamHI</td>
<td>805</td>
<td>1099</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1700</td>
<td>1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2450</td>
<td>2838</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077</td>
<td>14057</td>
</tr>
<tr>
<td>2</td>
<td>MS-BN1 – EcoRI</td>
<td>805</td>
<td>1159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1986</td>
<td>2450</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077</td>
<td>14057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077</td>
<td>14057</td>
</tr>
<tr>
<td>3</td>
<td>MS-BN1 – EcoRV</td>
<td>5077</td>
<td>14057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077</td>
<td>14057</td>
</tr>
<tr>
<td>4</td>
<td>MS-BN1 – HindIII</td>
<td>1700</td>
<td>2140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2450</td>
<td>2838</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2450</td>
<td>2838</td>
</tr>
<tr>
<td>6</td>
<td>Non-transgenic WOSR</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Control plasmid DNA</td>
<td>1700</td>
<td>1986</td>
</tr>
<tr>
<td></td>
<td>– BamHI</td>
<td>2450</td>
<td>2838</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5077</td>
<td>14057</td>
</tr>
</tbody>
</table>

(*) the lengths of these fragments are those predicted from the restriction map of the pTHW118 vector with BamHI.

4.2. Identification of the flanking regions

Flanking regions of the elite events MS-BN1 and RF-BN1 were first identified for spring OSR, in which the events were developed, and then checked for WOSR.

4.2.1. Identification of the flanking regions of MS-BN1

4.2.1.1. Right (5’) flanking region
The sequence of the right border flanking region of MS-BN1, a ligation-mediated polymerase chain reaction (Mueller et al. 1989, Science 780-786; Maxine et al., 1994, PCR Methods and Applications, 71-75) with extension capture (Törmanen et al., 1993, NAR 20:5487-5488) was used.

5

The oligonucleotides used for linker preparation were:

MDB248: (SEQ ID No. 3)
5'-CAT.GCC.CTG.ACC.CAG.GCT.AAG.TAT.TTT.AAC.TTT.AAC.CAC.TTT.GCT.CCG.ACA.GTC.CCA.TTG

MDB249: (SEQ ID No. 4)
5'-CAA.TGG.GAC.TGT.CGG.AGG.ACT.GAG.GGC.CAA.AGC.TTG.GCT.CTT.AGC.CTG.GGT.CAG.GGC.ATG

10

Preparation of the linker was followed by first strand synthesis from genomic MS-BN1 DNA digested with NcoI, using a biotinylated gene-specific primer:

<table>
<thead>
<tr>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated primer</td>
<td></td>
</tr>
<tr>
<td>MDB247</td>
<td></td>
</tr>
<tr>
<td>CCG.TCA.CCG.AGA.TCT.GAT.CTC.ACG.CG (SEQ ID No. 5)</td>
<td>322 ← 347</td>
</tr>
</tbody>
</table>

15

The linker was then ligated to the first strand DNA, which was then coupled to magnetic beads from which the non-biotinylated strand was eluted. This DNA was used in an upscaled PCR amplification using the following primers:

<table>
<thead>
<tr>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linker primer</td>
<td></td>
</tr>
<tr>
<td>MDB250</td>
<td></td>
</tr>
<tr>
<td>GCACTGAGGGCCAAAGCTTGCTGTC (SEQ ID No. 6)</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

| T-DNA primer       |                     |
| MDB251             |                     |
| GGA.TCC.CCC.GAT.GAG.CTA.AGC.TAG.C (SEQ ID No. 7) | 293 ← 317 |

20

This PCR yielded a fragment of about 1150 bp. This Right Border fragment was eluted out of an agarose gel and a nested PCR was done on a 100 fold dilution of this DNA using the following primers:
This yielded a fragment of about 1000 bp, which was eluted out of the agarose gel, purified, and ligated to the pGem®-T Vector. The recombinant plasmid DNA was screened using a standard PCR reaction with the following primers:

<table>
<thead>
<tr>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested linker primer MDB254</td>
<td>CTTAGCCTGGGGTCAGGCGATG (SEQ ID No. 8)</td>
</tr>
<tr>
<td>T-DNA primer MDB258</td>
<td>CGA.CGA.AGA.TGA.AGA.ATA.C (SEQ ID No. 9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW107</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP6 primer</td>
<td>TAA.TAC.GAC.TCA.CTA.TAG.GGC.GA (SEQ ID No. 10)</td>
</tr>
<tr>
<td>T7 primer</td>
<td>TTT.AGG.TGA.CAC.TAT.AGA.ATA.C (SEQ ID No. 11)</td>
</tr>
<tr>
<td>T-DNA primer MDB201</td>
<td>gCT.TGG.ACT.ATA.ATA.CCT.GAC (SEQ ID No. 12)</td>
</tr>
</tbody>
</table>

This yielded the following fragments: SP6-T7: 1224bp
SP6-MDB201: 1068bp
T7-MDB201: 1044bp

The right border fragment was purified and sequenced (SEQ ID No. 13) resulting in 963bp of which bp 1-867 corresponds to plant DNA and bp 868 to 953 corresponds to T-DNA of pTW107.

4.2.1.2. Left (3’) flanking region of MS-BN1

The sequence of the left border region flanking the inserted transgene in the MS-BN1 event were determined using the thermal asymmetric interlaced (TAIL-) PCR method as
described by Liu et al. (1995, The Plant Journal 8(3): 457-463). This method utilizes three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled. The specific primers were selected for annealing to the border of the transgene and based on their annealing conditions. A small amount (5µl) of unpurified secondary and tertiary PCR products were analyzed on a 1% agarose gel. The tertiary PCR product was used for preparative amplification, purified and sequenced on an automated sequencer using the DyeDeoxy Terminator cycle kit.

The following primers were used:

<table>
<thead>
<tr>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW107</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primer</td>
<td></td>
</tr>
<tr>
<td>MDB611</td>
<td>Gt.Cg.A SWg.TNT.WCA.A</td>
</tr>
<tr>
<td>(SEQ ID No. 14)</td>
<td></td>
</tr>
<tr>
<td>Primary TAIL</td>
<td>gTg.Cag.ggA.AgC.ggT.TAA.CTg.g</td>
</tr>
<tr>
<td>MDB259</td>
<td>(SEQ ID No. 15)</td>
</tr>
<tr>
<td></td>
<td>7164→4186</td>
</tr>
<tr>
<td>Second TAIL</td>
<td>CCT.TTg.Ag.TAA.ATg.TTg.g</td>
</tr>
<tr>
<td>MDB260</td>
<td>(SEQ ID No. 16)</td>
</tr>
<tr>
<td></td>
<td>4346→4366</td>
</tr>
<tr>
<td>Tertiary TAIL</td>
<td>gCg.AAT.gTA.TAT.TAT.ATg.CA</td>
</tr>
<tr>
<td>HCA24</td>
<td>(SEQ ID No. 17)</td>
</tr>
<tr>
<td></td>
<td>4738→4757</td>
</tr>
</tbody>
</table>

whereby: N=A,C,T or g; S=C or g; W=A or T

The fragment amplified using HCA24-MDB611 was ca. 540 bp of which 537 bp were sequenced (3'flank: SEQ ID No 18). The sequence between bp 1 and bp 180 comprised pTHW107 DNA, while the sequence between bp 181 and bp 537 corresponded to plant DNA.

4.2.1.3. Identification of the target site deletion

Using primers corresponding to sequences within the flanking regions of the transgene on the wildtype *Brassica napus* var. Drakkar as a template, the insertion site of the transgene was identified.
The following primers were used:

<table>
<thead>
<tr>
<th>Sequence (5’ → 3’)</th>
<th>Position in 5’flank</th>
<th>Position in 3’flank</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDS51 TgA.CAC.TTT.gAg.CCA.CTC.g</td>
<td>733 → 751</td>
<td>- - - -</td>
</tr>
<tr>
<td>(SEQ ID No. 19)</td>
<td>(SEQ ID No 18)</td>
<td></td>
</tr>
<tr>
<td>HCA48 GgA.ggg.TgT.TTT.Tgg.TTA.TC</td>
<td>- - - -</td>
<td>189 ← 208</td>
</tr>
<tr>
<td>(SEQ ID No. 20)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This yielded a 178 bp fragment (SEQ ID No 21) in which bp 132 to 150 corresponds to a target site deletion.

4.2.1.4. Identification of the MS-BN1 insertion region

Based on the identification of the flanking regions, and the target site deletion the insertion region of MS-BN1 could be determined (SEQ ID No 22):

- 1- 822: 5’ flanking region bp 46-867 of SEQ ID No 13
- 823- 841 target site deletion bp 132-150 of SEQ ID No 21
- 842-1198 3’ flanking region bp 181 to 537 of SEQ ID No 18

4.2.2. Identification of the flanking regions of RF-BN1

The border flanking regions of RF-BN1 were determined by Vectorette-PCR (Use of Vectorette and Subvectorette PCR to isolate transgene flanking DNA, Maxine J. Allen, Andrew Collick, and Alec J. Jeffreys PCR Methods and Applications - 1994 (4) pages 71 - 75) with RF-BN1 genomic DNA digested with HindIII as a template. The vectorette linker was made using the primers MDB248 (SEQ ID No. 3) and MDB249 (SEQ ID No. 4) primers described above.

4.2.2.1. Right (5’) flanking region of BN-RF1
The following primers were used:

<table>
<thead>
<tr>
<th>Prime</th>
<th>Sequence (5' → 3')</th>
<th>Position in pTHW118</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectorette primer MDB250</td>
<td>GCA.CG.CTG.AGG.GCC.AAA.GCT.TGG.CTC (SEQ ID No. 6)</td>
<td></td>
</tr>
<tr>
<td>Vectorette primer MDB254</td>
<td>CTT.AGC.CTG.GGT.CAG.GGC.ATG (SEQ ID No. 8)</td>
<td></td>
</tr>
<tr>
<td>T-DNA primer MDB251</td>
<td>GGA.TCC.CCC.GAT.GAG.CTA.AGC.TAG.C (SEQ ID No. 7)</td>
<td>293 ← 317</td>
</tr>
<tr>
<td>T-DNA primer MDB193</td>
<td>CTA.CGG.CAA.TGT.ACC.AGC (SEQ ID No. 23)</td>
<td>226 ← 243</td>
</tr>
<tr>
<td>T-DNA primer MDB258</td>
<td>CTA.CGG.CAA.TGT.ACC.AGC.TG (SEQ ID No. 9)</td>
<td>224 ← 243</td>
</tr>
<tr>
<td>T-DNA primer MDB201</td>
<td>GCT.TGG.ACT.ATA.ATA.CCT.GAC (SEQ ID No. 12)</td>
<td>143 ← 163</td>
</tr>
</tbody>
</table>

This yielded a 1077 bp fragment (SEQ ID No. 24) in which bp 1-881 corresponds to plant DNA and bp 882-1077 corresponds to T-DNA of pTW118.

4.2.2.2. Left (3’) flanking region of BN-RF1

To identify the 3’ flanking region of elite event BN-RF1, a TAIL PCR was performed as described above using an Arbitrary degenerate primer and primers located in the T-DNA in the vicinity of the left border.

The primers used were:

**Arbitrary Degenerate Primer:**

MDB286 \( \text{NTg.CG.A.SWg.ANA.Wg.A} \) (SEQ ID No. 25)  

whereby: \( N=A, C, T \) or \( g \); \( S=C \) or \( g \); \( W=A \) or \( T \)

**T-DNA primers:**

MDB314 \( \text{gTA.ggA.ggT.ggA.gAA.gAC.C} \) (SEQ ID No. 26)  

MDB315 \( \text{ggg.CTT.TCT.ACT.AgA.AAg.CTC.TCg.g} \) (SEQ ID No. 27)
A fragment of about 2000 bp was obtained. This fragment was cloned in a pGem®-T Vector and used as a template for a PCR reaction using the following primers:

Plant DNA primer:

MDB288 ATg.CAg.CAA.gAA.gCT.Tgg.Agg  (SEQ ID No. 29)

T-DNA primer:

MDB314 gTA.ggA.ggT.Tgg.gAA.gAC.C  (SEQ ID No. 26)

This yielded a fragment of about 1500 bp (SEQ ID No. 30) wherein bp 1-166 corresponds to T-DNA from plasmid pTW118 and bp 167-1441 corresponds to plant DNA.

4.2.2.3. Molecular analysis of the target-site deletion

The target site deletion was cloned with the TAIL-PCR method (described above), using wild type genomic DNA and plant DNA specific primers upstream of the T-DNA insert directed towards the insert:

Arbitrary Degenerate primer:

MDB286 NTg.CgA.SWg.ANA.WgA.A  (SEQ ID No. 25)

whereby: N=A,C,T or g; S=C or g; W=A or T

Plant DNA primers:

MDB269 ggTTTTTCggAggTCCgAgACg  (SEQ ID No. 31)
MDB283 CTTggACCCCTAggTAAATgC  (SEQ ID No. 32)
MDB284 gTACAAAAACTTggACCCCTAgg  (SEQ ID No. 33)

A fragment of 1068 bp (SEQ ID No. 34) was obtained, in which:

53-83: 5’ flanking region
84-133: target site deletion
134-1055: 3' flanking region

Upon insertion of the T-DNA, 51bp of the target site were deleted. Comparing the wild type locus sequence with the Rf3 locus revealed the presence of filler DNA at the Right border junction. The filler TCTCG sequence at the Right border is flanked by TCA at the 5'end and CGA at the 3'end. These triplets are also found at the breakpoint of the target site deletion and the T-DNA respectively. A search in more distal plant sequences revealed a possible origin of this filler DNA. The TCA.TCTCG.CGA sequence is also located in the plant DNA at the 3'end of the target site deletion. It is the core sequence of two 13bp identical repeats located 209bp downstream of the breakpoint of the target site deletion.

The insertion region for RF-BN1 can be defined as comprising the left flanking region, the target site deletion and the right flanking region as follows:

1-881: 5'flanking region (bp 1-881 of SEQ ID No 24)
882-932: target site deletion (bp 84-133 of SEQ ID No. 34)
933-2207 3’ flanking region (bp 167-1441 of SEQ ID No. 30)

4.3. genetic analysis of the locus

The genetic stability of the inserts for the two events was checked by molecular and phenotypic analysis in the progeny plants over several generations. Southern blot analyses of plants of the T₀, T₁ and T₂ generation were compared for both event MS-BN1 and RF-BN1. The patterns obtained were found to be identical for each of the events in the different generations. This proves that the molecular configuration of the transgenes in both MS-BN1 and RF-BN1 containing plants was stable.
The MS-BN1 and RF-BN1 events displayed Mendelian segregation for their respective transgenes as single genetic loci in at least three subsequent generations indicating that the inserts are stable.

On the basis of the above results MS-BN1 and MS-RF1 were identified as elite events.

4.4. Identification of the flanking sequences of MS-BN1 and RF-BN1 in WOSR

The flanking sequences of the elite events MS-BN1 and RF-BN1 in WOSR were determined using primers which were developed based on the flanking sequences of these events in spring oilseed rape.

MS-BN1 WOSR right (5') flanking sequence was determined using a T-DNA primer (SEQ ID No. 12) and a primer located in the MS-BN1 right border plant DNA:

VDS57: 5'-gCA.TgA.TCT.gCT. Cgg.gAT.ggC-3'  (SEQ ID No. 35)

This yielded a fragment of 909 bp (SEQ ID No. 36) with a sequence essentially similar to the sequence of SEQ ID No. 13 (starting from nucleotide 98).

MS-BN1 WOSR left (3') flanking sequence was determined using a T-DNA primer (SEQ ID No. 17) and a primer located in the MS-BN1 left border plant DNA:

HCA68: 5'-CCA.TAT.Acg.CCA.gAg.Agg.AC-3'  (SEQ ID No. 37)

This yielded a fragment of 522 bp (SEQ ID No. 38) with a sequence essentially similar to the sequence of SEQ ID No. 18.

RF-BN1 WOSR right (5') flanking sequence was determined using a T-DNA primer (SEQ ID No. 12) and a primer located in the RF-BN1 right border plant DNA (SEQ ID No. 31). This yielded a fragment of 694 bp (SEQ ID No. 39) with a sequence essentially similar to the sequence of SEQ ID No. 24 (from nucleotide 293 to 980).

RF-BN WOSR left border sequence was determined using a T-DNA primer (SEQ ID No. 26) and a primer located in the RF-BN1 left border plant DNA (SEQ ID No. 29). This yielded a fragment of 1450 bp of which 1279 were sequenced (SEQ ID No. 40). This
sequence was found to be essentially similar to the sequence of SEQ ID No. 30 (from nucleotide 141 to 1421).

Thus, left and right border sequences of the elite events MS-BN1 and RF-BN1 were confirmed to be essentially similar in SOSR and WOSR.

Example 5: Development of diagnostic tools for identity control

The following protocols were developed to identify any WOSR plant material comprising the elite event MS-BN1.

5.1. MS-BN1 and RF-BN1 Elite event Restriction map identification protocol

WOSR plants containing the elite event MS-BN1 can be identified by Southern blotting using essentially the same procedure as described in Example 4.1. Thus WOSR genomic DNA is 1) digested with at least two, preferably at least 3, particularly with at least 4, more particularly with all of the following restriction enzymes: EcoRI, EcoRV, Ndel, HpaI, AflIII 2) transferred to nylon membranes and 3) hybridized with the 3942 bp HindIII fragment of plasmid pTHW107. If, with respect to at least two of the restriction enzymes used, DNA fragments are identified with the same length as those listed in Table 3 of Example 4.1.1., the WOSR plant is determined to harbor elite event MS-BN1.

WOSR plants containing the elite event RF-BN1 can be identified by Southern blotting using essentially the same procedure as described in Example 4.1. Thus WOSR genomic DNA is 1) digested with at least two, preferably at least three, most preferably with all of the following restriction enzymes: BamHI, EcoRI, EcoRV, HindIII 2) transferred to nylon membranes and 3) hybridized with the 2182 bp HpaI fragment of plasmid pTHW118. If, with respect to at least two of the restriction enzymes used, DNA fragments are identified with the same length as those listed in Table 4 in Example 4.1.2., the WOSR plant is determined to harbor elite event RF-BN1.
5.2. MS-BN1 and RF-BN1 Elite event Polymerase Chain reaction identification protocol

A test run, with all appropriate controls, has to be performed before attempting to screen unknowns. The presented protocol might require optimization for components that may differ between labs (template DNA preparation, Taq DNA polymerase, quality of the primers, dNTP’s, thermocycler, etc.).

Amplification of the endogenous sequence plays a key role in the protocol. One has to attain PCR and thermocycling conditions that amplify equimolar quantities of both the endogenous and transgenic sequence in a known transgenic genomic DNA template. Whenever the targeted endogenous fragment is not amplified or whenever the targeted sequences are not amplified with the same ethidium bromide staining intensities, as judged by agarose gel electrophoresis, optimization of the PCR conditions may be required.

5.2.1. Template DNA

Template DNA is prepared from a leaf punch or a single seed according to Edwards et al. (Nucleic Acid Research, 19, p1349, 1991). When using DNA prepared with other methods, a test run utilizing different amounts of template should be done. Usually 50 ng of genomic template DNA yields the best results.

5.2.2. Assigned positive and negative controls

The following positive and negative controls should be included in a PCR run:

- Master Mix control (DNA negative control). This is a PCR in which no DNA is added to the reaction. When the expected result, no PCR products, is observed this indicates that the PCR cocktail was not contaminated with target DNA.
- A DNA positive control (genomic DNA sample known to contain the transgenic sequences). Successful amplification of this positive control demonstrates that the PCR was run under conditions which allow for the amplification of target sequences.

- A wildtype DNA control. This is a PCR in which the template DNA provided is genomic DNA prepared from a non-transgenic plant. When the expected result, no amplification of the transgene PCR product but amplification of the endogenous PCR product, is observed this indicates that there is no detectable transgene background amplification in a genomic DNA sample.

5.2.3. Primers

The following primers, which specifically recognize the transgene and a flanking sequence of MS-BN1 are used:

BNA01: 5'-gCT.Tgg.ACT.ATA.ATA.CCT.gAC-3' (SEQ ID 12) (target: transgene)
(MDB201)

BNA02: 5'-TgA.CAC.TTT.gAg.CCA.CTC.g-3' (SEQ ID 19) (target: plant DNA)
(VDS51)

To identify plant material comprising RF-BN1, the following primers, which specifically recognize the transgene and a flanking sequence of RF-BN1 are used:

BNA03: 5'-TCA.TCT.ACg.gCA.ATg.TAC.CAg-3' (SEQ ID 23) (target: transgene)
(MDB193)

BNA04: 5'-Tgg.ACC.CCT.Agg.TAA.ATg.CC-3' (SEQ ID 41) (target: plant DNA)
(MDB268)
Primers targeting an endogenous sequence are always included in the PCR cocktail. These primers serve as an internal control in unknown samples and in the DNA positive control. A positive result with the endogenous primer-pair demonstrates that there is ample DNA of adequate quality in the genomic DNA preparation for a PCR product to be generated. The endogenous primers used are:

BNA05: 5'-AAC.gAg.TgT.CAg.CTA.gAC.CAg.C-3'  (SEQ ID 42)
BNA06: 5'-CgC.AgT.TCT.gTg.AAC.ATC.gAC.C-3'  (SEQ ID 43)

5.2.4. Amplified fragments

The expected amplified fragments in the PCR reaction are:

For primer pair BNA05-BNA06: 394bp (endogenous control)
For primer pair BNA01-BNA02: 280bp (MS-BN1 Elite Event)
For primer pair BNA03-BNA04: 215bp (RF-BN1 Elite Event)

5.2.5. PCR conditions

The PCR mix for 50μl reactions contains:

5 μl template DNA
5 μl 10x Amplification Buffer (supplied with Taq polymerase)
1 μl 10 mM dNTP’s
1 μl BNA01 (MS-BN1) or BNA03 (RF-BN1)(10pmoles/μl)
1 μl BNA02 (RF-BN1) or BNA04 (RF-BN1) (10pmoles/μl)
0.5 μl BNA05 (10pmoles/μl)
0.5 μl BNA06 (10pmoles/μl)
0.2 µl Taq DNA polymerase (5 units/µl)
water up to 50 µl

The thermocycling profile to be followed for optimal results is the following:

4 min. at 95°C
Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 72°C
For 5 cycles
Followed by: 30 sec. at 92°C
30 sec. at 57°C
1 min. at 72°C
For 22 to 25 cycles
Followed by: 5 minutes at 72°C

5.2.6. Agarose gel analysis

Between 10 and 20µl of the PCR samples should be applied on a 1.5% agarose gel (Tris-borate buffer) with an appropriate molecular weight marker (e.g. 100bp ladder PHARMACIA).

5.2.7. Validation of the results

Data from transgenic plant DNA samples within a single PCR run and a single PCR cocktail should not be acceptable unless 1) the DNA positive control shows the expected PCR products (transgenic and endogenous fragments), 2) the DNA negative control is
negative for PCR amplification (no fragments) and 3) the wild-type DNA control shows the expected result (endogenous fragment amplification).

Lanes showing visible amounts of the transgenic and endogenous PCR products of the expected sizes, indicate that the corresponding plant from which the genomic template DNA was prepared, has inherited the MS-BN1 and/or RF-BN1 elite event. Lanes not showing visible amounts of either of the transgenic PCR products and showing visible amounts of the endogenous PCR product, indicate that the corresponding plant from which the genomic template DNA was prepared, does not comprise the elite event. Lanes not showing visible amounts of the endogenous and transgenic PCR products, indicate that the quality and/or quantity of the genomic DNA didn’t allow for a PCR product to be generated. These plants cannot be scored. The genomic DNA preparation should be repeated and a new PCR run, with the appropriate controls, has to be performed.

5.2.8. Use of discriminating PCR protocol to identify MS-BN1 and RF-BN1

WOSR leaf material from plants comprising either MS-BN1, RF-BN1 or another transgenic event was tested according to the above-described protocol. Samples from WOSR wild-type were taken as negative controls.

The results of the PCR analysis are illustrated in Figure 4 and 5. Figure 4 illustrates the result obtained with the elite event PCR identification protocol for MS-BN1 on two WOSR samples (lane 1 and 2). Lane 1 is recognized to contain the elite event as the 280 bp band is detected while the sample in lane 2 does not comprise MS-BN1.

Figure 5 illustrates the result obtained with the elite event PCR identification protocol for RF-BN1 on two WOSR samples (lane 1 and 2). Lane 1 is recognized to contain the elite event as the 215 bp band is detected while the sample in lane 2 does not comprise RF-BN1.
Example 6: Production of hybrid seed using MS-BN1 and RF-BN1 in WOSR

WOSR Plants comprising MS-BN1 which were male sterile were crossed with WOSR plants homozygous for RF-BN1. Hybrid seed was collected from MS-BN1 and deposited at the ATCC under ATCC accession number PTA-730.

This hybrid seed was replanted in the field. Plants were found to be 100% fertile and displaying optimal agronomic characteristics. Hybrid plants comprised either both the MS-BN1 and RF-BN1 or the RF-BN-1 event alone.

Example 7: Introduction of MS-BN1 and RF-BN1 into preferred cultivars of WOSR

Elite events MS-BN1 and RF-BN1 were introduced by repeated backcrossing of plants comprising event MS-BN1 or RF-BN1, respectively, into a number of agriculturally important WOSR cultivars.

It was observed that the introgression of the elite events into these cultivars did not significantly influence any of the desirable phenotypic or agronomic characteristics of these cultivars (no linkage drag) while expression of the transgene, as determined by glufosinate tolerance, meets commercially acceptable levels. This confirms the status of event MS-BN1 and RF-BN1 as elite events.

As used in the claims below, unless otherwise clearly indicated, the term "plant" is intended to encompass plant tissues, at any stage of maturity, as well as any cells, tissues, or organs taken from or derived from any such plant, including without limitation, any seeds, leaves, stems, flowers, roots, single cells, gametes, cell cultures, tissue cultures or protoplasts.

Seed comprising elite event MS-BN1 and elite event RF-BN1 or elite event RF-BN1 alone was deposited at the American Tissue Culture Collection under accession number: PTA-730.
CLAIMS

1. A plant of WOSR, or seed, plant cells or tissue thereof, characterized in that the
   genomic DNA of the WOSR plant, or seed, plant cells or tissues thereof is capable of
   yielding at least two sets of restriction fragments selected from the group of:
   i) one set of BamHI fragments, wherein one has a length of between 805 and 1099
      bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, one
      has a length between 2450 and 2838 bp, and one has a length between 5077 and
      14057 bp;
   ii) one set of EcoRI fragments, one with a length of between 805 and 1159 bp, one
      with a length between 1986 and 2450 bp, and two with a length of between 5077
      and 14057 bp;
   iii) one set of EcoRV fragments wherein both have a length of between 5077 and
        14057 bp;
   iv) one set of HindIII fragments, wherein one has a length of between 1700 and 2140
      bp, and two have a length between 2450 and 2838 bp;
   wherein each of the restriction fragments is capable of hybridizing under standard
   stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar
   sequence obtainable by Hpal digestion of the plasmid pTHW118.

2. The plant, seed, plant cells or tissue of claim 1, characterized in that the genomic
   DNA of said plant, seed, plant cells or tissues is capable of yielding at least three sets
   of restriction fragments selected from said group.

3. The plant, seed, plant cells or tissue of claim 2, characterized in that the genomic
   DNA of said plant, seed, plant cells or tissues is capable of yielding at least four sets
   of restriction fragments selected from said group.

4. The plant, seed, plant cells or tissue of claim 3, characterized in that the genomic
   DNA of said plant, seed, plant cells or tissues is capable of yielding all five sets of
   restriction fragments selected from said group.
5. The plant, seed, plant cells or tissue of claim 1, characterized in that the genomic DNA can be used to amplify a DNA fragment of between 195 and 235 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

6. The plant, seed, plant cells or tissue of claim 5, characterized in that the genomic DNA can be used to amplify a DNA fragment of 215 bp using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

7. The plant, seed, plant cells or tissue of claim 1, which is further characterized in that the genomic DNA of said plant, cell, tissue or seed is capable of yielding at least three sets of restriction fragments are selected from the group of:

i) One set of EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp;

ii) one set of EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp;

iii) one set of Hpal fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp;

iv) one set of AflIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, and one with a length of between 2140 and 2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp;

v) one set of NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp; wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107.
8. The plant, seed, plant cells or tissue of claim 7, characterized in that the genomic DNA of said plant, seed, plant cells or tissues is capable of yielding at least three sets of restriction fragments selected from said group.

9. The plant, seed, plant cells or tissue of claim 8, characterized in that the genomic DNA of said plant, seed, plant cells or tissues is capable of yielding at least four sets of restriction fragments selected from said group.

10. The plant, seed, plant cells or tissue of claim 9, characterized in that the genomic DNA of said plant, seed, plant cells or tissues is capable of yielding all five sets of restriction fragments selected from said group.

11. The plant, seed, plant cells or tissue of claim 7, further characterized in that the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.

12. The plant, plant cells or tissue of claim 1, which can be obtained from the seed deposited at the ATCC under accession number PTA-730.

13. The plant, seed, plant cells or tissue of any one of claims 1 to 12, which is a hybrid plant, seed, or hybrid plant cells or tissue.

14. The seed deposited at the ATCC under accession number PTA-730.

15. A WOSR plant or seed, plant cells or tissue thereof, characterized in that
   a) the genomic DNA of the WOSR plant, or seed, plant cells or tissues thereof is capable of yielding at least two sets of restriction fragments selected from the group of:
   i) one set of BamHI fragments, wherein one has a length of between 805 and 1099 bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, one
has a length between 2450 and 2838 bp, and one has a length between 5077 and 14057 bp;

ii) one set of EcoRI fragments, one with a length of between 805 and 1159 bp, one
with a length between 1986 and 2450 bp, and two with a length of between 5077
and 14057 bp;

iii) one set of EcoRV fragments wherein both have a length of between 5077 and
14057 bp;

iv) one set of HindIII fragments, wherein one has a length of between 1700 and 2140
bp, and two have a length between 2450 and 2838 bp;

wherein each of the restriction fragments is capable of hybridizing under standard
stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar
sequence obtainable by Hpal digestion of the plasmid pTHW118;

and/or

b) the genomic DNA can be used to amplify a DNA fragment of between 195 bp and 235
bp using a polymerase chain reaction with two primers having the nucleotide
sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

16. The plant, cell tissue or seed of claim 15, characterized in that the genomic DNA can
be used to amplify a DNA fragment of 215 bp using a polymerase chain reaction with
two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41
respectively.

17. The plant, seed, plant cells or tissue of claim 15, characterized in that the genomic
DNA of said plant, seed, plant cells or tissues is capable of yielding at least three sets
of restriction fragments selected from said group.

18. The plant, seed, plant cells or tissue of claim 17, characterized in that the genomic
DNA of said plant, seed, plant cells or tissues is capable of yielding at least four sets
of restriction fragments selected from said group.
19. The plant, seed, plant cells or tissue of claim 18, characterized in that the genomic DNA of said plant, seed, plant cells or tissues is capable of yielding all five sets of restriction fragments selected from said group.

20. A WOSR plant or seed, plant cells or tissue thereof, characterized in that
   a) the genomic DNA of said plant, cell, tissue or seed is capable of yielding at least three sets of restriction fragments selected from the group of:
      i) One set of EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp;
      ii) one set of EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp;
      iii) one set of Hpal fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp;
      iv) one set of AflIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, and one with a length of between 2140 and 2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp.;
      v) one set of NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp;
   wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107; and/or
   b) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.

21. The plant, cell tissue or seed of claim 20, characterized in that the genomic DNA can be used to amplify a DNA fragment of between 260 and 300bp using a polymerase
chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and
SEQ ID No 19 respectively.

22. The plant, seed, plant cells or tissue of claim 20, characterized in that the genomic
DNA of said plant, seed, plant cells or tissues is capable of yielding at least three sets
of restriction fragments selected from said group.

23. The plant, seed, plant cells or tissue of claim 22, characterized in that the genomic
DNA of said plant, seed, plant cells or tissues is capable of yielding at least four sets
of restriction fragments selected from said group.

24. The plant, seed, plant cells or tissue of claim 23, characterized in that the genomic
DNA of said plant, seed, plant cells or tissues is capable of yielding all five sets of
restriction fragments selected from said group.

25. A method for producing hybrid seed, said method comprising of
a) crossing a transgenic, male-sterile WOSR plant, characterized by one or both of the
following characteristics:
1) the genomic DNA of the WOSR plant, or seed, plant cells or tissues thereof is
capable of yielding at least two sets of restriction fragments selected from the group
of:
   i) one set of EcoRI fragments, one with a length of between 2140 and 2450 bp,
      preferably of about 2266 bp, and one with a length of more than 14 kbp;
   ii) one set of EcoRV fragments wherein one has a length of between 1159 and
       1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14
       kbp;
   iii) one set of Hpal fragments, one with a length of between 1986 and 2140 bp,
       preferably with a length of about 1990 bp, and one with a length of between
       2140 and 2450 bp, preferably of about 2229 bp;
   iv) one set of AflIII fragments, one with a length of between 514 and 805 bp,
       preferably with a length of about 522 bp, and one with a length of between
2140 and 2450 bp, preferably about 2250 bp, and one with a length of between
2450 and 2838 bp, preferably of about 2477 bp;
v) one set of NdeI fragments, both with a length of between 5077 and 14057 bp,
preferably one of about 6500 bp, and one with a length of about 10 kbp;
wherein each of the restriction fragments is capable of hybridizing under standard
stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase
sequence obtainable by HindIII digestion of the plasmid pTHW107.

and/or

2) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300
bp, using a polymerase chain reaction with two primers having the nucleotide
sequence of SEQ ID No 12 and SEQ ID No 19 respectively;
with a fertility restorer WOSR plant, comprising, stably integrated into its genome, a
fertility-restorer gene comprising:
- a DNA encoding a ribonuclease inhibitor
- a constitutive promoter; wherein said DNA is in the same transcriptional unit and
under the control of, said constitutive promoter.

b) harvesting said hybrid seed from said male sterile WOSR plant

26. The method of claim 25, wherein said fertility restorer plant is characterized by one or
both of the following characteristics

1) the genomic DNA of the WOSR plant, or seed, plant cells or tissues thereof is
capable of yielding at least two sets of restriction fragments selected from the group
of:
i) one set of BamHI fragments, wherein one has a length of between 805 and 1099
bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, one
has a length between 2450 and 2838 bp, and one has a length between 5077 and
14057 bp;
ii) one set of EcoRI fragments, one with a length of between 805 and 1159 bp, one
with a length between 1986 and 2450 bp, and two with a length of between 5077
and 14057 bp;
iii) one set of EcoRV fragments wherein both have a length of between 5077 and 14057 bp;
iv) one set of HindIII fragments, wherein one has a length of between 1700 and 2140 bp, and two have a length between 2450 and 2838 bp;
wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar sequence obtainable by Hpal digestion of the plasmid pTHW118;
and/or
2) the genomic DNA can be used to amplify a DNA fragment of between 195 and 235 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

27. A hybrid seed of WOSR obtainable according to the method of claim 25 or 26.

28. A process for producing a transgenic WOSR plant cell or plant which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of a WOSR cell characterized in that it is essentially similar to the sequence of SEQ ID No 22 and regeneration of a WOSR plant from the transformed WOSR cell.

29. The process of claim 28, wherein said recombinant DNA molecule is a male sterility gene and said regenerated plant is male sterile.

30. A process for producing a transgenic WOSR plant cell or plant which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of a WOSR cell characterized in that it is essentially similar to the sequence of SEQ ID No 34 and regeneration of a WOSR plant from the transformed WOSR cell.

31. The process of claim 30, wherein said recombinant DNA molecule is a fertility restorer gene and said regenerated plant is a fertility restorer plant, capable, when crossed with a male-sterile plant of producing male-fertile progeny.
32. A transgenic WOSR plant cell or plant obtainable by the method of any one of claims 28 to 31.

33. A method for identifying a transgenic plant, or cells or tissues thereof, comprising the elite event MS-BN1, which method comprises establishing one or both of the following characteristics of the genomic DNA of the transgenic plant, or its cells or tissues:

a) the genomic DNA of said plant, cell, tissue or seed is capable of yielding at least three restriction fragments or pairs of restriction fragments are selected from the group of:

i) one set of EcoRI fragments, one with a length of between 2140 and 2450 bp, preferably of about 2266 bp, and one with a length of more than 14 kbp;

ii) one set of EcoRV fragments wherein one has a length of between 1159 and 1700 bp, preferably of about 1.4 kbp and the other has a length of more than 14 kbp;

iii) one set of Hpal fragments, one with a length of between 1986 and 2140 bp, preferably with a length of about 1990 bp, and one with a length of between 2140 and 2450 bp, preferably of about 2229 bp;

iv) one set of AluIII fragments, one with a length of between 514 and 805 bp, preferably with a length of about 522 bp, and one with a length of between 2140 and 2450 bp, preferably about 2250 bp, and one with a length of between 2450 and 2838 bp, preferably of about 2477 bp;

v) one set of NdeI fragments, both with a length of between 5077 and 14057 bp, preferably one of about 6500 bp, and one with a length of about 10 kbp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 3942 bp fragment comprising the PTA29-barnase sequence obtainable by HindIII digestion of the plasmid pTHW107; and/or

b) the genomic DNA can be used to amplify a DNA fragment of between 260 and 300 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.
34. The method of claim 33, which comprises establishing whether the genomic DNA of the transgenic plant, or its cells or tissues can be used to amplify a DNA fragment of between 260 and 300 bp, using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 12 and SEQ ID No 19 respectively.

35. A kit for identifying a transgenic plant, its cells or tissues comprising the MS-BN1 elite event, said kit comprising PCR probes one of which recognizes a sequence within the transgene, another of which recognizes a plant DNA sequence within the 3’ flanking region of SEQ ID No. 13 or the 5’ flanking sequence of SEQ ID No. 18 of MS-BN1, for use in a PCR identification protocol.

36. The kit according to claim 35, for identifying a transgenic plant, its cells or tissues comprising the MS-BN1 elite event, said kit comprising the PCR probes having the nucleotide sequence of SEQ ID No. 12 and SEQ ID No. 19, respectively.

37. A method for identifying a transgenic plant, or cells or tissues thereof, comprising the elite event RF-BN1, which method comprises establishing one or both of the following characteristics of the genomic DNA of the transgenic plant, or its cells or tissues:

1) the genomic DNA of the WOSR plant, or seed, plant cells or tissues thereof is capable of yielding at least two sets of restriction fragments selected from the group of:
   i) one set of BamHI fragments, wherein one has a length of between 805 and 1099 bp, preferably of about 814 bp, one has a length between 1700 and 1986 bp, one has a length between 2450 and 2838 bp, and one has a length between 5077 and 14057 bp;
   ii) one set of EcoRI fragments, one with a length of between 805 and 1159 bp, one with a length between 1986 and 2450 bp, and two with a length of between 5077 and 14057 bp;
   iii) one set of EcoRV fragments wherein both have a length of between 5077 and 14057 bp;
iv) one set of HindIII fragments, wherein one has a length of between 1700 and
2140 bp, and two have a length between 2450 and 2838 bp;
wherein each of the restriction fragments is capable of hybridizing under standard
stringency conditions, with the 2182 bp fragment comprising the PTA29-barstar
sequence obtainable by Hpal digestion of the plasmid pTHW118;
and/or
2) the genomic DNA can be used to amplify a DNA fragment of between 195 and
235 bp, using a polymerase chain reaction with two primers having the nucleotide
sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

38. The method of claim 37, which comprises establishing whether the genomic DNA of
the transgenic plant, or its cells or tissues can be used to amplify a DNA fragment of
between 195 and 235 bp, using a polymerase chain reaction with two primers having
the nucleotide sequence of SEQ ID No 23 and SEQ ID No 41 respectively.

39. A kit for identifying a transgenic plant, its cells or tissues comprising the RF-BN1
elite event, said kit comprising PCR probes one of which recognizes a sequence
within the transgene, another of which recognizes a plant DNA sequence within the
3’ flanking region of SEQ ID No. 24 or the 5’ flanking sequence of SEQ ID No. 30
of RF-BN1, for use in a PCR identification protocol.

40. The kit according to claim 39, for identifying a transgenic plant, its cells or tissues
comprising the RF-BN1 elite event, said kit comprising the PCR probes having the
nucleotide sequence of SEQ ID No. 23 and SEQ ID No. 41.

41. A kit for identifying elite event MS-BN1 in biological samples, said kit comprising
at least one PCR primer or probe, which recognizes a sequence within the 3’ or 5’
flanking region of MS-BN1.
42. The kit of claim 41, wherein said at least one PCR primer recognizes a sequence within the plant DNA in SEQ ID No. 13.

43. The kit of claim 42, wherein said primer recognizing a sequence within the plant DNA in SEQ ID No. 13 comprises the sequence of SEQ ID No. 19.

44. The kit of any one of claims 41 to 43, which further comprises at least a second PCR primer or probe which recognizes a sequence within the foreign DNA of MS-BN2.

45. The kit of claim 44, wherein said primer recognizing a sequence within the foreign DNA of MS-BN1 comprises the sequence of SEQ ID No. 12.

46. A primer for use in a MS-BN1 PCR identification protocol, having a sequence which, under optimized PCR conditions specifically recognizes a sequence within the 5' or 3' flanking region of MS-BN1.

47. The primer of claim 46, having a sequence which has at least 80% sequence identity with a sequence within the plant DNA of SEQ ID No. 13 or SEQ ID No. 18 or the complement thereof.

48. The primer of claim 47, comprising the sequence of SEQ ID No. 19.

49. The primer comprising the sequence of SEQ ID No. 12.

50. A method for confirming seed purity, which method comprises the detection of a MS-BN1 specific DNA sequence with a specific primer or probe which specifically recognizes the 5' or 3' flanking region of MS-BN1, in seed samples.

51. A method for screening seeds for the presence of MS-BN1, which method comprises detection of a MS-BN1 specific DNA sequence with a specific primer or probe
which specifically recognizes the 5' or 3' flanking region of MS-BN1, in samples of seed lot.

52. A kit for identifying elite event RF-BN1 in biological samples, said kit comprising at least one PCR primer or probe, which recognizes a sequence within the 3' or 5' flanking region of RF-BN1.

53. The kit of claim 52, wherein said at least one PCR primer recognizes a sequence within the plant DNA in SEQ ID No. 24.

54. The kit of claim 53, wherein said primer recognizing a sequence within the plant DNA in SEQ ID No. 24 comprises the sequence of SEQ ID No. 41.

55. The kit of any one of claims 52 to 54, which further comprises at least a second PCR primer or probe which recognizes a sequence within the foreign DNA of MS-BN2.

56. The kit of claim 55, wherein said primer recognizing a sequence within the foreign DNA of RF-BN1 comprises the sequence of SEQ ID No. 23.

57. A primer for use in a RF-BN1 PCR identification protocol, having a sequence which, under optimized PCR conditions specifically recognizes a sequence within the 5' or 3' flanking region of RF-BN1.

58. The primer of claim 57, having a sequence which has at least 80% sequence identity with a sequence within the plant DNA of SEQ ID No. 24 or SEQ ID No. 30 or the complement thereof.

59. The primer of claim 58, comprising the sequence of SEQ ID No. 41.

60. The primer comprising the sequence of SEQ ID No. 23.
61. A method for confirming seed purity, which method comprises the detection of a RF-BN1 specific DNA sequence with a specific primer or probe which specifically recognizes the 5’ or 3’ flanking region of RF-BN1, in seed samples.

62. A method for screening seeds for the presence of RF-BN1, which method comprises detection of a RF-BN1 specific DNA sequence with a specific primer or probe which specifically recognizes the 5’ or 3’ flanking region of RF-BN1, in samples of seed lot.
Figure 1
Figure 2
Figure 4
SEQUENCE LISTING

AVENTIS CROPSCIENCE N.V.

Hybrid Winter Oilseed Rape and Methods for Producing Same.

EE-BN1

43

PatentIn Ver. 2.0

1

4946

DNA

Artificial Sequence

Description of Artificial Sequence: T-DNA of plasmid pTHW107

misc_feature

(964)...(4906)

Hind III fragment

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WO 01/41558

PCT/EP00/12872

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<223> pGEM®-T vector
<400> 13
ccgccgcgcc atggcgcgcg gattctttagc ctgggtcaggg cgtgcctagg tgtgcattca 60
agactttctc gcgcgaatta ctaaatcatca caagttcagtgc atgatcctgc cgggttgcttgc 120
aagaaaaatc gaaccacatga caatatccac agttgtaaggt tttttacccag tagacaataata 180
cacctggttt taacatatag taaacttaatt atataggaaga tgttcctatt cagaaaataaa 240
tatagttata tataaaaaat tttatccgccc actcggatt gcagagaaatat ataaatgtt 300
ggctgcttag accatctccca atgtattttct caatctttac cttctaaataa aagggatctct 360
taatagagg tgctgtttgc tccaaatgtat ttcttttaaaa tagagatcctc tacatatagta 420
gcaaatata gagaatgttt attttcttct ctataaataag aggagaaatat agcaattctct 480
atattagagg ccaaatagta gatbsgttgtg aagtagttttcg cctctaaatgtt ctattataga 540
ggtgaaataa gagggtttgt ggagatgttc ttaactatattt catagtaggt gaaaacttga 600
aactagaag gttttggttg tagaggtgga aacccctctct ttgtagaaatac atacacatgc 660
catttagtaa actaggttgac atagatttttttngtgcagaactataaagaa ataattatgttt 720
tggatagaggt ttgacactctc gcgacaccct gaaagcaacaa ttttttaaaaat ttggttgatg 780
cgttcgacat aacattgtag acvsttttga taatatcata ttaactacag tagaataaatg 840
attccgctt tgcattttttcttcaggtaatcctgcgcctgc gatcgcggtc gtcagttcaca 900
tggtgcataaa gagaaggaat ggtgttagatg ttaattctcctc tcctgaagataa atat 953

<210> 14
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 611

<400> 14
ngtgcgswgt ntwca 16

<210> 15
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 259

<400> 15
ngtgcagggaa gcggttactgg 22

<210> 16
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 260
<400> 16
cctttggagt aaatgttgtt gg 22

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 24

<400> 17
gcgaatgtat attatatgca 20

<210> 18
<211> 537
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sequence comprising the 3' flanking region of MS-BN1

<400> 18
gcgaatgtat attatatgca taatattat attaaatgtg tataatcatg tataaatcaat 60
gtataaatca tataaatgtt tagaaaaagt aaacaattaa tataatgcccct catttattgta 120
aaaatatctcta ataataacga ccgatcccg ccgatcccg gcgaactctt gatccgtaaa 180
tttgttatga taaccaaaaa cacccctcctt tttattataaa agtagggat agctatagtctg 240
ttatccggtt tttgatgtag atattaatcc cgttttatca aacgtcatgtt gatgtattttt 300
tttgtttccgtt ttcatctctaa tccaaagcaca gttgtagttta ttctcattttt cccaaaaaaa 360
aggttccccggtt tttgtgtag ac tattcctgt tttatatattttt cccctatcag atgaaaggttc 420
aatccgttgaa taacgtcttt atataatcttta ataatctaaa ataatctaatcttta ttttattcta 480
ttaaatgca attanactat gttctctcttg gcgtatatgg ttctctgca aacactc 537

<210> 19
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 51

<400> 19
tgacactttg agccactcg 19

<210> 20
<211> 20
<212> DNA
Artificial Sequence

Description of Artificial Sequence: primer 40

gaggggtgtt tttggttatc

Description of Artificial Sequence: sequence comprising the target site deletion of MS-BN1

gacactttga gccactcgaag gagacaatтtt taaaaactttg tgggatgtgag tggccataaa 60
ccttgagggac gcctttgatca tattttata actacaagact gaaatagatt cgacaatttgc 120
aatattttctc ttgtrrtctaa ttcatatagga ttggtgtatga taacccaaaa cacccttc 178

description of Artificial Sequence: insertion region MS-BN1

cattgggttga ttccaaagact ttctcggccc aataactaat catcacaagct catgcatgat 60
cattttgttaa gtttctttttt tgggctgttgttg ctgaaactagaa gaaatagctgtt 120
acccgatgc ttgagccctg gattttaaaac ttattatatat ttattatatat tattatatatat 180
dgacactttga gccactcgaag gagacaatтtt taaaaactttg tgggatgtgag tggccataaa 60
ccttgagggac gcctttgatca tattttata actacaagact gaaatagatt cgacaatttgc 120
aatattttctc ttgtrrtctaa ttcatatagga ttggtgtatga taacccaaaa cacccttc 178

description of Artificial Sequence: insertion region MS-BN1

cattgggttga ttccaaagact ttctcggccc aataactaat catcacaagct catgcatgat 60
cattttgttaa gtttctttttt tgggctgttgttg ctgaaactagaa gaaatagctgtt 120
acccgatgc ttgagccctg gattttaaaac ttattatatat ttattatatat tattatatatat 180

tgggctgttgttg ctgaaactagaa gaaatagctgtt 360
cattgggttga ttccaaagact ttctcggccc aataactaat catcacaagct catgcatgat 60
cattttgttaa gtttctttttt tgggctgttgttg ctgaaactagaa gaaatagctgtt 120
acccgatgc ttgagccctg gattttaaaac ttattatatat ttattatatat tattatatatat 180

tgggctgttgttg ctgaaactagaa gaaatagctgtt 360
cattgggttga ttccaaagact ttctcggccc aataactaat catcacaagct catgcatgat 60
cattttgttaa gtttctttttt tgggctgttgttg ctgaaactagaa gaaatagctgtt 120
acccgatgc ttgagccctg gattttaaaac ttattatatat ttattatatat tattatatatat 180

tgggctgttgttg ctgaaactagaa gaaatagctgtt 360
cattgggttga ttccaaagact ttctcggccc aataactaat catcacaagct catgcatgat 60
cattttgttaa gtttctttttt tgggctgttgttg ctgaaactagaa gaaatagctgtt 120
acccgatgc ttgagccctg gattttaaaac ttattatatat ttattatatat tattatatatat 180

tgggctgttgttg ctgaaactagaa gaaatagctgtt 360
ttttgttcgt tttcattaca atccagaca agtaggtttt attacatttt accaaaaaaaa 1020
aaggttttgt ttaggtgaa cattgtgtcg gtttatattt aatttgattct attc aaaggt 1080
caatccgtat ttaaacaagta aactagttcttt tatataaatct taaatctcaac gatcttttcat 1140
ttttaaattg cattancta tgtctctcttg gggtatatag gttcttttga aaacactc 1198

<210> 23
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 193

<400> 23
tcatctacgg caatgtacca gc 22

<210> 24
<211> 1077
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sequence
comprising the 5' flanking region of RF-BN1

<220>
<221> misc_feature
<222> (1)..(45)
<223> pGEM®-T vector

<220>
<221> misc_feature
<222> (1061)..(1077)
<223> pGEM®-T vector

<400> 24
gagctctccc atatgtcga cctgcagggc gccgcactag tgaattcttag cctgggtcag 60
ggcattgcat gtctgatggt acatgtctaa atgctatatt ctgtttaaaa gttttaaat 120
catttttctga tggaacctaa tccgatttta agagtaactg acaagtcaaa ttagcaca 180
caatataata gtagtaattg gcattttgta ttgtaaatata tcaaaacagtt aagtttaaa 240
aaaaaaaaata cacaaacaata atgaagactt gcgcggagaca gtgcgcgtgcg aaggttttcg 300
gaggctcggag acaggtccaa aaatacattt tacataaat atttttcata tatatata 360
taaacttcc aaaaagttga atattacat aaacgttttc taattttttc tccacaaata 420
tttataaact aatatatatg atctgacaa aaaaagtgtga attgttttga taatatcaca 480
gatacaatt ttgattgaa atatgtgtag ctgctaaaaa agtaaatctt agaatattaa 540
ttaactatag taacatatat tgaatattaa taaaaatattt tataaatattt ctcataaata 600
taaatataaa atctaatcata tagcataaa aaagaagact aatgtgggtac aaaaaatat 660
cagTTTTTTT gaagtagaat ctttatagtt ttatataaa tatagaaaa aagtttcatgaa 720
acctagttaay ttaaggagaa gtcacaattca aatacaata aaaataaat ctatctaaa 780
aatatgtta actaccatgc aaaaagtattt tttttgtaat tagaaacccct gaaatgtgta 840
caaaaccttg gaccctaggt aatgccttttt ttcattctcgc gataagaaaaa ggcataattgt 900
agatgttaat tcctcattgtt aagaaatat atgtttaataa tttattgata aataacaaag 960
tcaggtatta tagtccaaagc aaaaacataa atttattgat gcaaagttaaa attcagaaat 1020
atttcataaa ctgattatat cagctgttac atgcgcgtgag aatccggcgcc catggcg 1077

<210> 25
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 286

<400> 25
ntgcagawga nawgaa 16

<210> 26
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 314

<400> 26
gtagaggtt gggagaagcc 19

<210> 27
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 315

<400> 27
gggctttctta ctagaaagct ctccgg 25

<210> 28
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 316
<400> 28
cagataggga agtgatgtag gagg
  24

<210> 29
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 288

<400> 29
atgcagcaag aagcttgag g
  21

<210> 30
<211> 1501
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sequence
  comprising the 3' flanking region of RF-BNL

<220>
<221> misc_feature
<222> (1)..<(16)
<223> pGEM®-T vector

<220>
<221> misc_feature
<222> (1458)..<(1501)
<223> pGEM®-T vector

<400> 30
ccatggccgc gggattgtag gaggttgga agacagggcc agaagaagat ttatctgact 60
cgttttttgt atagttttca atgttcataa aggaagatgg agacttgaga a tgtt ttttttttt 120
ggacctttgt tagcttttgtt gggcggttttt ttttttgat caataacttt gtgggctta 180
tgggccgataa gggctgcgcat gtgtgatggt acatgctaa a tgctatatatt gtttttgat 240
tgtttaaatg attttttgat ggaacttaat ccaagtttaa gagtaactga caagtaatg 300
tagcacaac aataaaaatg tagtaatgag cactttttgt gttttaatat caaaaaacata 360
aagtccacaa aaaaaatcacc aaaaaatatg tcaagacttc gcggagagcag ttgcgtgcga 420
tagttttcgg gcagttgcagc cagttccccaa aataatatttt atatttttatattattatatat 480
atatatatat atataacattt caaaaagttgg aattattca taaaaagttt ctaaaatctttc 540
ttttatttttt ttttatttttt tttttttttt ttttttttttt ttttttttttt ttttttttttt 600
tataaacm aagttccaa aatatttttt ggatagtttgag cagctgtcaca aagtttttataa 660
tttagattta aatataactag aatagactat atatggaaa aattatatataat tttttatat 720
tttctataaaatatatat catagcatat aaaaaagagata ctaagttggag tcaaratatatt agagtttgttag atcttatag ttttatattt aataagttg 840
aaatgatcac aaacctagttt accattaacca gaagtcacat tccaaatatc aatatataa 900
aaacctatct aaaaaaatat gttaactacc atgacaaagt aaaaaaaaaa gtaattagaa 960
acctggaat ttgtaaaaaaa cttggacccc taggtaaatt ccttgaagag tatacctatta 1020
gctgcgacaa acttggtgctc atatatattttt ctcctttactt tatatcatac actaataatan 1080
gnagatgact taattaattta ttcatattca tgcagctaa ttcaagaaaaa agaaaaaaaaa 1140
cattatctca actatatatct ccgcacaccc ctcggagata acaggatata tgtcattat 1200
gaatgcttga actcatctcgc ggaactcactc tcgcatcgct tatagccaca aagatccaac 1260
cctctctcct aatcatataat cagtagtaca atacataaag atatgtgag ccatatgcc 1320
gtctagctact gtatgtgaca tgaagagag ccccaaatgt tagtgcactc caacaataag 1380
gctgcgacac gcctcctctg ataggtgatca gcccgtcccttt ttgctctctc aatatccttc 1440
caatgcttttt gctgcataaa tcacttagtgc ggccgctgtgc aggtgcgacca tatgggagag 1500

<210> 31
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 269

<400> 31
gttttctgga gttccgagac g 21

<210> 32
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 283

<400> 32
cctggacccc taggtaaatgc 21

<210> 33
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 284

<400> 33
gtacaaact tgtgaccccccta gg 22

<210> 34
<211> 1068
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: sequence comprising the target site deletion of RF-BN1

<400> 34
cgcgttcggg gctctcccat atggtacacc tgcagccgcc cgcactagtg attcttgagc 60
cctctagttg atgcttttt taaaaagcttc taagcaggtg tctgggccgg ggcagtcgccga 120
gaaaaaaga tatttcctca gaaagatcct tatttagcgc gacaaactgt tgctctatatt 180
ttctctctct taccttatc cataacatca taataaaga aattattcat 240
tttcattctga gtaattccaa gaaaaaaga aaactattata tctaaactta tattcagca 300
cacccctgga gataacagga tataattttat taatgaatgtcttgatctagcttcgaact 360
catctttattgc gcgatatacg ccacagagatc caacccctct cttcaatttt ataactgtgtc 420
tcaatactgat ctatagtattg tgacacata tgctctctag tacatgtggtg tatatgtaga 480
ggagccaaactgcagtgctctcctgccctgcttgacatgcacacatcctttgctctctgctcggatc 540
cagcaggtgc ctttgtgattcttcctcaattttc ctccacagttt cttctctgct ggacaccaacc 600
ttctctgtctctttcattgata acaaaatctca acgagacgag aaccccttaattcaggaaga 660
cacaaaaaatctctaagcagagtggtgtgag aagtactagag atagtgttgag tgacggagag 720
aagatgtagcatgtaaactgtaatcggtcagagctgctcaaggtgttgtctgatccatctattaca 780
tacacgtacactcatcctcattgaataatgaga attaaagagac ctgctctctgctcttctatt 840
gagatcactctctctcttcactcattttaaagcggagagctgctctcttcttctttgctctatt 900
taagtagtag ctctctctctccattttaaagcggagagctgctctcttcttctttgctctatt 960
taagtagtag ctctctctctccattttaaagcggagagctgctctcttcttctttgctctatt 1020
cacccctagg cgcattgtgt tcatttctct cgcacactccc gcggcct 1088

<210> 35
<211> DNA
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer 57

<400> 35
gcatgatcctg ctggagatgg c 21

<210> 36
<211> DNA
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: sequence comprising the 5' flanking region of MS-BN1 in WOSR
<400> 36
tgcatgatct gtcgggagt gccgaagaaaa atcgaaccca tgcacatatg cacagtgtga
agtttttac cagtagacaa ataccaaccttg ttgaataactt aatatataga
agatgtcct attcagaaaa taatatagtt atatatataaa attttttatg gcgacctcgag
120
gtagccacaga aatataaaat tttggtctgcgt tagaccatct ccaatgtatt tctctatattt
tacctcttaa ataaaggaacc tctataaatag aggtgggttt tactccaagtg tattttttta
300
aaatatagat ctctcatatag agagcaaaat atagaggaat tttatatcttt cctctataaaa
360
tagaggagaat aacagtcaactctctatattag aggcacaaat agagatgggt cggagtcatt
420
ttgcctctaca atgccttataagaggtgaa aataaggttggt ttggggagatg cttctactat
480
tttcctagta ggtgaaacct tgaacactgaa aggcttttggagtgactggt ggaacacctc
540
tcgtttagata aacatcacaagc cctattttag ttagacatgatt tttgagtcag
600
ataaccttttaa gaataatatag tttgggtgatga aggcttggaga ctgttaagccat ctcgaaggac
660
aaatatttaaaa aacctgtgggg atgtcgtggc ccataaaccct ggaggacctg ttgatcataat
720
tctattaactgt acgtacagtaa tctagatcggat ctttctcttctgct ctagcgcggcc
780
gtccaaactg gctgtcggagt acatgtgctga taagaaaaag caatttttag atgtagtacct
840
cctccctgtga aagaaataattag ttcataatatt tttgcttataa aataaaactg cagttattat
900
agttcaacg
909

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 68

<400> 37
ccatatacgcc cagagaggac
20

<210> 38
<211> 522
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sequence comprising the 3' flanking region of MS-BN1 in WOSR

<400> 38
gcgaatgtat attatatgca taattatat attaaatgtg tataatcaatg tataatcaat
60
gtataacttc tttatatgtg tagaaactaggt aaccaatattaa tatagccggcc tattttgtga
120
aaaaacctcta atataacggg cggatccccgg ggaatcgggg gggaacgtta gaccctagga
180
 ttgtgttaga taacccaaag caccctccct tttatatataa aggtaggggt agcataactg
240
 ttatccggttt tgtgatagag atattaatcc cgtttttatca agtacagttt gatgtatatttt
300
 tttggtcggttt tttattacaca ctcgaagacgg ttgaggttta tttcatttta ccaaaaaaa
360
aggttgggttt tgtgtagaacc attttgctccgg tttttttataa attttgattct atccaaagt
420
cattccgtat ttaacaaagta aactagtcttt tttattttct ttaatcttaac gataacttggg
480
<210> 39
<211> 694
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: sequence comprising the 5' flanking region of RF-BNL in WOSR

<400> 39
gtttttgcgg ggtcgcggac gagt tttaaa atacagat taataaatatatc ttttcatata 60
tatatata ataacaattc aaaaagttgta attattacat aacagtttttc taatat ttttct 120
tcacaacaatt cttactcttat atactagac aaaaaagtttta atatattgttat 180
ataaatccag attagttgta atcttattgta aagagtaagtc atatagctaa aagatatact 240
tgaatttattatatattc atatatataa gttatctata tatttaaa gttatataaatc 300
tccatataat atatatatat atataataac aaaaaggttttta tatttttattataattta 360
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taatagcataa aaggtgatggcc 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A. CLASSIFICATION OF SUBJECT MATTER

| IPC     | A01H5/00 | A01H5/10 | C12Q1/68 | C12N15/09 | C12N15/82 |

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

| IPC     | A01H | C12N |

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

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of box C.

Patient family members are listed in annex.

* Special categories of cited documents:

| A* | document defining the general state of the art which is not considered to be of particular relevance |
| E* | earlier document but published on or after the international filing date |
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Date of the actual completion of the international search

23 April 2001

Date of mailing of the international search report

11/05/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rivierenbuurt
Tel: (+31-70) 340-2040, Tx: 31 651 epo nl
Fax: (+31-70) 340-3316

Authorized officer

Bilang, J
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<td></td>
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<td>EP 1090999 A</td>
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<td></td>
<td>HU 214927 B</td>
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<td>US 5792929 A</td>
</tr>
<tr>
<td></td>
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|                                        |                | AU 3124899 A            | 19-08-1999      |
|                                        |                | AU 621113 B             | 05-03-1992      |
|                                        |                | AU 3537189 A            | 24-11-1989      |
|                                        |                | AU 5224596 A            | 26-09-1996      |
|                                        |                | CA 1340324 A            | 19-01-1999      |
|                                        |                | DE 68927721 D           | 13-03-1997      |
|                                        |                | DE 68927721 T           | 26-06-1997      |
|                                        |                | DK 668489 A             | 28-02-1990      |
|                                        |                | WO 8910396 A            | 02-11-1989      |
|                                        |                | EP 0737749 A            | 16-10-1996      |
|                                        |                | ES 2097745 T            | 16-04-1997      |
|                                        |                | GR 3023224 T            | 30-07-1997      |
|                                        |                | HK 1008051 A            | 30-04-1999      |
|                                        |                | HU 52553 A, B           | 28-07-1990      |
|                                        |                | IE 81154 B              | 03-05-2000      |
|                                        |                | IL 90095 A              | 28-10-1999      |
|                                        |                | IL 117780 A             | 28-10-1999      |
|                                        |                | JP 2000037146 A         | 08-02-2000      |
|                                        |                | JP 2503988 T            | 22-11-1990      |
|                                        |                | JP 3020530 B            | 15-03-2000      |
|                                        |                | PT 90420 A, B           | 10-11-1989      |
|                                        |                | US 5652354 A            | 29-07-1997      |
|                                        |                | ZA 8903136 A            | 28-12-1990      |

| EP 0757102 A                           | 05-02-1997     | AU 710740 B             | 30-09-1999      |
|                                        |                | AU 6739896 A            | 05-03-1997      |
|                                        |                | CA 2200496 A            | 20-02-1997      |
|                                        |                | WO 9706267 A            | 20-02-1997      |
|                                        |                | EP 0795019 A            | 17-09-1997      |
|                                        |                | JP 11500627 T           | 19-01-1999      |
|                                        |                | US 6074876 A            | 13-06-2000      |