METHODS AND MEANS TO MODIFY A PLANT GENOME AT A NUCLEOTIDE SEQUENCE COMMONLY USED IN PLANT GENOME ENGINEERING

FIELD OF THE INVENTION

The invention relates to the field of agronomy. More particularly, the invention provides methods and means to introduce a targeted modification, including insertion, deletion or substitution, at a precisely localized nucleotide sequence in the genome of a transgenic plant, wherein the nucleotide sequence is comprised within an element or DNA fragment frequently used in plant transgenesis, such as a commonly used selectable marker gene. The modifications are triggered in a first step by induction of a double stranded break at the recognition nucleotide sequence using meganucleases derived from naturally occurring meganucleases which have been re-designed to recognize the recognition site and cleave it.

BACKGROUND ART

The need to introduce targeted modifications in plant genomes, including the control over the location of integration of foreign DNA in plants has become increasingly important, and several methods have been developed in an effort to meet this need (for a review see Kumar and Fladung, 2001, *Trends in Plant Science*, 6, pp155-159). These methods mostly rely on the initial introduction of a double stranded DNA break at the targeted location.

Activation of the target locus and/or repair or donor DNA through the induction of double stranded DNA breaks via rare-cutting endonucleases, such as I-SceI. has been shown to increase the frequency of homologous recombination by several orders of magnitude. (Puchta *et al.*, 1996, *Proc. Natl. Acad. Sci. U.S.A.*, 93, pp5055-5060; Chilton and Que, *Plant Physiol.*, 2003; D'Halluin *et al.* 2008 *Plant Biotechnol. J.* 6, 93-102).

WO96/14408 describes an isolated DNA encoding the enzyme I-SceI. This DNA sequence can be incorporated in cloning and expression vectors, transformed cell lines and transgenic animals. The vectors are useful in gene mapping and site-directed insertion of genes.

WO00/46386 describes methods of modifying, repairing, attenuating and inactivating a gene or other chromosomal DNA in a cell through an I-SceI induced double strand break. Also disclosed are methods of treating or prophylaxis of a genetic disease in an individual in need thereof. Further disclosed are chimeric restriction endonucleases.

In addition, methods have been described which allow the design of rare cleaving endonucleases to alter substrate or sequence-specificity of the enzymes, thus allowing to induce a double stranded break at a locus of interest without being dependent on the presence of a recognition site for any of the natural rare-cleaving endonucleases. Briefly, chimeric restriction enzymes can be prepared using hybrids between a zinc-finger domain designed to recognize a specific nucleotide sequence and the non-specific DNA-cleavage domain from a natural restriction enzyme, such as FokI. Such methods have been described e.g. in WO 03/080809, WO94/18313 or WO95/09233 and in Isalan et al., 2001, *Nature Biotechnology* 19, 656- 660; Liu et al. 1997, *Proc. Natl. Acad. Sci.* USA 94, 5525-5530). Another way of producing custom-made meganucleases, by selection from a library of variants, is described in WO2004/067736. Custom made meganucleases or redesigned meganucleases with altered sequence specificity and DNA-binding affinity may also be obtained through rational design as described in WO2007/047859.

WO2007/049095 describes "LADGLIDADG" homing endonuclease variants having mutations in two separate subdomains, each binding a distinct part of a modified DNA target half site, such that the endonuclease variant is able to cleave a chimeric DNA target sequence comprising the nucleotides bound by each subdomain.

WO2007/049156 and WO2007/093836 describe I-CreI homing endonuclease variants having novel cleavage specificity and uses thereof.

WO2007/047859 describes rationally designed meganucleases with altered sequence specificity and DNA binding affinity.

WO2006/105946 described a method for the exact exchange in plant cells and plants of a target DNA sequence for a DNA sequence of interest through homologous recombination, whereby the selectable or screenable marker used during the homologous recombination phase for temporal selection of the gene replacement events can subsequently be removed without leaving a foot-print and without resorting to *in vitro* culture during the removal step, employing the therein described method for the removal of a selected DNA by microspore specific expression of a double stranded break inducing rare cleaving endonuclease.

US provisional patent application 60/828,042 and European patent application 06020370.0, and WO2008/037436 describe variants of the methods and means of WO2006/105946 wherein the removal step of a selected DNA fragment induced by a double stranded break inducing rare cleaving endonuclease is under control of a germline-specific promoter. Other embodiments of the method relied on non-homologous endjoining at one end of the repair DNA and homologous recombination at the other end.

Gao *et al.* 2009, *The Plant Journal* , pp 1-11 describe heritable targeted mutagenesis in maize using a re-designed endonuclease.

Since the re-designed meganucleases are derived from naturally occurring endonucleases, the available potential recognition sites are not entirely random but appear to have some degree of resemblance to the nucleotide sequence originally recognized by the naturally occurring endonuclease upon which the re-designed meganuclease is based. As stated by Gao et al, 2009 (*supra*) the structure-based protein design method to modify the DNA-binding characteristics of I-CreI are based on visual inspection of the I-CreI-DNA co-crystal structure leading to a prediction of a large number of amino acid substitutions that change I-CreI base preference at particular positions in its recognition site. Individual

amino acid substitutions were evaluated experimentally, and those that conferred the desired change in base preference were added to a database of mutations that can be "mixed and matched" to generate derivatives of I-CreI that recognize highly divergent DNA sites. In theory, the combinatorial diversity available using the current mutation database is sufficient to target an engineered endonuclease approximately every 1000 bp in a random DNA sequence.

Accordingly, there still remains a need for functional re-designed meganucleases which can recognize a recognition site in an DNA element or region previously introduced into a transgenic plant as a commonly used part of a transgene, and induce a double branded DNA break in that region with sufficient efficiency, thereby triggering the events required for e.g. insertion of foreign DNA, deletion or substitution by homologous recombination or non-homologous endjoining at the double stranded break site. Identification of such a pair of recognition site and re-designed meganuclease, enhances the available tools to modify a plant genome in a targeted manner, by allowing insertion, deletion or substitution of the DNA in the vicinity of the induced double stranded DNA break at the location of a previously introduced transgene, without having to resort to presence of historically introduced recognition sites for rare-cleaving endonucleases such as e.g. I-SceI (which does not occur naturally in plant cells).

These and other problems are solved as described hereinafter in the different detailed embodiments of the invention, as well as in the claims.

SUMMARY OF THE INVENTION

In one embodiment of the invention, a method is provided for introducing a foreign DNA molecule at a predefined site in a genome of a transgenic plant cell comprising the steps of

a. inducing a double stranded DNA break at the predefined site;
b. introducing the foreign DNA molecule in the plant cell;

c. selecting a plant cell wherein the foreign DNA is introduced at the predefined site; and
d. optionally regenerating the plant cell into a plant

characterized in that the predefined site is a nucleotide sequence different from a recognition site for a natural occurring meganuclease and that the predefined site is a nucleotide sequence commonly introduced as part of a transgene in a transgenic plant and wherein double stranded DNA break is induced by introduction of a non-naturally occurring single chain meganuclease or a pair of non-naturally occurring meganucleases which recognizes or recognize in concert the predefined site and induces or induce the double stranded break.

In another embodiment the invention provides a method for introducing a foreign DNA molecule at a predefined site in a genome of a plant cell comprising the steps of

a. inducing a double stranded DNA break at the predefined site;
b. introducing the foreign DNA molecule in the plant cell;
c. selecting a plant cell wherein the foreign DNA is introduced at the predefined site; and
d. optionally regenerating the plant cell into a plant

characterized in that the predefined site is comprised within a phosphinotricin acetyl transferase coding region from S. hygroscopicus (bar coding region), which may have the nucleotide sequence of SEQ ID No 3 and that the double stranded DNA break is induced by introduction of a single chain meganuclease or a pair of meganucleases which recognizes or recognize in concert the predefined site and induces or induce the double stranded break. The predefined site may comprise the nucleotide sequence of SEQ ID No 1 or SEQ ID No 2.

In yet another embodiment, a method is provided for introducing a foreign DNA molecule at a predefined site in a genome of a plant cell comprising the steps of

a. inducing a double stranded DNA break at the predefined site;
b. introducing the foreign DNA molecule in the plant cell;

c. selecting a plant cell wherein the foreign DNA is introduced at the predefined site;

d. optionally regenerating the plant cell into a plant

characterized in that the predefined site comprises the nucleotide sequence of SEQ ID No 1 or SEQ ID No. 2 and that the double stranded DNA break is induced by introduction of a single chain meganuclease or a pair of meganucleases which recognizes or recognize in concert the predefined site and induces or induce the double stranded break such as a meganuclease or the pair of meganucleases is/are derived from I-CreI (represented by SEQ ID No. 16) and wherein the following amino acids are present in one of the subunits : - S at position 32; Y at position 33; E at position 38; R at position 40; K at position 66; Q at position 80; T at position 42; R at position 77; R at position 68; R at position 70; Q at position 44; I at position 24; S at position 26; S at position 28 and R at position 30 or R at position 70; T at position 44; I at position 24; S at position 26; S at position 28; N at position 30; S at position 32; R at position 33; Q at position 38; Q at position 80; R at position 40; K at position 66; T at position 42; R at position 77 and R at position 68 (positions corresponding to I-Cre-I amino acid sequence). Examples of such meganuclease are proteins comprising the amino acid sequence of SEQ ID No. 5 and SEQ ID 6, respectively, encoded by a nucleic acid comprising the nucleotide sequence of SEQ ID No. 4 from nucleotide position 2004 to nucleotide position 2525 or to 2522, or the nucleotide sequence of SEQ ID No. 4 from nucleotide position 4885 to nucleotide position 5405 or to 5403. A single chain meganucleases according to the invention can be a protein comprising the amino acid sequence of SEQ ID No. 18, encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID No. 17 from nucleotide position 1267 to 1605 and 1795 to 2541, or a protein comprising the amino acid sequence of SEQ ID No.18 from amino acid position 1 to 167 and 208 to 362, encoded by a nucleotide sequence comprising the nucleotide sequence of SEQ ID No. 17 from nucleotide position 1267 to 1605, 1795 to 1956 and 2071 to 2541.

In any of the embodiments, the foreign DNA may be comprised within a repair DNA, the repair DNA comprising at least one flanking nucleotide sequence homologous to the upstream or downstream sequence of the nucleotide sequence of SEQ ID No. 1 or SEQ

ID No. 2. The foreign DNA may comprises a selectable marker gene and/or a plant expressible gene of interest such as of a herbicide tolerance gene, an insect resistance gene, a disease resistance gene, an abiotic stress resistance gene, a enzyme involved in oil biosynthesis, carbohydrate biosynthesis, a enzyme involved in fiber strength or fiber lenght, an enzyme involved in biosynthesis of secondary metabolites. The foreign DNA may also be integrated as such, i.e. without flanking sequences with homology to the region around the predefined target site (without any further DNA), for integration by non-homologous end-joining

The meganuclease or the pair of meganucleases may be expressed from a chimeric gene or a pair of chimeric genes, each comprising a plant expressible promoter operably linked to a coding region encoding the meganuclease or one of the pair of meganucleases, and further operationally linked to a DNA region involved in transcription termination and polyadenylation functional in a plant cell.

The invention further provides, plant cells and plants and seeds or propagating parts wherein the foreign DNA has been introduced into the predefined site, which have been obtained by the methods herein provided.

The invention also provides a method of growing a plant wherein the foreign DNA has been introduced into the predefined site, which has been obtained by the methods herein provided comprising the step of applying a chemical to the plant or substrate wherein the plant is grown.

Yet another embodiment of the invention concerns a process for producing a plant comprising foreign DNA integrated at the bar coding region comprising the step of crossing a plant consisting essentially of the plant cells obtained by the methods of the invention with another plant or with itself and optionally harvesting seeds.

The invention also concerns a process comprising the step of applying a chemical compound on a plant or a seed of a plant wherein the foreign DNA has been introduced into the predefined site, which has been obtained by the methods herein provided.

Another embodiment of the invention relates to the use of a meganuclease or a pair of meganucleases as herein described to introduce a foreign DNA into the bar coding region in a plant cell.

Yet another embodiment of the invention relates to the use of a custom made meganuclease to introduce a foreign DNA of interest at a predefined site in a plant cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic representation of the recognition site and interactions with amino acids of the different meganuclease monomeric units BAY 39 and BAY40.

Figure 2: Amino acid sequence of BAY 39/40 monomeric unit 2 ("40") (note that the amino acid sequence comprises a SV40 nuclear localization signal (amino acids 1 to 10)).

Figure 3: Amino acid sequence of BAY 39/40 monomeric unit 1 ("39") (note that the amino acid sequence comprises an SV40 nuclear localization signal (amino acids 1 to 10)).

Figure 4: Amino acid sequence of the single chain BAY 39/40 meganuclease (note that the amino acid sequence comprises an SV40 nuclear localization signal (amino acids 1-12) and a linker region (amino acids 168 to 205)).

Figure 5: Alignment of the nucleotide sequence of PCR amplicons around the recognition site of the bar coding regions, in phosphinotricin sensitive lines derived from a transgenic plant comprising a plant expressible bar gene and plant expressible genes for the monomeric units of BAY39/40 . 1. nucleotide sequence of a control sample; 2. nucleotide

sequence of a phosphinotricin tolerant line; 3-9: nucleotide sequences of phosphinotricin sensitive lines.

DESCRIPTION OF DIFFERRENT EMBODIMENTS OF THE INVENTION

The current invention is based on the observation that functional re-designed meganucleases can be obtained which specifically recognize and cleave a nucleotide sequence (SEQ ID No. 1 and SEQ ID No. 2 – Figure 1), which can be found in the nucleotide sequence of the coding region of the phosphinotricin acetyltransferase gene from *Streptomyces hygroscopicus* (bar gene) (Thompson, C., Movva, R., Tizard, R., Crameri, R., Davies, J., Lauwereys, M. ans Botterman, J. (1987) Characterization of the herbicide-resistance gene bar from Streptomyces hygroscopicus. *The EMBO Journal* 6: 2519-2523 (Accession X05822), which nucleotide sequence is present in a commonly used selectable marker gene in plant transgenesis.

SEQ ID No. 3 represents the nucleotide sequence of the bar gene. The complement of the recognition site of SEQ ID No. 1 (SEQ ID No. 2) corresponds to the nucleotide sequence of SEQ ID No. 3 from nucleotide 132 to 153. The herein described meganucleases are thus capable of recognizing and cleaving a nucleotide sequence in transgenic plants comprising a plant-expressible gene which has a plant expressible promoter operable linked to a DNA region encoding the phosphinotricin acetyltransferase gene from *Streptomyces hygroscopicus* (bar) and followed by a 3' transcription termination and polyadenylation region functional in plants, the bar coding region comprising the nucleotide sequence which is the complement of the nucleotide sequence of SEQ ID No. 1, such as SEQ ID No. 3.

The bar coding region has been incorporated in a number of transgenic plants which have been, are or will be commercialized including plants comprising the following events:

Chicory (Cichorium intybus):

- Events RM3-3, RM3-4, RM3-6 as described in regulatory file 97-148-01p

Oilseed rape (Brassica napus)

- Event MS1 as described in regulatory files DD95-04 (CA) or 98-278-01p (US)
- Event MS8 as described in regulatory files DD96-17 (CA) or 98-278-01p (US) or WO 2001/041558
- Event RF1 as described in regulatory files DD95-04 (CA) or 98-278-01p (US)
- Event RF2 as described in regulatory files DD95-04 (CA) or 98-278-01p (US)
- Event RF3 as described in regulatory files DD96-17 (CA) or 98-278-01p (US) or WO 2001/041558
- Events PHY14, PHY35, PHY36 as described in Japanese deregulatory files

Cotton (Gossypium hirsutum)

- Event LLcotton 25 as described in regulatory files 02-042-01p (US) or WO 2003/013224
- Event T303-40 as described in WO2008/122406
- Event GHB119 as described in regulatory file 08-340-01p (US) or WO2008/151780

Corn (Zea mays)

- Event TC-6275 (=DAS-06275-8) as described in regulatory file 03-181-01p (US)
- Event Bt176 as described in regulatory file 94-319-01p (US)
- Event B16 (=DLL25) as described in US deregulation dossier 95-145-01p or WO9506128
- Event DBT418 as described in US deregulation dossier 96-291-01p (US)
- Event ZMA101
- Event CBH351 as described in US deregulation dossier 97-265-01p (US)
- Event MS3 as described in US deregulation file 95-228-01p (US)
- Event MS6 as described in US deregulation file 98-349-01p (US)

Rice (Oryza sativa)

- Event LLRice62 as described in US deregulation dossier 98-329-01p or WO 2001/083818

- Event LLRICE601 as described in US deregulation dossier 06-234-01p or US patent application 2008289060

Soybean (Glycine max)

- Events W62 and W98 described in regulatory file 96-068-01p(US)

Transgenic plants containing these events therefore contain a recognition sequence for the meganucleases herein described and are suitable subjects for the methods of the invention. Furthermore, the plant expressible bar gene is used generally as a selectable marker and numerous transgenic plants have been generated which are also suitable subjects for the methods of the invention.

Accordingly, in one embodiment, the invention relates to a method for introducing a foreign DNA molecule at a predefined or preselected site in a (nuclear) genome of a transgenic plant cell comprising the steps of

a. inducing a double stranded DNA break at the predefined site;
b. introducing the foreign DNA molecule in said plant cell; and
c. selecting a plant cell wherein the foreign DNA is introduced at the predefined site;

wherein the predefined site is a nucleotide sequence different from a recognition site for a natural occurring meganuclease and is a nucleotide sequence commonly introduced as part of a transgene in a transgenic plant and wherein double stranded DNA break is induced by introduction of a non-naturally occurring single chain meganuclease or a pair of non-naturally occurring meganuclease monomeric units which recognizes or recognize together the predefined site and induces or induce the double stranded break.

As used herein, "a nucleotide sequence commonly introduced as a part of a transgene in plants" refers to a nucleotide sequence of a DNA region that has been used previously as an element of a chimeric gene introduced in plants, whereby transgenic plants are readily available, particularly whereby the transgenic plants have been, are or will be commercialized and regulatory approvals have been applied for and are publicly

available. Several databases are available which summarize and provide information on applications for regulatory approvals including the GM crop database of the Center of Environmental risk assessment which can be consulted online (http://www.cera-gmc.org/?action=gm_crop_database&) or the summary list of the Petitions of Nonregulated Status Granted or Pending by APHIS, available online at http://www.aphis.usda.gov/brs/not_reg.html.

DNA regions commonly introduced as part of a transgene in plants include promoter regions such as the 35S promoter of the CaMV 35S transcript (Odell *et al.* (1985), *Nature* 313 : 810-812); the FMV 35S promoter (Richins R.D., Scholthof H.B., Shepherd R.J. (1987) Sequence of the figwort mosaic virus (caulimovirus group). *Nucleic Acids Research* 15: 8451-8466); the promoter of the small subunit of Arabidopsis thaliana Rubisco gene (Krebbers E., Seurinck J., Herdies L., Cashmore A. R., Timko M. P. (1988). Four genes in two diverged subfamilies encode the ribulose-1,5-bisphosphate carboxylase small subunit polypeptides of Arabidopsis thaliana. *Plant Molecular Biology*, 11, 745-759); the Casava Vein Mosaic Virus promoter (Verdaguer et al (1996) *Plant Mol. Biol.* 31: 1129 or Verdaguer et al (1998) *Plant Mol. Biol.* 37: 1055); the Actin2 promoter from Arabidopsis (An Y.Q., McDowell J.M., Huang S., McKinney E.C., Chambliss S., Meagher R.B. (1996) Strong, constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissues. *The Plant Journal* 10: 107-121) or rice (McElroy D., Zhang W., Cao J., Wu R. (1990) Isolation of an efficient actin promoter for use in rice transfomation. *The Plant Cell* 2: 163-171); the Histone H3 promoter or histone H4 promoter (Chaboute M, Chaubet N, Philipps G, Ehling M and Gigot C (1987) Genomic organization and nucleotide sequences of two histone H3 and two histone H4 genes of Arabidopsis thaliana. *Plant Mol. Biol.* 8: 179-191); the promoter of the maize (Zea mays) ubiquitin-1 gene (Christensen et al (1992) *Plant Mol. Biol.* 18: 675); 5' UTR leader sequences such as the cab22L leader (Harpster M, Townsend J, Jones J, Bedbrook J and Dunsmuir P.(1988) Relative strengths of the 35S cauliflower mosaic virus, 1', 2' and nopaline synthase promoters in transformed tobacco, sugarbeet and oilseed rape callus tissue. *Mol Gen Genet.* 212:182-190); or 5' tev (Carrington J and Freed D (1990) Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J*

Virol 64(4): 1590-1597); a 3' end of the nopaline synthase gene (Depicker A., Stachel S., Dhaese P., Zambryski P., Goodman H.M. (1982). Nopaline synthase: transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* 1, 561-573); a 3' end of the octopine synthase gene (De Greve H., Dhaese P., Seurinck J., Lemmers M., Van Montagu M., Schell J. (1982). Nucleotide sequence and transcript map of the Agrobacterium tumefaciens Ti plasmid-encoded octopine synthase gene. *Journal of Molecular and Applied Genetics*, 1, 499-511); the CaMV35S terminator (Sanfaçon et al (1991) *Genes Dev.* 5: 141) transcription termination and polyadenylation region of gene 7 of the octopine type T-DNA vector (D'Haese *et al*, 1983, *The EMBO Journal*, 2, 419-426) and selectable markers such as bar (Thompson, C., Movva, R., Tizard, R., Crameri, R., Davies, J., Lauwereys, M. ans Botterman, J. (1987) Characterization of the herbicide-resistance gene bar from Streptomyces hygroscopicus. *The EMBO Journal* 6: 2519-2523 (Accession X05822)); pat (Wohlleben,W., Arnold,W., Broer,I., Hillemann,D., Strauch,E. and Puhler,A. Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from Streptomyces viridochromogenes Tu494 and its expression in Nicotiana tabacum. *Gene* 70 (1), 25-37 (1988)); 2mepsps (sequence 4 from patent US6566587 or EMBL number AR337832); CP4 (Padgette S.R., Re D., Barry G., Eichholtz D., Delannay X., Fuchs R.L., Kishore G.M., Fraley R.T. (1996). New weed control opportunities: development of soybeans with a Roundup Ready gene. In Herbicide-Resistant Crops: Agricultural, Environmental, Econ...., neo Accession V00618; Beck et al (1982) *Gene* 19(3) p327-336); or hpt (Kaster et al., (1983), NAR 11, 6895-6911).

A preferred DNA region in the context of this invention is the nucleotide sequence of the coding region of the bar gene as mentioned above.

The redesigned meganucleases described herein are based on the naturally occurring meganuclease I-CreI for use as a scaffold. I-CreI is a homing endonuclease found in the chloroplasts of *Chlamydomonas rheinhardti* (Thompson *et al.* 1992, *Gene* 119, 247-251). This endonuclease is a homodimer that recognizes a pseudo-palindromic 22 bp DNA site in the 23SrRNA gene and creates a double stranded DNA break that is used for the introduction of an intron. I-CreI is a member of a group of endonucleases carrying a

single LAGLIDADG motif. LAGLIDADG enzymes contain one or two copies of the consensus motif. Single-motif enzymes, such as I-CreI function as homodimers, whereas double-motif enzymes are monomers with two separate domains. Accordingly, when re-designing meganucleases derived from an I-CreI scaffold to recognize a 22 bp nucleotide sequence of interest, two monomeric units are designed, each recognizing a part of the 22 bp recognition site, which are needed in concert to induce a double stranded break at the 22 bp recognition site (WO2007/047859). Concerted action may be achieved by linking the two monomeric units into one single chain meganuclease, or may also be achieved by promoting the formation of heterodimers, as described e.g. in WO2007/047859.

The amino acid sequence of a naturally occurring I-CreI monomer is provided as SEQ ID No. 16. To re-design I-CreI monomeric units such that the heterodimers thereof recognize the nucleotide sequence of SEQ ID No. 1 and/or 2 the following amino acids are present at the mentioned positions:

1. in meganuclease unit 1:
    a. S at position 32;
    b. Y at position 33;
    c. E at position 38;
    d. R at position 40;
    e. K at position 66;
    f. Q at position 80;
    g. T at position 42;
    h. R at position 77;
    i. R at position 68;
    j. R at position 70;
    k. Q at position 44;
    l. I at position 24;
    m. S at position 26;
    n. S at position 28;
    o. R at position 30.

2. in meganuclease unit 2:
    p. R at position 70;
    q. T at position 44;
    r. I at position 24;
    s. S at position 26;
    t. S at position 28;
    u. N at position 30;
    v. S at position 32;
    w. R at position 33;
    x. Q at position 38;
    y. Q at position 80;
    z. R at position 40;
    aa. K at position 66;
    bb. T at position 42;
    cc. R at position 77;
    dd. R at position 68.

A schematic representation thereof is provided in Figure 1.

The re-designed double stranded break inducing enzyme may comprise, but need not comprise, a nuclear localization signal (NLS), such as the NLS of SV40 large T-antigen [Raikhel, *Plant Physiol.* 100: 1627-1632 (1992) and references therein] [Kalderon *et al. Cell* 39: 499-509 (1984)]. The nuclear localization signal may be located anywhere in the protein, but is conveniently located at the N-terminal end of the protein. The nuclear localization signal may replace one or more of the amino acids of the double stranded break inducing enzyme. It should be noted that if the re-designed meganuclease has been provided with a NLS at the N-terminus of the protein, such as a 10 or 12 amino acid NLS of SV40, the amino acid positions would be shifted (increased) accordingly. Likewise, in the event two monomeric units are linked into a single chain meganuclease, the position of the second unit will also be shifted. The corresponding amino acid positions with regard to the I-CreI amino acid sequence can also be identified by determining the

optimal alignment as described below. It will be clear that in the single chain redesigned meganuclease the order of the units is irrelevant, i.e. whether the above unit 1 and 2 occur indeed within that order in the single amino acid chain or unit 2 precedes unit one in the single amino acid chain does not make a difference in order for the two units combined to be able to recognize the target sequence.

Re-designed meganucleases suitable for the invention may comprise an amino acid sequence as represented in SEQ ID No. 5 and 6 (monomeric units which can cleave the recognition site as a heterodimer) or may comprise an amino acid sequence as represented in SEQ ID No. 18 (single chain meganuclease comprising two units represented by amino acid 1 to 167 and from 208 to 362 respectively, linked by a linker sequence represented by amino acids 168 to 205) or may comprise an amino acid sequence comprising the amino acid sequence of SEQ ID No. 18 from 1 to 167 and 206 to 362 (respectively unit 1 and 2 of the single chain meganuclease without the linker).

Conveniently, the redesigned meganuclease(s) can be provided by expression of a plant expressible recombinant gene(s) encoding such meganuclease(s). To this end, a DNA region comprising a nucleotide sequence encoding a re-designed meganuclease or meganuclease monomeric unit can be operably linked to a plant-expressible promoter and a DNA region involved in transcription termination and polyadenylation and introduced into a plant or plant cells. The recombinant gene(s) encoding redesigned meganuclease(s) may be introduced transiently or stably.

For the purpose of the invention, the term "plant-operative promoter" and “plant-expressible promoter” mean a promoter which is capable of driving transcription in a plant, plant tissue, plant organ, plant part, or plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell.

Promoters that may be used in this respect are constitutive promoters, such as the promoter of the cauliflower mosaic virus (CaMV) 35S transcript (Hapster et al.,1988,

Mol. Gen. Genet. 212: 182-190), the CaMV 19S promoter (U.S. Pat. No. 5,352,605; WO 84/02913; Benfey et al., 1989, *EMBO J.* 8:2195-2202), the subterranean clover virus promoter No 4 or No 7 (WO 96/06932), the Rubisco small subunit promoter (U.S. Pat. No. 4,962,028), the ubiquitin promoter (Holtorf et al., 1995, *Plant Mol. Biol.* 29:637-649), T-DNA gene promoters such as the octopine synthase (OCS) and nopaline synthase (NOS) promoters from *Agrobacterium*, and further promoters of genes whose constitutive expression in plants is known to the person skilled in the art.

Further promoters that may be used in this respect are tissue-specific or organ-specific promoters, preferably seed-specific promoters, such as the 2S albumin promoter (Joseffson et al., 1987, *J. Biol. Chem.* 262:12196-12201), the phaseolin promoter (U.S. Pat. No. 5,504,200; Bustos et al., 1989, *Plant Cell* 1.(9):839-53), the legumine promoter (Shirsat et al., 1989, *Mol. Gen. Genet.* 215(2):326-331), the "unknown seed protein" (USP) promoter (Baumlein et al., 1991, *Mol. Gen. Genet.* 225(3):459-67), the napin promoter (U.S. Pat. No. 5,608,152; Stalberg et al., 1996, *Planta* 199:515-519), the Arabidopsis oleosin promoter (WO 98/45461), the *Brassica* Bce4 promoter (WO 91/13980), and further promoters of genes whose seed-specific expression in plants is known to the person skilled in the art.

Other promoters that can be used are tissue-specific or organ-specific promoters like organ primordia-specific promoters (An et al., 1996, *Plant Cell* 8: 15-30), stem-specific promoters (Keller et al., 1988, *EMBO J.* 7(12): 3625-3633), leaf-specific promoters (Hudspeth et al., 1989, *Plant Mol. Biol.* 12: 579-589), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al., 1989, *Genes Dev.* 3: 1639-1646), tuber-specific promoters (Keil et al., 1989, *EMBO J.* 8(5): 1323-1330), vascular tissue-specific promoters (Peleman et al., 1989, *Gene* 84: 359-369), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone-specific promoters (WO 97/13865), and the like.

Nucleotide sequences encoding re-designed meganucleases suitable for the invention may comprise the nucleotide sequence of SEQ ID No. 4 from nucleotide position 2004 to

nucleotide position 2525 or 2522 or the nucleotide sequence of SEQ ID No. 4 from nucleotide position 4885 to nucleotide position 5405 or 5403. To facilitate cloning and other recombinant DNA techniques, it may be advantageous to include an intron functional in plants into the region encoding a meganuclease, particularly a single chain meganuclease. Such an intron may for example comprise the nucleotide sequence of SEQ ID No. 17 from nt position 1606 to 1794.

The DNA region encoding the re-designed meganuclease may be optimized for expression in plants by adapting GC content, codon usage, elimination of unwanted nucleotide sequences. The coding region may further be optimized for expression in plants and the synthetic coding region may have a nucleotide sequence which has been designed to fulfill the following criteria:

a) the nucleotide sequence encodes a functional redesigned homing endonuclease as herein described;
b) the nucleotide sequence has a GC content of about 50% to about 60%;
c) the nucleotide sequence does not comprise a nucleotide sequence selected from the group consisting of GATAAT, TATAAA, AATATA, AATATT, GATAAA, AATGAA, AATAAG, AATAAA, AATAAT, AACCAA, ATATAA, AATCAA, ATACTA, ATAAAA, ATGAAA, AAGCAT, ATTAAT, ATACAT, AAAATA, ATTAAA, AATTAA, AATACA and CATAAA;
d) the nucleotide does not comprise a nucleotide sequence selected from the group consisting of CCAAT, ATTGG, GCAAT and ATTGC;
e) the nucleotide sequence does not comprise a sequence selected from the group consisting of ATTTA, AAGGT, AGGTA, GGTA or GCAGG;
f) the nucleotide sequence does not comprise a GC stretch consisting of 7 consecutive nucleotides selected from the group of G or C;
g) the nucleotide sequence does not comprise a AT stretch consisting of 5 consecutive nucleotides selected from the group of A or T; and

h) the nucleotide sequence does not comprise codons coding for Leu, Ile, Val, Ser, Pro, Thr, Ala that comprise TA or CG duplets in positions 2 and 3 (i.e. the nucleotide sequence does not comprise the codons TTA, CTA, ATA, GTA, TCG, CCG, ACG and GCG).

An example of such an optimized sequence is represented by SEQ ID No. 17 from nt position 1267 to 1605 and from nt position 1795 to 2541 (wherein the nucleotide sequence encoding the linker present between the two meganuclease units is represented by nt 1957 to 2070).

It will also be clear that the terms used to describe the method such as "introduction of a DNA fragment" as well as "regeneration of a plant from the cell" do not imply that such DNA fragment necessarily needs to be introduced by transformation techniques. Indeed, it will be immediately clear to the person skilled in the art that the DNA molecule of interest may also be introduced by breeding or crossing techniques from one plant to another.

However, it will be clear that the DNA molecule of interest may be introduced into the plant cells by any method known in the art, including *Agrobacterium*-mediated transformation but also by direct DNA transfer methods. The transforming DNA molecule can be transferred into plant cells using any conventional method, including but not limited to direct DNA transfer method. As used herein "direct DNA transfer" is any method of DNA introduction into plant cells which does not involve the use of natural *Agrobacterium spp.* and which is capable of introducing DNA into plant cells. This includes methods well known in the art such as introduction of DNA by electroporation into protoplasts, introduction of DNA by electroporation into intact plant cells or partially degraded tissues or plant cells, introduction of DNA through the action of agents such as PEG and the like, into protoplasts, use of silicon whiskers, and bombardment with DNA coated microprojectiles.

The capability of inducing a double stranded break at a preselected site opens up several potential applications. Foreign DNA of interest may be introduced into the preselected site either by homologous recombination, or in the process of non-homologous endjoining. The double stranded break may also be used to induce the formation of small deletions or insertions at the preselected site, thereby potentially inactivating the chimeric gene comprising the nucleotide sequence of the preselected site. The double stranded break at the preselected site will also facilitate replacement of a DNA region in the vicinity of that site for a DNA region of interest e.g. as described in WO 06/105946, WO08/037436 or WO08/148559.

To insert foreign DNA by homologous recombination at the preselected site, the foreign DNA may be comprised within a repair DNA, wherein the foreign DNA is flanked by at least one flanking DNA region having a nucleotide sequence which is similar to the nucleotide sequence of the DNA region upstream or downstream of the preselected site. The repair DNA may comprise the foreign DNA to be inserted flanked by two flanking DNA regions, upstream and downstream of the foreign DNA and which are similar to nucleotide sequence of the DNA region upstream or downstream of the preselected sites. Alternatively, the foreign DNA may be integrated as such, i.e. without flanking sequences with homology to the region around the predefined target site (without any further DNA), for integration by non-homologous end-joining.

As used herein "a flanking DNA region" is a DNA with a nucleotide sequences having homology to the DNA regions respectively upstream or downstream of the target DNA sequence or preselected site. This allows to better control the insertion of the foreign DNA or the DNA molecule of interest. Indeed, integration by homologous recombination will allow precise joining of the foreign DNA fragment to the plant nuclear genome up to the nucleotide level.

The flanking DNA regions may vary in length, and should be at least about 10 nucleotides in length. However, the flanking region may be as long as is practically possible (e.g. up to about 100-150 kb such as complete bacterial artificial chromosomes (BACs)).

Preferably, the flanking region will be about 50 bp to about 2000 bp. Moreover, the regions flanking the foreign DNA of interest need not be identical to the DNA regions flanking the preselected site and may have between about 80% to about 100% sequence identity, preferably about 95% to about 100% sequence identity with the DNA regions flanking the preselected site. The longer the flanking region, the less stringent the requirement for homology. Furthermore, it is preferred that the sequence identity is as high as practically possible in the vicinity of the location of exact insertion of the foreign DNA. Furthermore, to achieve exchange of the target DNA sequence without changing the DNA sequence of the adjacent DNA sequences, the flanking DNA sequences should preferably be identical to the DNA regions flanking the preselected site.

Moreover, the regions flanking the foreign DNA of interest need not have homology to the regions immediately flanking the preselected site, but may have homology to a DNA region of the nuclear genome further remote from that preselected site. Insertion of the foreign DNA will then result in a removal of the target DNA between the preselected insertion site and the DNA region of homology. In other words, the target DNA located between the homology regions will be substituted for the foreign DNA of interest. Thus, by choosing the appropriate configuration of the foreign DNA for repair of the double stranded DNA break, by introducing a foreign DNA molecule according to the methods of the invention, in addition to insertions, one can also make targeted replacements or targeted deletions of the genomic region located between the homology regions.

The foreign DNA to be inserted may also comprise a selectable or screenable marker, which may or may not be removed after insertion.

"Selectable or screenable markers" as used herein have there usual meaning in the art and include, but are not limited to plant expressible phosphinotricin acetyltransferase, neomycine phosphotransferase, glyphosate oxidase, glyphosate tolerant EPSP enzyme, nitrilase gene, mutant acetolactate synthase or acetohydroxyacid synthase gene, β-glucoronidase (GUS), R-locus genes, green fluorescent protein and the likes.

The selection of the plant cell or plant wherein the selectable or screenable marker and the rest of the foreign DNA molecule has been introduced by homologous recombination through the flanking DNA regions can e.g. be achieved by screening for the absence of sequences present in the transforming DNA but located outside of the flanking DNA regions. Indeed, presence of sequences from the transforming DNA outside the flanking DNA regions would indicate that the origination of the transformed plant cells is by random DNA insertion. To this end, selectable or screenable markers may be included in the transforming DNA molecule outside of the flanking DNA regions, which can then be used to identify those plant cells which do not have the selectable or screenable markers located outside of the transforming DNA and which may have arisen by homologous recombination through the flanking DNA regions. Alternatively, the transforming DNA molecule may contain selectable markers outside the flanking DNA regions that allow selection for the absence of such genes (negative selectable marker genes).

It will be clear that the methods according to the invention allow insertion of any DNA of interest including DNA comprising a nucleotide sequence with a particular nucleotide sequence signature e.g. for subsequent identification. The DNA of interest may also be one or more plant expressible gene(s) including but not limited to a herbicide tolerance gene, an insect resistance gene, a disease resistance gene, an abiotic stress resistance gene, an enzyme involved in oil biosynthesis or carbohydrate biosynthesis, an enzyme involved in fiber strength and/or length, an enzyme involved in the biosynthesis of secondary metabolites.

Herbicide-tolerance genes include a gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Examples of such EPSPS genes are the AroA gene (mutant CT7) of the bacterium Salmonella typhimurium (Comai et al., 1983, Science 221, 370-371), the CP4 gene of the bacterium Agrobacterium sp. (Barry et al., 1992, Curr. Topics Plant Physiol. 7, 139-145), the genes encoding a Petunia EPSPS (Shah et al., 1986, Science 233, 478-481), a Tomato EPSPS (Gasser et al., 1988, J. Biol. Chem. 263, 4280-4289), or an Eleusine EPSPS (WO 01/66704). It can also be a mutated EPSPS as described in for example EP 0837944, WO 00/66746, WO 00/66747 or WO02/26995.

Glyphosate-tolerant plants can also be obtained by expressing a gene that encodes a glyphosate oxido-reductase enzyme as described in U.S. Patent Nos. 5,776,760 and 5,463,175. Glyphosate-tolerant plants can also be obtained by expressing a gene that encodes a glyphosate acetyl transferase enzyme as described in for example WO 02/36782, WO 03/092360, WO 05/012515 and WO 07/024782. Glyphosate-tolerant plants can also be obtained by selecting plants containing naturally-occurring mutations of the above-mentioned genes, as described in for example WO 01/024615 or WO 03/013226. EPSPS genes that confer glyphosate tolerance are described in e.g. US Patent Application Nos 11/517,991, 10/739,610, 12/139,408, 12/352,532, 11/312,866, 11/315,678, 12/421,292, 11/400,598, 11/651,752, 11/681,285, 11/605,824, 12/468,205, 11/760,570, 11/762,526, 11/769,327, 11/769,255, 11/943801 or 12/362,774. Other genes that confer glyphosate tolerance, such as decarboxylase genes, are described in e.g. US patent applications 11/588,811, 11/185,342, 12/364,724, 11/185,560 or 12/423,926.

Other herbicide tolereance genes may encode an enzyme detoxifying the herbicide or a mutant glutamine synthase enzyme that is resistant to inhibition, e.g. described in US Patent Application No 11/760,602. One such efficient detoxifying enzyme is an enzyme encoding a phosphinothricin acetyltransferase (such as the bar or pat protein from Streptomyces species). Phosphinothricin acetyltransferases are for example described in U.S. Patent Nos. 5,561,236; 5,648,477; 5,646,024; 5,273,894; 5,637,489; 5,276,268; 5,739,082; 5,908,810 and 7,112,665.

Herbicide-tolerance genes may also confer tolerance to the herbicides inhibiting the enzyme hydroxyphenylpyruvatedioxygenase (HPPD). Hydroxyphenylpyruvatedioxygenases are enzymes that catalyze the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. Plants tolerant to HPPD-inhibitors can be transformed with a gene encoding a naturally-occurring resistant HPPD enzyme, or a gene encoding a mutated or chimeric HPPD enzyme as described in WO 96/38567, WO 99/24585, and WO 99/24586, WO 2009/144079, WO 2002/046387, or US 6,768,044. Tolerance to HPPD-inhibitors can also be obtained by transforming plants with genes encoding certain enzymes enabling the formation of homogentisate

despite the inhibition of the native HPPD enzyme by the HPPD-inhibitor. Such plants and genes are described in WO 99/34008 and WO 02/36787. Tolerance of plants to HPPD inhibitors can also be improved by transforming plants with a gene encoding an enzyme having prephenate deshydrogenase (PDH) activity in addition to a gene encoding an HPPD-tolerant enzyme, as described in WO 2004/024928. Further, plants can be made more tolerant to HPPD-inhibitor herbicides by adding into their genome a gene encoding an enzyme capable of metabolizing or degrading HPPD inhibitors, such as the CYP450 enzymes shown in WO 2007/103567 and WO 2008/150473.

Still further herbicide tolerance genes encode variant ALS enzymes (also known as acetohydroxyacid synthase, AHAS) as described for example in Tranel and Wright (2002, Weed Science 50:700-712), but also, in U.S. Patent No. 5,605,011, 5,378,824, 5,141,870, and 5,013,659. The production of sulfonylurea-tolerant plants and imidazolinone-tolerant plants is described in U.S. Patent Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270. Other imidazolinone-tolerance genes are also described in for example WO 2004/040012, WO 2004/106529, WO 2005/020673, WO 2005/093093, WO 2006/007373, WO 2006/015376, WO 2006/024351, and WO 2006/060634. Further sulfonylurea- and imidazolinone-tolerance genes are described in for example WO 07/024782 and US Patent Application No 61/288958.

Insect resistance gene may comprise a coding sequence encoding:
1) an insecticidal crystal protein from Bacillus thuringiensis or an insecticidal portion thereof, such as the insecticidal crystal proteins listed by Crickmore et al. (1998, Microbiology and Molecular Biology Reviews, 62: 807-813), updated by Crickmore et al. (2005) at the Bacillus thuringiensis toxin nomenclature, online at: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/), or insecticidal portions thereof, e.g., proteins of the Cry protein classes Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, Cry1F, Cry2Ab, Cry3Aa, or Cry3Bb or insecticidal portions thereof (e.g. EP 1999141 and WO 2007/107302), or such proteins encoded by synthetic genes as e.g. described in and US Patent Application No 12/249,016 ; or

2) a crystal protein from Bacillus thuringiensis or a portion thereof which is insecticidal in the presence of a second other crystal protein from Bacillus thuringiensis or a portion thereof, such as the binary toxin made up of the Cry34 and Cry35 crystal proteins (Moellenbeck et al. 2001, Nat. Biotechnol. 19: 668-72; Schnepf et al. 2006, Applied Environm. Microbiol. 71, 1765-1774) or the binary toxin made up of the Cry1A or Cry1F proteins and the Cry2Aa or Cry2Ab or Cry2Ae proteins (US Patent Appl. No. 12/214,022 and EP 08010791.5); or

3) a hybrid insecticidal protein comprising parts of different insecticidal crystal proteins from Bacillus thuringiensis, such as a hybrid of the proteins of 1) above or a hybrid of the proteins of 2) above, e.g., the Cry1A.105 protein produced by corn event MON89034 (WO 2007/027777); or

4) a protein of any one of 1) to 3) above wherein some, particularly 1 to 10, amino acids have been replaced by another amino acid to obtain a higher insecticidal activity to a target insect species, and/or to expand the range of target insect species affected, and/or because of changes introduced into the encoding DNA during cloning or transformation, such as the Cry3Bb1 protein in corn events MON863 or MON88017, or the Cry3A protein in corn event MIR604; or

5) an insecticidal secreted protein from Bacillus thuringiensis or Bacillus cereus, or an insecticidal portion thereof, such as the vegetative insecticidal (VIP) proteins listed at: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html, e.g., proteins from the VIP3Aa protein class; or

6) a secreted protein from Bacillus thuringiensis or Bacillus cereus which is insecticidal in the presence of a second secreted protein from Bacillus thuringiensis or B. cereus, such as the binary toxin made up of the VIP1A and VIP2A proteins (WO 94/21795); or

7) a hybrid insecticidal protein comprising parts from different secreted proteins from Bacillus thuringiensis or Bacillus cereus, such as a hybrid of the proteins in 1) above or a hybrid of the proteins in 2) above; or

8) a protein of any one of 5) to 7) above wherein some, particularly 1 to 10, amino acids have been replaced by another amino acid to obtain a higher insecticidal activity to a target insect species, and/or to expand the range of target insect species affected, and/or because of changes introduced into the encoding DNA during cloning or transformation

(while still encoding an insecticidal protein), such as the VIP3Aa protein in cotton event COT102; or

9) a secreted protein from Bacillus thuringiensis or Bacillus cereus which is insecticidal in the presence of a crystal protein from Bacillus thuringiensis, such as the binary toxin made up of VIP3 and Cry1A or Cry1F (US Patent Appl. No. 61/126083 and 61/195019), or the binary toxin made up of the VIP3 protein and the Cry2Aa or Cry2Ab or Cry2Ae proteins (US Patent Appl. No. 12/214,022 and EP 08010791.5);

10) a protein of 9) above wherein some, particularly 1 to 10, amino acids have been replaced by another amino acid to obtain a higher insecticidal activity to a target insect species, and/or to expand the range of target insect species affected, and/or because of changes introduced into the encoding DNA during cloning or transformation (while still encoding an insecticidal protein).

An "insect-resistant gene as used herein, further includes transgenes comprising a sequence producing upon expression a double-stranded RNA which upon ingestion by a plant insect pest inhibits the growth of this insect pest, as described e.g. in WO 2007/080126, WO 2006/129204, WO 2007/074405, WO 2007/080127 and WO 2007/035650.

Abiotic stress tolerance genes include

1) a transgene capable of reducing the expression and/or the activity of poly(ADP-ribose) polymerase (PARP) gene in the plant cells or plants as described in WO 00/04173, WO/2006/045633, EP 04077984.5, or EP 06009836.5.

2) a transgene capable of reducing the expression and/or the activity of the PARG encoding genes of the plants or plants cells, as described e.g. in WO 2004/090140.

3) a transgene coding for a plant-functional enzyme of the nicotineamide adenine dinucleotide salvage synthesis pathway including nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase, nicotinamide adenine dinucleotide synthetase or nicotine amide phosphorybosyltransferase as described e.g. in EP 04077624.7, WO 2006/133827, PCT/EP07/002433, EP 1999263, or WO 2007/107326.

Enzymes involved in carbohydrate biosynthesis include those described in e.g. EP 0571427, WO 95/04826, EP 0719338, WO 96/15248, WO 96/19581, WO 96/27674, WO 97/11188, WO 97/26362, WO 97/32985, WO 97/42328, WO 97/44472, WO 97/45545, WO 98/27212, WO 98/40503, WO99/58688, WO 99/58690, WO 99/58654, WO 00/08184, WO 00/08185, WO 00/08175, WO 00/28052, WO 00/77229, WO 01/12782, WO 01/12826, WO 02/101059, WO 03/071860, WO 2004/056999, WO 2005/030942, WO 2005/030941, WO 2005/095632, WO 2005/095617, WO 2005/095619, WO 2005/095618, WO 2005/123927, WO 2006/018319, WO 2006/103107, WO 2006/108702, WO 2007/009823, WO 00/22140, WO 2006/063862, WO 2006/072603, WO 02/034923, EP 06090134.5, EP 06090228.5, EP 06090227.7, EP 07090007.1, EP 07090009.7, WO 01/14569, WO 02/79410, WO 03/33540, WO 2004/078983, WO 01/19975, WO 95/26407, WO 96/34968, WO 98/20145, WO 99/12950, WO 99/66050, WO 99/53072, US 6,734,341, WO 00/11192, WO 98/22604, WO 98/32326, WO 01/98509, WO 01/98509, WO 2005/002359, US 5,824,790, US 6,013,861, WO 94/04693, WO 94/09144, WO 94/11520, WO 95/35026 or WO 97/20936 or enzymes involved in the production of polyfructose, especially of the inulin and levan-type, as disclosed in EP 0663956, WO 96/01904, WO 96/21023, WO 98/39460, and WO 99/24593, the production of alpha-1,4-glucans as disclosed in WO 95/31553, US 2002031826, US 6,284,479, US 5,712,107, WO 97/47806, WO 97/47807, WO 97/47808 and WO 00/14249, the production of alpha-1,6 branched alpha-1,4-glucans, as disclosed in WO 00/73422, the production of alternan, as disclosed in e.g. WO 00/47727, WO 00/73422, EP 06077301.7, US 5,908,975 and EP 0728213, the production of hyaluronan, as for example disclosed in WO 2006/032538, WO 2007/039314, WO 2007/039315, WO 2007/039316, JP 2006304779, and WO 2005/012529.

The invention also provides a method for introducing a deletion at a predefined or preselected site in a (nuclear) genome of a transgenic plant cell comprising the steps of

a. inducing a double stranded DNA break at the predefined site; and
b. selecting a plant cell having a deletion at said predefined site;

wherein the predefined site is a nucleotide sequence different from a recognition site for a natural occurring meganuclease and is a nucleotide sequence commonly introduced as part of a transgene in a transgenic plant and wherein double stranded DNA break is induced by introduction of a non-naturally occurring single chain meganuclease or a pair of non-naturally occurring meganuclease monomeric units which recognizes or recognize together the predefined site and induces or induce the double stranded break.

It is also an embodiment of the invention to provide chimeric genes encoding re-designed meganucleases as herein described, wherein the chimeric gene comprise a plant expressible promoter operably linked to a DNA region encoding a protein comprising an amino acid sequence corresponding to the amino acid sequence of I-CreI as a scaffold comprising a S at position 32; Y at position 33; E at position 38; R at position 40; K at position 66; Q at position 80; T at position 42; R at position 77; R at position 68; R at position 70; Q at position 44; I at position 24; S at position 26; S at position 28 and R at position 30 or R at position 70; T at position 44; I at position 24; S at position 26; S at position 28; N at position 30; S at position 32; R at position 33; Q at position 38; Q at position 80; R at position 40; K at position 66; T at position 42; R at position 77 and R at position 68 (positions with respect to the amino acid sequence of I-CreI, corresponding amino acid positions in redesigned meganucleases can be determined by alignment), such as a protein comprising the amino acid sequence of SEQ ID 5 or SEQ ID 6 or a protein comprising the amino acid sequence of SEQ ID No. 18, or a protein comprising the amino acid sequence of SEQ ID NO 18 from position 1 to 167 and the amino acid sequence of SEQ ID NO 18 from position 206 to 362.

It will be appreciated that the means and methods of the invention may be used in any plant including corn, tobacco, cereal plants including wheat, oat, barley, rye, rice, turfgrass, sorghum, millet or sugarcane plants. The methods of the invention can also be applied to any plant (Angiospermae or Gymnospermae) including but not limited to cotton, canola, oilseed rape, soybean, vegetables, potatoes, Lemna spp., Nicotiana spp., Arabidopsis, alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, wheat, asparagus, beet and sugar beet, broccoli,

cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, oilseed rape, pepper, potato, pumpkin, radish, spinach, squash, tomato, zucchini, almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut and watermelon.

It is also an object of the invention to provide plant cells and plants generated according to the methods of the invention. Gametes, seeds, embryos, either zygotic or somatic, progeny or hybrids of plants comprising the DNA insertion events, which are produced by traditional breeding methods, are also included within the scope of the present invention. Such plants may contain a heterologous or foreign DNA sequence inserted at or instead of a target sequence, and will only be different from their progenitor plants by the presence of this heterologous DNA or DNA sequence post exchange.

The plants obtained by the methods described herein may be further crossed by traditional breeding techniques with other plants to obtain progeny plants comprising the targeted DNA insertion events obtained according to the present invention.

The plants and seeds according to the invention may be further treated with a chemical compound, such as a chemical compound selected from the following lists:

- Fruits/Vegetables Herbicides: Atrazine, Bromacil, Diuron, Glyphosate, Linuron, Metribuzin, Simazine, Trifluralin, Fluazifop, Glufosinate, Halosulfuron Gowan, Paraquat, Propyzamide, Sethoxydim, Butafenacil, Halosulfuron, Indaziflam
- Fruits/Vegetables Insecticides: Aldicarb , Bacillus thuriengiensis, Carbaryl, Carbofuran, Chlorpyrifos, Cypermethrin, Deltamethrin, Abamectin, Cyfluthrin/beta-cyfluthrin, Esfenvalerate, Lambda-cyhalothrin, Acequinocyl, Bifenazate, Methoxyfenozide, Novaluron, Chromafenozide, Thiacloprid, Dinotefuran, Fluacrypyrim, Spirodiclofen, Gamma-cyhalothrin, Spiromesifen, Spinosad, Rynaxypyr, Cyazypyr, Triflumuron,Spirotetramat, Imidacloprid, Flubendiamide, Thiodicarb, Metaflumizone, Sulfoxaflor, Cyflumetofen,

Cyanopyrafen, Clothianidin, Thiamethoxam, Spinotoram, Thiodicarb, Flonicamid, Methiocarb, Emamectin-benzoate, Indoxacarb, Fenamiphos, Pyriproxifen, Fenbutatin-oxid

- Fruits/Vegetables Fungicides: Ametoctradin, Azoxystrobin, Benthiavalicarb, Boscalid, Captan, Carbendazim, Chlorothalonil, Copper, Cyazofamid, Cyflufenamid, Cymoxanil, Cyproconazole, Cyprodinil, Difenoconazole, Dimetomorph, Dithianon, Fenamidone, Fenhexamid, Fluazinam, Fludioxonil, Fluopicolide, Fluopyram, Fluoxastrobin, Fluxapyroxad, Folpet, Fosetyl, Iprodione, Iprovalicarb, Isopyrazam, Kresoxim-methyl, Mancozeb, Mandipropamid, Metalaxyl/mefenoxam, Metiram, Metrafenone, Myclobutanil, Penconazole, Penthiopyrad, Picoxystrobin, Propamocarb, Propiconazole, Propineb, Proquinazid, Prothioconazole, Pyraclostrobin, Pyrimethanil, Quinoxyfen, Spiroxamine, Sulphur, Tebuconazole, Thiophanate-methyl, Trifloxystrobin
- Cereals herbicides: 2.4-D, amidosulfuron, bromoxynil, carfentrazone-e, chlorotoluron, chlorsulfuron, clodinafop-p, clopyralid, dicamba, diclofop-m, diflufenican, fenoxaprop, florasulam, flucarbazone-na, flufenacet, flupyrsulfuron-m, fluroxypyr, flurtamone, glyphosate, iodosulfuron, ioxynil, isoproturon, mcpa, mesosulfuron, metsulfuron, pendimethalin, pinoxaden, propoxycarbazone, prosulfocarb, pyroxsulam, sulfosulfuron, thifensulfuron, tralkoxydim, triasulfuron, tribenuron, trifluralin, tritosulfuron
- Cereals Fungicides: Azoxystrobin, Bixafen, Boscalid, Carbendazim, Chlorothalonil, Cyflufenamid, Cyproconazole, Cyprodinil, Dimoxystrobin, Epoxiconazole, Fenpropidin, Fenpropimorph, Fluopyram, Fluoxastrobin, Fluquinconazole, Fluxapyroxad, Isopyrazam, Kresoxim-methyl, Metconazole, Metrafenone, Penthiopyrad, Picoxystrobin, Prochloraz, Propiconazole, Proquinazid, Prothioconazole, Pyraclostrobin, Quinoxyfen, Spiroxamine, Tebuconazole, Thiophanate-methyl , Trifloxystrobin
- Cereals Insecticides: Dimethoate, Lambda-cyhalthrin, Deltamethrin, alpha-Cypermethrin, ß-cyfluthrin, Bifenthrin, Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid, Acetamiprid, Dinetofuran, Clorphyriphos, Pirimicarb, Methiocarb, Sulfoxaflor

- Maize Herbicides: Atrazine, Alachlor, Bromoxynil, Acetochlor, Dicamba, Clopyralid, (S-)Dimethenamid, Glufosinate, Glyphosate, Isoxaflutole, (S-)Metolachlor, Mesotrione, Nicosulfuron, Primisulfuron, Rimsulfuron, Sulcotrione, Foramsulfuron, Topramezone, Tembotrione, Saflufenacil, Thiencarbazone, Flufenacet, Pyroxasulfon
- Maize Insecticides: Carbofuran, Chlorpyrifos, Bifenthrin, Fipronil, Imidacloprid, Lambda-Cyhalothrin, Tefluthrin, Terbufos, Thiamethoxam, Clothianidin, Spiromesifen, Flubendiamide, Triflumuron, Rynaxypyr, Deltamethrin, Thiodicarb, ß-Cyfluthrin, Cypermethrin, Bifenthrin, Lufenuron, Tebupirimphos, Ethiprole, Cyazypyr, Thiacloprid, Acetamiprid, Dinetofuran, Avermectin
- Maize Fungicides: Azoxystrobin, Bixafen, Boscalid, Cyproconazole, Dimoxystrobin, Epoxiconazole, Fenitropan, Fluopyram, Fluoxastrobin, Fluxapyroxad, Isopyrazam, Metconazole, Penthiopyrad, Picoxystrobin, Propiconazole, Prothioconazole, Pyraclostrobin, Tebuconazole, Trifloxystrobin
- Rice Herbicides: Butachlor, Propanil, Azimsulfuron, Bensulfuron, Cyhalofop, Daimuron, Fentrazamide, Imazosulfuron, Mefenacet, Oxaziclomefone, Pyrazosulfuron, Pyributicarb, Quinclorac, Thiobencarb, Indanofan, Flufenacet, Fentrazamide, Halosulfuron, Oxaziclomefone, Benzobicyclon, Pyriftalid, Penoxsulam, Bispyribac, Oxadiargyl, Ethoxysulfuron, Pretilachlor, Mesotrione, Tefuryltrione, Oxadiazone, Fenoxaprop, Pyrimisulfan
- Rice Insecticides: Diazinon, Fenobucarb, Benfuracarb, Buprofezin, Dinotefuran, Fipronil, Imidacloprid, Isoprocarb, Thiacloprid, Chromafenozide, Clothianidin, Ethiprole, Flubendiamide, Rynaxypyr, Deltamethrin, Acetamiprid, Thiamethoxam, Cyazypyr, Spinosad, Spinotoram, Emamectin-Benzoate, Cypermethrin, Chlorpyriphos, Etofenprox, Carbofuran, Benfuracarb, Sulfoxaflor
- Rice Fungicides: Azoxystrobin, Carbendazim, Carpropamid, Diclocymet, Difenoconazole, Edifenphos, , Ferimzone, Gentamycin, Hexaconazole, Hymexazol, Iprobenfos (IBP), Isoprothiolane, Isotianil, Kasugamycin, Mancozeb, Metominostrobin, Orysastrobin, Pencycuron, Probenazole, Propiconazole, Propineb, Pyroquilon, Tebuconazole, Thiophanate-methyl, Tiadinil, Tricyclazole, Trifloxystrobin, Validamycin

- Cotton Herbicides: Diuron, Fluometuron, MSMA, Oxyfluorfen, Prometryn, Trifluralin, Carfentrazone, Clethodim, Fluazifop-butyl, Glyphosate, Norflurazon, Pendimethalin, Pyrithiobac-sodium, Trifloxysulfuron, Tepraloxydim, Glufosinate, Flumioxazin, Thidiazuron
- Cotton Insecticides: Acephate, Aldicarb, Chlorpyrifos, Cypermethrin, Deltamethrin, Abamectin, Acetamiprid, Emamectin Benzoate, Imidacloprid, Indoxacarb, Lambda-Cyhalothrin, Spinosad, Thiodicarb, Gamma-Cyhalothrin, Spiromesifen, Pyridalyl, Flonicamid Flubendiamide, Triflumuron, Rynaxypyr, Beta-Cyfluthrin, Spirotetramat
- Clothianidin, Thiamethoxam, Thiacloprid, Dinetofuran, Flubendiamide, Cyazypyr, Spinosad, Spinotoram, gamma Cyhalothrin, 4-[[(6-Chlorpyridin-3-yl)methyl](2,2-difluorethyl)amino]furan-2(5H)-on, Thiodicarb, Avermectin, Flonicamid, Pyridalyl, Spiromesifen, Sulfoxaflor
- Cotton Fungicides: Azoxystrobin, Bixafen, Boscalid, Carbendazim, Chlorothalonil, Copper, Cyproconazole, Difenoconazole, Dimoxystrobin, Epoxiconazole, Fenamidone, Fluazinam, Fluopyram, Fluoxastrobin, Fluxapyroxad, Iprodione, Isopyrazam, Isotianil, Mancozeb, Maneb, Metominostrobin, Penthiopyrad, Picoxystrobin, Propineb, Prothioconazole, Pyraclostrobin, Quintozene, Tebuconazole, Tetraconazole, Thiophanate-methyl, Trifloxystrobin
- Soybean Herbicides: Alachlor, Bentazone, Trifluralin, Chlorimuron-Ethyl, Cloransulam-Methyl, Fenoxaprop, Fomesafen, Fluazifop, Glyphosate, Imazamox, Imazaquin, Imazethapyr, (S-)Metolachlor, Metribuzin, Pendimethalin, Tepraloxydim, Glufosinate
- Soybean Insecticides: Lambda-cyhalothrin, Methomyl, Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid, Acetamiprid, Dinetofuran, Flubendiamide, Rynaxypyr, Cyazypyr, Spinosad, Spinotoram, Emamectin-Benzoate, Fipronil, Ethiprole, Deltamethrin, ß-Cyfluthrin, gamma and lambda Cyhalothrin, 4-[[(6-Chlorpyridin-3-yl)methyl](2,2-difluorethyl)amino]furan-2(5H)-on, Spirotetramat, Spinodiclofen, Triflumuron, Flonicamid, Thiodicarb, beta-Cyfluthrin

- Soybean Fungicides: Azoxystrobin, Bixafen, Boscalid, Carbendazim, Chlorothalonil, Copper, Cyproconazole, Difenoconazole, Dimoxystrobin, Epoxiconazole, Fluazinam, Fluopyram, Fluoxastrobin, Flutriafol, Fluxapyroxad, Isopyrazam, Iprodione, Isotianil, Mancozeb, Maneb, Metconazole, Metominostrobin, Myclobutanil, Penthiopyrad, Picoxystrobin, Propiconazole, Propineb, Prothioconazole, Pyraclostrobin, Tebuconazole, Tetraconazole, Thiophanate-methyl, Trifloxystrobin
- Sugarbeet Herbicides: Chloridazon, Desmedipham, Ethofumesate, Phenmedipham, Triallate, Clopyralid, Fluazifop, Lenacil, Metamitron, Quinmerac, Cycloxydim, Triflusulfuron, Tepraloxydim, Quizalofop
- Sugarbeet Insecticides: Imidacloprid, Clothianidin, Thiamethoxam, Thiacloprid, Acetamiprid, Dinetofuran, Deltamethrin, ß-Cyfluthrin, gamma/lambda Cyhalothrin, 4-[[(6-Chlorpyridin-3-yl)methyl](2,2-difluorethyl)amino]furan-2(5H)-on, Tefluthrin, Rynaxypyr, Cyaxypyr, Fipronil, Carbofuran
- Canola Herbicides: Clopyralid, Diclofop, Fluazifop, Glufosinate, Glyphosate, Metazachlor, Trifluralin Ethametsulfuron, Quinmerac, Quizalofop, Clethodim, Tepraloxydim
- Canola Fungicides: Azoxystrobin, Bixafen, Boscalid, Carbendazim, Cyproconazole, Difenoconazole, Dimoxystrobin, Epoxiconazole, Fluazinam, Fluopyram, Fluoxastrobin, Flusilazole, Fluxapyroxad, Iprodione, Isopyrazam, Mepiquat-chloride, Metconazole, Metominostrobin, Paclobutrazole, Penthiopyrad., Picoxystrobin, Prochloraz, Prothioconazole, Pyraclostrobin, Tebuconazole, Thiophanate-methyl, Trifloxystrobin, Vinclozolin
- Canola Insecticides: Carbofuran, Thiacloprid, Deltamethrin, Imidacloprid, Clothianidin, Thiamethoxam, Acetamiprid, Dinetofuran, ß-Cyfluthrin, gamma and lambda Cyhalothrin, tau-Fluvaleriate, Ethiprole, Spinosad, Spinotoram, Flubendiamide, Rynaxypyr, Cyazypyr, 4-[[(6-Chlorpyridin-3-yl)methyl](2,2-difluorethyl)amino]furan-2(5H)-on

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined may comprise additional DNA regions etc.
As used herein, "plant part" includes any plant organ or plant tissue, including but not limited to fruits, seeds, embryos, meristematic regions, callus tissue, leaves, roots, shoots, flowers, gametophytes, sporophytes, pollen, and microspores.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970). The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA molecules will be clear from the context of the application.

The following non-limiting Examples describe the use of a re-designed meganuclease to modify plants at the site of a bar coding region already present in the plant genome.

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

All patents, patent applications and publications mentioned herein are hereby incorporated by reference, in their entireties, for all purposes.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No. 1: nucleotide sequence of the recognition site of the re-designed meganucleases BAY 39/BAY40

SEQ ID No. 2: nucleotide sequence of the complement of the recognition site of the re-designed meganucleases BAY 39/BAY40

SEQ ID No. 3: nucleotide sequence of the bar gene coding region

SEQ ID No. 4: nucleotide sequence of the vector pCV177 expressing a pair of heterodimer meganucleases BAY 39 and BAY40

SEQ ID No. 5: amino acid sequence of the meganuclease BAY39/40 monomeric unit 2 ("40")

SEQ ID No. 6: amino acid sequence of the meganuclease BAY39/40 monomeric unit 1 ("39")

SEQ ID No. 7: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (control)

SEQ ID No. 8: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT tolerant line)

SEQ ID No. 9: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 1)

SEQ ID No. 10: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 2)

SEQ ID No. 11: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 3)

SEQ ID No. 12: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 4)

SEQ ID No. 13: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 5)

SEQ ID No. 14: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 6)

SEQ ID No. 15: nucleotide sequence of the PCR amplicon of the bar coding region around the BAY39/40 recognition site (PPT sensitive line 7)

SEQ ID No. 16: amino acid sequence of I-CreI natural variant (monomer)

SEQ ID No. 17: nucleotide sequence of the vector pCV170 expressing a single chain BAY39/BAY40 meganuclease

SEQ ID No. 18: amino acid sequence of the single chain BAY39/40 meganuclease

EXAMPLES

All re-designed meganucleases described herein have been designed by Precision BioSciences Inc., 104 T.W. Alexander Drive, Research Triangle Park, NC27713.

Example 1: Description of the T-DNA vectors encoding re-designed meganucleases according to the invention.

Using conventional recombinant DNA techniques a chimeric gene encoding a pair of re-designed meganuclease monomers which as a heterodimer recognize the nucleotide sequence of SEQ ID No. 1 or 2 (hd BAY39/40) was constructed, comprising the following operably linked DNA fragments:

- a DNA region encoding the CaMV35S promoter (SEQ ID No 6 from nt position 1516 to nt position 1933, such as SEQ ID No 4 from nt position 1516 to nt position 1997)
- a DNA region comprising the BAY 39/40 monomeric unit 2 encoding region, operably linked to a SV40 NLS at the N-terminus (SEQ ID No 4

from nt position 2004 to 2525 including the stop codon, or to 2522 excluding the stopcodon)

- a DNA region involved in 3' end transcription termination and polyadenylation from nopaline synthase gene (SEQ ID No 4 from nt position 2530 to 2783)
- a DNA region encoding the CaMV35S promoter (SEQ ID No 4 from nt position 4397 to nt position 4814, such as SEQ ID No 4 from nt position 4397 to nt position 4878)
- a DNA region comprising the BAY 39/40 monomeric unit 1, operably linked to a SV40 NLS at the N-terminus (SEQ ID No 4 from nt position 4885 to 5405, including the stopcodon, or to 5403, excluding the stop codon)
- a DNA region involved in 3' end transcription termination and polyadenylation from nopaline synthase gene (SEQ ID No 4 from nt position 5411 to 5664).

The nucleotide sequence of the resulting plasmid is represented in SEQ ID No. 4.

Using conventional recombinant DNA techniques a chimeric gene encoding a single chain re-designed meganuclease which recognize the nucleotide sequence of SEQ ID No. 1 or 2 (sc BAY39/40) was constructed, comprising the following operably linked DNA fragments:

- a DNA region encoding the CaMV35S promoter (SEQ ID No 17 from nt position 691 to nt position 1223)
- leader sequence from *Arabidopsis thaliana* rbcS ATS1A gene; SEQ ID No 17 from nt position 1224 to nt position 1266; Krebbers et al. 1988 Plant Molecular Biology 11:745-759)
- a DNA region encoding the N-terminal region of the single chain BAY 39/40 meganuclease, operably linked to a SV40 NLS at the N-terminus, optimized for expression in tobacco (SEQ ID No 17 from nt position 1267 to 1605)

- a DNA region encoding the second intron of the potato light inducible tissue-specific ST-LS1 gene (SEQ ID No 17 from nt position 1606 to 1794; X04753; Eckes et al. 1986 Mol. Gen. Genet. 205, 14-22)
- a DNA region encoding the C-terminal region of the single chain BAY 39/40 meganuclease (SEQ ID No 17 from nt position 1795 to 1798, including the stop codon, or to 2541, excluding the stop codon), including DNA region encoding a linker sequence (SEQ ID No 17 from nt position 1757 to 2070), optimized for expression in tobacco.
- a DNA region involved in 3' end transcription termination and polyadenylation from 35S gene (SEQ ID No. 17 from nt position 2545 to 2678).

The nucleotide sequence of the resulting plasmid is represented in SEQ ID No. 17.

Example 2: Description of the target tobacco line and assay.

In order to develop an assay for double stranded DNA break induction, a phosphinotricin (PPT) tolerant tobacco transgenic plant line was selected that contained a bar coding region under control of a plant-expressible promoter.

This transgenic line was used as starting material in a transformation wherein the chimeric genes encoding hd BAY39/40 meganucleases were either stably or transiently introduced together with a plant expressible chimeric gene comprising a hygromycinphosphotransferase conferring resistance to hygromycine.

After double stranded DNA break induction at the recognition site in the bar coding region through expression of the plant expressible chimeric genes encoding the BAY39/40 heterodimer, the break can be repaired by non-homologous end-joining in the absence of repair DNA, resulting in deletion or insertion of one or more base pairs, thereby disrupting the bar coding region, resulting in phosphinotricin sensitivity.

Several plant lines exhibiting phosphinotricine sensitivity and hygromycin resistance were selected. From these plant lines a DNA fragment was amplified by PCR using primers located at either side of the recognition site (SEQ ID No 1 or 2) in the bar coding region, and the nucleotide sequence of the amplicons was determined. An alignment of the different nucleotide sequences is represented in Figure 5.

It is clear that in the PPT sensitive plant lines (3 to 9), the recognition site for BAY39/40 has been altered by deletion(3 to 8) or insertion (9) whereas no alteration was found in PPT resistant plant lines (2).

From these experiments it can thus be concluded that hdBAY39/40 exhibits cleavage activity at the preselected site.

Example 3: Targeted insertion by non-homologous end-joining.

Co-delivery of pCV177 comprising the chimeric genes encoding hd BAY39/40 meganucleases or pCV170 comprising the chimeric gene encoding the sc BAY39/40 with repair DNA comprising a selectable marker such as a plant-expressible chimeric gene comprising a 2mepsp coding region (without further homology to the target region) to plant cells comprising a plant expressible chimeric bar gene integrated in their genome and selection of phosphinotricin sensitive plants tolerant to the selection compound such as glyphosate allows the identification of the plant cells wherein repair DNA sequences are integrated in the bar coding region.

Example 4: Targeted insertion by homologous end-joining.

Co-delivery of either pCV177, comprising the chimeric genes encoding hd BAY39/40 meganucleases or pCV170, comprising the chimeric gene encoding the sc BAY39/40, with repair DNA comprising a selectable marker such as a plant-expressible chimeric gene comprising a 2mepsp coding region flanked upstream by flanking sequences comprising a nucleotide sequence with sequence similarity to the bar coding region of

SEQ ID No 3 from nucleotide 1 to nucleotide 132 and flanked downstream by flanking sequences comprising a nucleotide sequence with sequence similarity to the bar coding region of SEQ ID No 3 from nucleotide 154 to nucleotide 552, into plant cells comprising a plant expressible chimeric bar gene integrated in their genome and selection of phosphinotricin sensitive plants tolerant to the selection compound such as glyphosate allows the identification of the plant cells wherein the repair DNA is integrated in the bar coding region.

Example 5: Targeted double stranded DNA break induction using BAY39/40 single chain and heterodimeric meganuclease in cotton.

Embryogenic calli from PPT-resistant cotton plants containing a chimeric gene comprising the bar gene under control of the CSVMV promoter were grown on M100 substrate (MS salts, B5 vitamins, MES 0.5 g/L, $MgCl_2.6H_2O$ 0.94 g/L, gelrite 2g/L, glucose 30 g/L, pH 5.8) with 2g/L active carbon. These calli were subjected to microparticle bombardment, using a BioRAD PPS_1000/He Biolistic Particle delivering system essentially as described by Sanford et al., 1992 whereby the particles were coated with either the pCV177 vector encoding the heterodimeric BAY39/40 meganuclease or the pCV170 vector encoding the single chain BAY39/40 meganuclease. A co-delivery was done of the meganuclease vector with a vector containing the 2mEPSPS gene under control of a plant-expressible promoter conferring glyphosate tolerance as a selectable marker gene. After bombardment, the calli were transferred to a medium containing 1mM glyphosate, resulting in about 3000 glyphosate resistant embryogenic calli. Of these, 85 events appeared PPT sensitive, of which 79 events were further molecularly analyzed. These 79 events were characterized for genotype by PCR using primers flanking the target site and subsequent sequencing of the PCR product. The absence of a PCR product is indicative of a large deletion around the target site (table 1).

pCV170 (sc)			
PCR product obtainable	# events	change at target site	# events
no	11	large deletion	11
yes	8	no mutation	6
		replacement/insertion	1
		deletion	1

pCV177 (hd)			
PCR product obtainable	# events	change at target site	# events
no	33	large deletion	33
yes	27	no mutation	19
		insertion	4
		deletion	4

Table 1: Characterization of PPT-sensitive glyphosate-resistant transformation events.

Thus, these results demonstrate that both the single chain as well as the heterodimeric BAY39/40 meganuclease are capable of inducing a targeted double stranded DNA break at the desired position and that targeted deletion, replacement and insertion events can be obtained using these meganucleases in cotton.

CLAIMS

1. A method for introducing a foreign DNA molecule at a predefined site in a genome of a plant cell comprising the steps of
   a. inducing a double stranded DNA break at said predefined site;
   b. introducing said foreign DNA molecule in said plant cell; and
   c. selecting a plant cell wherein said foreign DNA is introduced at said predefined site;

   characterized in that said predefined site is comprised within a DNA region encoding a phosphinotricin acetyltransferase as encoded by Streptomyces hygroscopicus (bar coding region) and that said double stranded DNA break is induced by introduction of a single chain meganuclease or a pair of meganucleases which recognizes or recognize in concert said predefined site and induces or induce said double stranded break.

2. The method according to claim 1, wherein said predefined site comprises the nucleotide sequence of SEQ ID No. 1 or SEQ ID No. 2.

3. The method according to claim 1 or claim 2, wherein said bar coding region comprises the nucleotide sequence of SEQ ID No.: 3.

4. A method for introducing a foreign DNA molecule at a predefined site in a genome of a plant cell comprising the steps of
   a. inducing a double stranded DNA break at said predefined site;
   b. introducing said foreign DNA molecule in said plant cell; and
   c. selecting a plant cell wherein said foreign DNA is introduced at said predefined site;

   characterized in that said predefined site comprises the nucleotide sequence of SEQ ID No. 1 or SEQ ID No. 2 and that said double stranded DNA break is induced by introduction of a single chain meganuclease or a pair of

meganucleases which recognizes or recognize in concert said predefined site and induces or induce said double stranded break.

5. The method according to any one of claims 1 to 4, wherein said meganuclease or said pair of meganucleases is/are derived from I-CreI and wherein the following amino acids are present in meganuclease unit 1:
    a. S at position 32;
    b. Y at position 33;
    c. E at position 38;
    d. R at position 40;
    e. K at position 66;
    f. Q at position 80;
    g. T at position 42;
    h. R at position 77;
    i. R at position 68;
    j. R at position 70;
    k. Q at position 44;
    l. I at position 24;
    m. S at position 26;
    n. S at position 28;
    o. R at position 30.

    and wherein the following amino acids are present in meganuclease unit 2:
    p. R at position 70;
    q. T at position 44;
    r. I at position 24;
    s. S at position 26;
    t. S at position 28;
    u. N at position 30;
    v. S at position 32;
    w. R at position 33;
    x. Q at position 38;

y. Q at position 80;
z. R at position 40;
aa. K at position 66;
bb. T at position 42;
cc. R at position 77;
dd. R at position 68.

6. The method according to any one of claims 1 to 5, wherein said pair of meganucleases obligatory forms heterodimers or wherein said meganuclease is a single chain meganuclease comprising two domains derived from I-CreI covalently connected by a linker.

7. The method according to any one of claims 1 to 6, wherein said pair of meganucleases comprises the amino acid sequence of SEQ ID No. 5 and SEQ ID No. 6, respectively, or said single chain meganuclease comprises the amino acid sequence of SEQ ID No. 18 from position 1 to 167 and from position 206 to 362.

8. The method according to any one of claims 1 to 7, wherein said pair of meganucleases is encoded by a nucleic acid comprising the nucleotide sequence of SEQ ID No. 4 from nucleotide position 2004 to nucleotide position 2525 or to 2522 and the nucleotide sequence of SEQ ID No. 4 from nucleotide position 4885 to nucleotide position 5405 or to 5403, or said single chain meganuclease is encoded by a nucleic acid molecule comprising the nucleotide sequence of SEQ ID No. 17 from nucleotide position 1267 to 1605 and from 1795 to 1956 and from 2071 to 2541, or said single chain meganuclease is encoded by a nucleic acid molecule comprising the nucleotide sequence of SEQ ID No. 17 from nucleotide position 1267 to 1605 and from 1795 to 2541.

9. The method according to any one of claims 1 to 8, wherein said meganuclease or said pair of meganucleases is encoded by a nucleic acid which has been codon-optimized for expression in said cell of said plant.

10. The method according to any one of claims 1 to 9, wherein said foreign DNA is introduced by direct DNA transfer.

11. The method according to any one of claims 1 to 10, wherein said foreign DNA is introduced without any further DNA.

12. The method according to any one of claims 1 to 11, wherein said foreign DNA is comprised within a repair DNA, said repair DNA comprising at least one flanking nucleotide sequence homologous to the upstream or downstream sequence of the nucleotide sequence of SEQ ID No. 1.

13. The method according to any one of claims 1 to 12, wherein said meganuclease or said pair of meganucleases is expressed from a chimeric gene or a pair of chimeric genes, each comprising a plant expressible promoter operably linked to a coding region encoding said meganuclease or one of said pair of meganucleases, and further operationally linked to a DNA region involved in transcription termination and polyadenylation functional in a plant cell.

14. The method according to claim 13, wherein said chimeric gene is introduced transiently into said plant cell.

15. The method according to claim 14, wherein said chimeric gene is introduced stably into said plant cell.

16. The method according to any one of claims 1 to 15, wherein said foreign DNA comprises a selectable marker gene.

17. The method according to any one of claims 1 to 16, wherein said foreign DNA comprises a plant expressible gene of interest.

18. The method according to claim 17, wherein said plant expressible gene of interest is selected from the group of a herbicide tolerance gene, an insect resistance gene, a disease resistance gene, an abiotic stress resistance gene, an enzyme involved in oil biosynthesis, carbohydrate biosynthesis, an enzyme involved in fiber strength or fiber length, an enzyme involved in biosynthesis of secondary metabolites.

19. The method according to any one of the preceding claims wherein said plant cell is further regenerated into a plant.

20. A plant cell wherein said foreign DNA has been introduced into said predefined site, obtained by the method according to any one of claims 1 to 19.

21. A plant wherein said foreign DNA has been introduced into said predefined site, obtained by the method according to claim 19.

22. A seed or propagating part of a plant comprising said foreign DNA integrated at said predefined site, from the plant of claim 21.

23. A method of growing a plant according to claim 21, comprising the step of applying a chemical to said plant or substrate wherein said plant is grown.

24. A process for producing a plant comprising foreign DNA integrated at the bar coding region, comprising the step of crossing a plant consisting essentially of the plant cells of claim 20 with another plant or with itself and optionally harvesting seeds.

25. A process of growing a plant in the field comprising the step of applying a chemical compound on a plant of claim 21.

26. A process of producing treated seed comprising the step applying a chemical compound on a seed of plant according to claim 22.

27. Use of a meganuclease or a pair of meganucleases as described in any one of claims 1 to 15 to introduce a foreign DNA into the bar coding region.

28. Use of a custom made meganuclease to introduce a foreign DNA of interest at a predefined site in a plant cell.

29. A method for introducing a foreign DNA molecule at a predefined site in a genome of a plant cell comprising the steps of
    a. inducing a double stranded DNA break at said predefined site;
    b. introducing said foreign DNA molecule in said plant cell; and
    c. selecting a plant cell wherein said foreign DNA is introduced at said predefined site;

    characterized in that said double stranded DNA break is induced by introduction of a non-naturally occurring single chain meganuclease or a pair of non-naturally occurring meganucleases which recognizes or recognize in concert said predefined site and induces or induce said double stranded break.

30. A method for introducing a foreign DNA molecule at a predefined site in a genome of a transgenic plant cell comprising the steps of
    a. inducing a double stranded DNA break at said predefined site;
    b. introducing said foreign DNA molecule in said plant cell; and
    c. selecting a plant cell wherein said foreign DNA is introduced at said predefined site;

    characterized in that said predefined site is a nucleotide sequence different from a recognition site for a natural occurring meganuclease, said predefined site being a nucleotide sequence commonly introduced as part of a transgene in a transgenic plant and wherein double stranded DNA break is induced by introduction of a non-naturally occurring single chain meganuclease or a pair

of non-naturally occurring meganucleases which recognizes or recognize in concert said predefined site and induces or induce said double stranded break.