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(56) Fremdragne publikationer:
EP-A1- 2 274 973
WO-A1-2014/144155
WO-A1-2014/194190
US-A1- 2015 059 010
BALTES N J ET AL: "DNA replicons for plant genome engineering", THE PLANT CELL, AMERICAN SOCIETY OF PLANT BIOLOGISTS, US, Bd. 26, Nr. 1, 17. Januar 2014 (2014-01-17), Seiten 151-163, XP002752140, ISSN: 1040-4651, DOI: 10.1105/TPC.113.119792 [gefunden am 2014-01-17]
Thomas P Quinn ET AL: "A Streamlined Method for the Production, Screening, and Application of sgRNAs for CRISPR/Cas Gene Editing", , 2014, XP055290306, Mountain View, CA, USA Gefunden im Internet: URL:http://info.clontech.com/rs/clontech/i mages/633702_Guide_It_PS_0514_web.pdf [gefunden am 2016-07-21]
TAKESHI MARUYAMA ET AL: "Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining", NATURE BIOTECHNOLOGY, Bd. 33, Nr. 5, 20. April 2015 (2015-04-20) , Seiten 538-542, XP055290186, ISSN: 1087-0156, DOI: 10.1038/nbt.3190

Fortsættes ...

ALI ZAHIR ET AL: "Efficient Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System", MOLECULAR PLANT, Bd. 8, Nr. 8, 6. März 2015 (2015-03-06), Seiten 1288-1291, XP055290127,
HUI-LI XING ET AL: "A CRISPR/Cas9 toolkit for multiplex genome editing in plants", BMC PLANT BIOLOGY, BIOMED CENTRAL, LONDON, GB, Bd. 14, Nr. 1, 29. November 2014 (2014-11-29), Seite 327, XP021205803, ISSN: 1471-2229, DOI: 10.1186/S12870-014-0327-Y
HYUN YOUBONG ET AL: "Site-directed mutagenesis in Arabidopsis thaliana using dividing tissue-targeted RGEN of the CRISPR/Cas system to generate heritable null alleles", PLANTA, SPRINGER VERLAG, DE, Bd. 241, Nr. 1, 1. Oktober 2014 (2014-10-01), Seiten 271-284, XP035417502, ISSN: 0032-0935, DOI: 10.1007/S00425-014-2180-5 [gefunden am 2014-10-01]
THOMAS B JACOBS ET AL: "Targeted genome modifications in soybean with CRISPR/Cas9", BMC BIOTECHNOLOGY, BIOMED CENTRAL LTD. LONDON, GB, Bd. 15, Nr. 1, 12. März 2015 (2015-03-12), Seite 16, XP021219338, ISSN: 1472-6750, DOI: 10.1186/S12896-015-0131-2
W. JIANG ET AL: "Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice", NUCLEIC ACIDS RESEARCH, Bd. 41, Nr. 20, 2. September 2013 (2013-09-02), Seiten e188-e188, XP055219328, GB ISSN: 0305-1048, DOI: 10.1093/nar/gkt780
HUI ZHANG ET AL: "The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation", PLANT BIOTECHNOLOGY JOURNAL, Bd. 12, Nr. 6, 23. Mai 2014 (2014-05-23), Seiten 797-807, XP055269351, GB ISSN: 1467-7644, DOI: 10.1111/pbi.12200
LUISA BORTESI ET AL: "The CRISPR/Cas9 system for plant genome editing and beyond", BIOTECHNOLOGY ADVANCES, Bd. 33, Nr. 1, 20. Dezember 2014 (2014-12-20), Seiten 41-52, XP055217852, ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2014.12.006
ANONYMOUS: "A Streamlined Method for the Production, Screening, and Application of sgRNAs for CRISPR/Cas9 Gene Editing", BIOTECHNIQUES, Bd. 57, Nr. 3, September 2014 (2014-09), Seite 157, XP055290305,
BERND ZETSCHE ET AL: "Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System", CELL, Bd. 163, Nr. 3, 25. September 2015 (2015-09-25), Seiten 759-771, XP055267511, US ISSN: 0092-8674, DOI: 10.1016/j.cell.2015.09.038 in der Anmeldung erwähnt
BENT ANDREW F: "Arabidopsis in planta transformation. Uses, mechanisms, and prospects for transformation of other species", PLANT PHYSIOLOGY, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, USA, vol. 124, no. 4, 1 December 2000 (2000-12-01), pages 1540-1547, XP002416813, ISSN: 0032-0889, DOI: 10.1104/PP.124.4.1540

Field

The present invention relates in particular to methods for the manufacture of a maize plant, comprising providing and introducing at least one gRNA as well as a CRISPR nuclease or a catalytically active fragment thereof and/or an effector domain or at least one recombinant construct, comprising a gRNA as well as a CRISPR nuclease or a catalytically active fragment and/or an effector domain or the sequences encoding therefor, as well as at least one regulatory sequence and/or a localization sequence, into a plant target structure comprising at least one meristematic cell, whereupon a maize plant comprising a targeted modification of a target region of a nucleic acid, can be obtained directly, wherein the at least one recombinant construct is not integrated chromosomally or extrachromosomally. In addition, appropriate recombinant constructs and vectors as well as methods for introducing these constructs and vectors into a plant target structure of interest are disclosed. Finally, the use of a recombinant construct for the specific modification of a target region of a nucleic acid in a plant cell is disclosed, as well as plants, plant material or a plant cell which can be obtained or is obtained by the method in accordance with the invention. Furthermore, an *in vitro* screening method is disclosed as a preliminary test, in order to readily determine, with a high output, the functionality of a gRNA or an encoding sequence for a gRNA with respect to the targeted modification of a specific target region of a nucleic acid in a plant cell, together with a CRISPR nuclease, comprising, inter alia, a Cas and/or Cpf1 nuclease, or variations or catalytically active fragments thereof, or a catalytically active fragment thereof. The methods disclosed herein are suitable in particular for the targeted introduction, modification, or elimination of a desired trait in a plant, in particular within the framework of the targeted trait development, in order to ensure highly specific and efficient genome editing.

Background of the Invention

Genome editing constitutes a molecular biological method by means of which specific modifications such as insertions, deletions or point mutations or combinations thereof can be introduced into the genome of a living organism. To this end, specific molecular instruments are required which firstly have nuclease activity, but above all can be guided to the target sequence to be modified with sufficient specificity to programme and carry out a specific and site-directed mutagenesis. In the past few years in plant biotechnology, specific genome editing has developed into an alternative to conventional cultivation and to transgenic strategies. However, tools which are currently available, such as zinc finger nucleases (ZFNs) or "transcription activator-like effector nucleases" (TALENs) are only used in plant

biotechnology to a limited extent because of their occasional low efficiency and also because of the complex and costly design of the constructs.

A further molecular tool which has been widely used in recent years for precise and site-directed genome modification is the CRISPR nuclease-based system. These nucleases, including inter alia Cas (CRISPR associated gene) nuclease and Cpf1 nucleases, form part of the system described now in the literature as "CRISPR" (clustered regularly interspaced short palindromic repeat). This system was originally identified in 1987 when the *iap* gene of *E. coli* was analysed and naturally occurring repeat sequences in the bacterial genome were identified. Later on it was discovered that these palindromic DNA repeat sequences of 20 to 50 nucleotides followed a pattern. The acronym CRISPR was then adopted (Jansen, R. et al., "Identification of genes that are associated with DNA repeats in prokaryotes", Mol. Microbiol., 2002, 43(6), 1565-1575), whereupon research focused even more closely upon bacteria. Finally, it was reported that the CRISPR locus constitutes a type of bacterial immune system and could confer immunity against phages (Barrangou et al., "CRISPR provides acquired resistance against viruses in procaryotes" Science 2007, 315:1709.1712), by installing the invading phage DNA as a protospacer into a CRISPR locus, transcribing the locus and finally activating the CRISPR-mediated silencing mechanism.

Functional characterization gradually led to the system being exploited as a universal tool for genome modification of higher organisms. In the meantime, a large number of CRISPR systems have been described (see Van der Oost et al., "Unravelling the structural and mechanistic basis of CRISPR-Cas systems" Nature 2014, 482:331-338, Makarova et al., "An updated evolutionary classification of CRISPR-Cas systems", Nature Reviews Microbiology 13, 722-736); as yet, the analyses are still far from complete.

A further genome editing system with significant potential is now available thanks to the discovery and exploitation of the bacterial type II CRISPR system.

Five types (I-V) of CRISPR systems have been described so far (Barrangou et al., 2007, Science, 315(5819):1709-12.; Brouns et al., 2008, Science, 321(5891):960-4.; Marraffini und Sontheimer, 2008, Science, 322(5909):1843-5; Makarova et al., Nature Rev. Microbiol., 13, 722-736, 2015), wherein each system comprises a cluster of CRISPR-associated genes (Cas or others) and a corresponding CRISPR array. These characteristic CRISPR arrays are composed

of repetitive sequences (direct repetitions, so-called repeats), in which short sections of non-repetitive sequences ("spacers") are embedded, wherein the spacers originate from short fragments of foreign genetic material (protospacers). The CRISPR arrays are subsequently transcribed into short CRISPR RNAs (crRNAs), wherein the crRNAs direct the Cas proteins or other effector nucleases of a CRISPR system to the respective target nucleic acid molecules, where cleavage occurs by means of Watson-Crick base pairing. The Type I and Type III CRISPR systems use complexes of Cas proteins and crRNAs for the recognition and subsequent cleaving of target nucleic acids (Wiedenheft et al., 2011, Nature, 477(7365):486-9). Type II CRISPR systems, on the other hand, recognise and cleave target DNA in their natural form in conjunction with the RNA-directed nuclease Cas9 with two non-encoded RNAs, the crRNA, and a trans-activating RNA (tracrRNA) (Garneau et al., 2010; Sapranauskas et al., 2011, Nucleic Acids Res., 39(21):9275-82; Deltcheva et al., 2011, Nature, 471(7340):602-7). A possible Type IV CRISPR system has also been proposed (Makarova et al., Biol. Direct, 6 (38), 2011).

The immune response mediated by CRISPR/Cas in natural systems requires CRISPR-RNA (crRNA), wherein the maturation of this guide RNA, which controls the specific activation of the Cas nuclease, varies significantly between the various CRISPR systems which have been characterized so far. Firstly, the invading DNA, also known as a spacer, is integrated between two adjacent repeat regions at the proximal end of the CRISPR locus. Type II CRISPR systems encode a Cas9 nuclease as a key enzyme for the interference step. This involves both a crRNA and also a trans-activating RNA (tracrRNA) as the guide motif. These hybridize and form double-stranded (ds) RNA regions which are recognized by RNAseIII and can be cleaved in order to form mature crRNAs. These then in turn associate with the Cas molecule in order to direct the nuclease specifically to the target region of a nucleic acid.

A recombinant CRISPR/Cas system, or, in general, a CRISPR/nuclease system, enables targeted DNA recognition and/or bonding through a small, individually tailored, non-encoding RNA (guide RNA or gRNA) in combination with a possibly modified nuclease, and the optional generation of a single-stranded or double-stranded break. Recombinant gRNA molecules can comprise both the variable DNA recognition region and also the nuclease interaction region and thus can be specifically designed, independently of the specific target nucleic acid and the desired nuclease (Jinek et. al., 2012, supra). As a further safety mechanism, PAMs (protospacer adjacent motifs) must be present in the target nucleic acid region; these are

DNA sequences which in type II CRISPR system follow on directly from the Cas9/RNA complex-recognized DNA. The PAM sequence for the Cas9 from *Streptococcus pyogenes* is therefore "NGG" or "NAG" (Standard IUPAC nucleotide code) (Jinek et al., "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity", Science 2012, 337: 816-821). The PAM sequence for Cas9 from *Staphylococcus aureus* is "NNGRRT" or "NNGRR(N)". Further variant CRISPR/Cas9 systems have been reported. Thus, a *Neisseria meningitidis* Cas9 cleaves at the PAM sequence NNNNGATT. A *Streptococcus thermophilus* Cas9 cleaves at the PAM sequence NNAGAAW. Furthermore, by using modified Cas polypeptides, specific single-stranded breaks can be obtained. The combined use of Cas nickases with various recombinant gRNAs can also induce highly specific DNA double-stranded breaks by means of double-stranded DNA nicking. By using two gRNAs, the specificity of the DNA binding and thus the DNA cleavage can be optimized.

In addition to the CRISPR/Cas system, so-called CRISPR/Cpf1 systems have also been described recently. These are suitable as tools for targeted genome editing in a manner analogous to that of CRISPR/Cas systems (see Zetsche et al., "Cpf1 Is a Singel RNA-Guides Endonuclease of a Class 2 CRISPR-Cas System", Cell, 163, pp. 1-13, October 2015). The CRISPR/Cpf1 system is also referred to as a Type V CRISPR system (Makarova et al., Nature Rev. Microbiol., 2015, supra). Unlike a Cas9 nuclease of a Type II CRISPR/Cas system, a Cpf1 nuclease requires no additional trans-activating tracr RNAs. Cpf1 recognizes T-rich PAM sequences, and cleaves the target DNA, producing "sticky ends," i.e. overhangs, while Cas9 leaves "blunt ends." Like Cas nucleases, Cpf1 nucleases contain an RuvC-like endonuclease domain, but lack a second HNH endonuclease domain (Makarova & Koonin, Methods Mol. Biol., 1311, 47-75, 2015). While Type I, II and IV CRISPR systems are currently referred to as Class 1 systems, Type II and Type V systems are regarded as Class 2 (cf. Makarova et al., Nature Rev. Microbiol.,2015, supra).

A DNA double-stranded break inside a plant cell is repaired either by means of "non-homologous end joining" (NHEJ) or "homologous recombination" ((HR), also referred to as "homology-directed repair" (HDR)). Furthermore, in plants, so-called alternative end joining (AEJ) pathways have been described (Charbonnel C, Allain E, Gallego ME, White CI (2011) Kinetic analysis of DNA double-strand break repair pathways in Arabidopsis. DNA Repair (Amst) 10: 611-619).

It is therefore proposed in US 2015/082478 A1 that a separate HDR DNA repair vector be used in order to introduce a double-stranded break, which was previously obtained through a recombinant CRISPR/Cas system. The modification of complex eukaryotic genomes constitutes a more significant challenge than the genetic modification of bacterial genomes as, due to the complexity of this genome, molecular tools have to be provided which can effect a specific genome modification without unwanted off-target effects, i.e. unwanted mutations or modifications within the genome or the non-genomic DNA of the target cell.

US 8,697,359 B1 discloses that CRISPR/Cas technology can be used to modify eukaryotic genomes, in particular mammalian genomes, and preferably for therapeutic purposes. This involves suppressing the expression of a target gene by specific introduction of a Cas9 endonuclease as well as a guide RNA (gRNA) in a programmable manner. This gRNA is an essential element of every Cas9 CRISPR system as it guides the actual Cas nuclease specifically to the (genomic) target DNA. To this end, in addition, a tracr (trans-activating CRISPR RNA) sequence and a tracr mate sequence are disclosed for Cas9-CRISPR systems which can be included in the gRNA, wherein the tracr sequences hybridize and can thus be recognized. However, the use of CRISPR technology to modify complex plant genomes and the molecular tools required for this is not disclosed.

WO 2015/026885 A1, on the other hand, focuses on the use of CRISPR/Cas technology in plants. Here, however, only an overall strategy and appropriate molecular tools are disclosed which necessarily require the subculture, selection and regeneration of plant calluses following the successful introduction of the CRISPR/Cas tools and thus do not allow a plant or plant material containing the desired DNA modification to be obtained directly.

An overview of the current use of CRISPR/Cas technology for the genome editing of plant genomes can be found in Bortesi & Fischer ("The CRISPR/Cas9 system for plant genome editing and beyond", Biotechnology Advances, 33, Pages 41-52, 20/12/2014). This reports, inter alia, on the problems involved in providing specific gRNAs for targeting in maize. Furthermore, the problems related to the high off-target mutation rates are discussed; these are not only observed when using CRISPR/Cas in mammalian cells, but are also observed when using them in plant cells; here, the design of the individual CRISPR/Cas tool play a decisive role in obtaining a targeted modification of the respective target cell/respective target organism without off-target effects. Furthermore, Bortesi & Fischer recognise that the CRISPR/Cas

system can also be used for the epigenetic modification of DNA since the CRISPR/Cas system can also be used to cleave methylated DNA, then state that no applications in plants are known as yet.

Furthermore, Guilinger *et al.* describe FokI nucleases which are used in the manner of nickases, and thus produce a higher specificity (Guilinger et al.; Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification, doi:10.1038/nbt.2909). However, Guilinger et al. only present data for human cells, and not for plant cells.

Mali *et al.* (2013) report on the use of the CRISPR/Cas system in human cells, wherein nuclease-null variants of Cas9 or aptamer-coupled gRNAs are used which may be fused to transcriptional activator domains (Mali, P., et al. (2013). "CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering). However, Mali *et al.* do not mention how and to what extent a corresponding system could be used in plant cells.

In summary, many of the strategies used today are only temporary or transient in the plant field, meaning that mutations occur in already differentiated cells, for example in leaves, but these mutations cannot be inherited via the germline. Furthermore, strategies exist which require a stable integration of the coding sequences into the genome along with the required regulatory sequences such as promoters and terminators, via which a stable mutation can then be produced which is inherited from generation to generation. However, the CRISPR/Cas tools are still contained in the genome of the plant and thus also in potential plant products such as fruit and seeds; this has an undesirable impact on the risk level of these products.

Accordingly, using CRISPR/Cas in plant biotechnology is inefficient, with constructs difficult and time-consuming to design and off-target effects a frequent occurrence. In addition, many of the current strategies are based on either integrating the CRISPR/Cas tools into the genome of a plant cell in a stable manner or introducing the CRISPR/Cas tools into cells of a differentiated tissue, for example leaves. As a consequence, with the stable strategy, the individual tools such as Cas9 are inherited by the descendants, and not just the specific DNA modifications effected by them. Upon transformation into differentiated cells and tissue, the mutation introduced by the CRISPR/Cas tools is only effective in the relevant cells, but cannot be inherited via the germline. Specifically with regard to the targeted development of positive

traits in plants, comprising resistances, in particular to pests and environmental factors, e.g. cold, drought, saline content, increased yield, or herbicide resistances, the creation of reliable methods for silencing RNA inside a plant cell and the targeted activation, deactivation, or modification of genomic material is of major economic interest.

Regarding the selection of suitable gRNAs, *in silico* tools already exist for the identification and subsequent production of suitable gRNAs (see: www.dna20.com/eCommerce/cas9/input), but there are currently no specific tools that could be used in important crop plants, which always have complex genomes. Furthermore, the available tools provide no information regarding the actual effectiveness of gRNA identified *in silico* in subsequent *in vitro* or *in vivo* test within a plant cell.

There is therefore a continuing need for the establishment of transient and also optionally inducible methods and constructs based, inter alia, on gRNAs and CRISPR nucleases or gRNAs and other effector domains, in order to carry out a desired modification of a target sequence in a target plant cell, wherein only the modification of the target sequence but not the construct is passed on to descendants. In addition, there is a substantial need for a CRISPR-based method which offers the possibility of direct germline modification in a plant cell or plant tissue so that the modification can be inherited and seeds can be harvested directly from the plant resulting from the modified plant cell or tissue which contain the specific genome modification without the need for difficult and expensive intermediate steps. Finally, there is a need for specifically broadening RNA-directed DNA modification systems which are provided by the CRISPR/Cas tools, in which not only genomic target structures but any nucleic acid can not be modified as the target structure in a controlled manner in the genome of a cell, in the cytosol or in plastids. In this regard, there is currently a demand for suitable insertion systems which allow for the targeted insertion of CRISPR/Cas tools and thus allow for the targeted modification of a target region inside a plant target structure.

Furthermore, there is a demand for efficient *in vitro* screening methods, by means of which it is possible to determine and reliably predict the effectiveness of a gRNA inside a plant cell in an *in vitro* assay with a high output, in order to avoid costly and lengthy attempts with plant material.

The ultimate goal is to optimize the precision of a genome editing approach, in particular for the modification of larger eukaryotic genomes, comprising plant genomes and genomes from animal organisms, in order to reduce the rate of off-target effects, and, ideally, to bring about an optimal repair of a targeted, inserted double-stranded break by providing a repair template along with the actual genome modification tools.

Summary of the Invention

The object of the present invention is therefore to provide methods and molecular tools which facilitate the transient transformation of plant tissue or plant cells, especially meristematic cells, and thus allow the controlled modification of any target region of a nucleic acid in any cellular compartment of a plant cell. Likewise, one object is to create suitable insertion methods for the molecular tools in accordance with the present disclosure. In addition, suitable *in vitro* screening assays or tests should be created, such that suitable functional gRNAs can be identified *in vitro* in advance, in order to be able to efficiently reduce the subsequent effort *in planta* or *in vitro* in plant tissue.

Combining the aforementioned advantages into one system with broad application in plant biotechnology is an aim of the invention.

With a particular focus on creating tools which facilitate the cultivation of diverse monocotyledonous and dicotyledonous plants such that traits of interest can quickly be inserted in a plant genome, removed therefrom or modified therein.

This object is accomplished by the methods which are the subject matter of the accompanying claims and as covered in detail in the description, the figures and the accompanying sequence listing. In particular, the object is achieved by the provision of a method comprising the transformation or transfection of at least one meristematic cell. Furthermore, the object is achieved by the provision of recombinant constructs that comprise specifically modified CRISPR tools and/or further effector domains. Finally, the objective is achieved by the provision of appropriate regulatory sequences and localization sequences which allow the recombinant construct of interest to be directed in a controlled manner into any compartment of a plant target structure of interest.

In this manner, at least one specific modification in any target region of a nucleic acid can be obtained in any compartment of a plant cell, in particular a meristematic plant cell. Since the at least one meristematic cell modified in this manner can pass on the specific modification in the target region of a nucleic acid directly by means of subsequent cell division and differentiation to its descendants and/or has the potential for a completely modified plant organism to mature from the meristematic cell, a plant or plant material or a plant cell can be provided without having to carry out additional complex cultivation or crossing and selection steps (in particular complex backcrossing procedures). Rather, a plant, plant material or a plant cell can be immediately and directly obtained from the at least one meristematic target cell modified in this manner. In this manner, it is possible to produce or provide or activate gRNA(s) and/or CRISPR nuclease(s) or one or more catalytically active fragment(s) thereof and/or other effector domain(s) on a transient basis in the meristems, wherein these recombinant macromolecules are subsequently preferably degraded, i.e. after the gRNA(s) and/or CRISPR nuclease(s) or catalytically active fragments thereof and/or other effector domain(s) have carried out the desired effect due to the absence of any integration thereof into the genome or endogenous extrachromosomal DNA; this may be of benefit as regards regulatory aspects and the risk assessment of the plant product. The CRISPR nucleases or catalytically active fragments thereof used herein may also contain one or more mutation(s) in the catalytic domains responsible for (double-stranded or single-stranded) DNA cleavage. This results in a broad spectrum of application for the CRISPR nuclease or the catalytically active fragment thereof and, in the case of Cas-based nickases for example, in a higher binding specificity, since two CRISPR/Cas constructs are used in order to cleave both strands of the DNA double strand at the desired site. Cas-null variants are also proposed herein, as well as their combined use with other effector domains to optimize a specific nucleic acid modification.

Furthermore, it was found that by exploiting the mechanism of action of the CRISPR tools, other effector domains such as DNA-modifying, RNA-modifying or histone-modifying or DNA-binding, RNA-binding or histone-binding polypeptides or nucleic acids comprising, for example, any type of monomeric, dimeric or multimeric nuclease, including nickases, transcriptional activators and repressors, phosphatases, glycosylases or enzymes which can effect epigenetic modifications, such as methylases, acetylases, methyltransferases or histone deacetylases, aptamers, comprising single-stranded DNA or RNA sequences as well as peptides, fluorescent proteins, bioluminescence proteins, marker nucleic acid sequences or marker amino acid sequences and the like, and combinations thereof can be used in accordance

with the method provided herein, expanding known targeted genome editing to general nucleic acid editing which is not *per se* limited to genomic DNA.

Regarding the aspect of editing genomic DNA disclosed herein, a DNA repair template or HDR template is provided, which can be inserted in a targeted manner, together with the CRISPR tools, into a target cell or target structure of interest in order to out-compete the error-prone endogenous NHEJ repair system, and to furthermore be able to insert a desired nucleic acid at the site of a double-strand break. The terms DNA repair template and HDR template are used interchangeably.

The present disclosure thus offers the possibility of expanding the CRISPR/Cas mechanism to facilitate not only the nucleolytic cleavage of DNA, but also any modification of genomic DNA (for example epigenetic modification) and of RNA in plant cells (for example mRNA).

By using other regulatory sequences comprising promoters, terminators, transcription factor binding sites or introns, and/or localization sequences comprising nuclear, mitochondrial and plastid localization sequences, the present disclosure also provides the possibility of modifying any target region of a nucleic acid of a plant target structure in a specific manner, wherein mitochondrial and plastid DNA, for example, can also be targeted for editing. The present disclosure also provides the possibility of the specific modification of RNA (for example mRNA), wherein the gRNA-directed sequence recognition which is the basis of the CRISPR/Cas system can be exploited and "reprogrammed" in accordance with the present disclosure in order to broaden the field of application of CRISPR/Cas technology.

Methods and constructs are also provided herein, by means of which gRNA and/or CRISPR nuclease or the catalytically active fragment thereof already linked to a further effector domain is/are provided on a recombinant construct.

Furthermore, a method is provided in which the at least one gRNA as well as the at least one CRISPR nuclease or the catalytically active fragment thereof and/or the at least one further effector domain are provided separately on different recombinant constructs. In accordance with this method, the gRNA component may be provided as DNA or RNA, the CRISPR nuclease or variant thereof or the catalytically active fragment thereof may be provided as DNA

or RNA or as a polypeptide sequence and the effector domain may be provided as DNA or RNA or as a polypeptide sequence.

An additional object is to provide the possibility of providing specific constructs which may be inducible or tissue-specific or organelle-specific, and of minimizing unwanted off-target effects by establishing plant-specific constructs and methods. Finally, it is an object of the present invention to design methods and constructs which provide the possibility of efficient gene knock-ins in addition to, for example, the possibility of specific gene knock-outs, insertions of genetic fragments, specific epigenetic modifications, the introduction of point mutations, acetylation, methylation, phosphorylation, glycosylation, marking by resistance markers or fluorescent proteins, activation or repriming of transcription, specific cleavage of double-stranded or single-stranded nucleic acids, the binding of nucleic acids and the like, so that the field of application in plant cultivation is broadened. It is desirable from a cultivation and regulatory standpoint to ensure that a trait effected by the modification can be inherited by at least one generation while ensuring the simultaneous absence of the constructs of the CRISPR/Cas system required for this in the resulting plant or the resulting plant cells.

Lastly, it is an object of the present invention to create an *in vitro* screening method for identifying a gRNA or a gRNA encoding sequence in an *in vitro* assay to identify a gRNA or a gRNA encoding sequence, which, together with a CRISPR nuclease or catalytically active fragment thereof, is suitable for the targeted modification of a target region of a nucleic acid in a plant cell.

Specific aspects and embodiments of the present invention are provided in the following detailed description and examples, the figures, the sequence listing and in particular the accompanying patent claims.

Brief Description of the Drawings

Figure 1 A-F (Fig. 1 A-F) show maize embryos of various sizes. In the embryos analysed here, the meristem is the disc-shaped structure in the centre of the embryo. Depending on the size and the stage of development of the embryos, the meristem is at different stages of development and is easier or harder to detect. The meristem is also marked with an asterisk (*).

Figure 2 A and B (Fig. 2 A and B) show a direct comparison of the meristems of a 0.5 mm (A) and a 1 mm (B) maize embryo. In both cases, the meristem is the disc-shaped structure in the centre of the embryo. In the 1 mm embryo, the meristem is already surrounded by a great deal of leaf tissue. This makes accessing the meristem more difficult. As a result, smaller embryos with an exposed meristem are preferred.

Figure 3 A-D (Fig. 3 A-D) show prepared meristems in maize seedlings. Since the meristem in seedlings is completely surrounded by leaves, it has to be exposed so that it can be accessed for bombardment, microinjection etc. To this end, the outer tissue structures are completely removed so that the meristem (arrows) is exposed.

Figure 4 A-C (Fig. 4 A-C) show prepared meristems in older maize plants. Since the meristem in older plants is completely surrounded by leaves (as it is in seedlings), it has to be prepared so that it can be accessed for bombardment, microinjection etc. To this end, the outer tissue structures are completely removed so that the meristem (arrows) is exposed.

Figure 5 A and B (FIG. 5 A and B) shows the biolistic test bombardment of the maize embryo meristems. FIG. 5 (A) shows an embryo with the disc-shaped meristem structure (highlighted by a double ring and an arrow). FIG. 5 (B) shows the fluorescence (white areas in the black/white image) after the test bombardment. A fluorescent protein coding gene was used for the test bombardment. A clear expression of the protein in the meristematic regions (double ring) can be detected.

FIG. 6 shows the preparation of tassel meristems in adult maize plants. The meristems (arrow) are exposed on one side through a window-like aperture. The recombinant constructs can then be introduced by bombardment, microinjection and similar methods. The advantage is that the plant is not significantly damaged and the meristems are not completely exposed (about 1-2 days later, the tassel meristem can no longer be seen in the opening, since it is moved further up), reducing oxidation and further damage.

Figure 7 A and B (FIG. 7 A and B) shows the biolistic test bombardment of an exposed maize tassel meristem maize. FIG. 7 (A) shows the meristem tissue of the maize tassel. FIG. 7 (B) shows the fluorescence (white areas in the black/white image) after the test bombardment. A

fluorescent protein coding gene was used for the test bombardment. A clear expression of the protein in the meristematic tissues can be detected.

Figure 8 shows the results of an *in vitro* assay for assessing the efficiency of a gRNA of interest. The original plant in this case is a maize plant and the target gene is the hmg13 gene (HMG transcription factor 13; GRMZM2G066528). The figure shows the results of a separation in a 1% gel with the default parameter of 100 V as visualized using the fluorescence of ethidium bromide. The molecular weight marker (given in base pairs; GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific Inc., USA; SM1331) 20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 400, 300, 200, 75 bp) is provided in bands 3, 6, 10, 12, and 14. The results for the gRNA 14 (SEQ ID NO: 41), gRNA 16 (SEQ ID NO: 42), molecular marker, gRNA 37 (SEQ ID NO: 43), gRNA 38 (SEQ ID NO: 44), molecular marker, gRNA 39 (SEQ ID NO: 45), gRNA 43 (SEQ ID NO: 46), gRNA 18 (SEQ ID NO: 47), molecular marker, gRNA 52 (SEQ ID NO: 48), molecular marker, gRNA 39 (SEQ ID NO: 45) and gRNA 43 (SEQ ID NO: 46) are shown from left to right in the other bands. The given SEQ ID NOS indicate the respective individual protospacer regions in each gRNA; the remaining regions of all of the gRNAs used are identical.

Figure 9 A and B (Fig. 9 A and B) show the results of a test bombardment of maize embryos at different pressure levels (indicated in psi: pounds per square inch). The maize embryos were bombarded 7-10 days after pollination. The microscopic analysis was carried out 2 days later. An expression vector was used as the plasmid, which encodes, inter alia, a fluorescent marker. 9(A) shows six images of the bombardment at 1350 psi. FIG. 9(B) shows four images of the bombardment at 1550 psi. Significantly more fluorescence can be seen in the images along the bottom, as indicated by the brighter regions in the black-and-white image. An increased fluorescence/brightness, i.e. an increased efficiency in the insertion, may however be accompanied by reduced germination.

Figure 10 A and B (Fig. 10 A and B) show two views of a maize embryo and meristematic tissue located therein. This data was initially visualised with a fluorescence marker. Figure 10 A and B show the accumulation of fluorescence in the original assay (marked with stars) Figure 10 (A) shows a top down view of the embryo, Figure 10(B) shows a deeper layer in which the fluorescence can also be seen in the meristematic region (marked with stars). The images were taken with a laser scanning microscope. The vector used for the bombardment was an

expression vector, which encodes a fluorescent protein inter alia. The embryo layers have been dyed with a suitable dye.

Figure 11 A and B (Fig. 11 A and B) show the horizontal bombardment of the exposed meristems in older maize plants (5-10 day old seedlings) in accordance with example 3. Since the meristem in older plants is completely surrounded by leaves (as it is in seedlings), it had to be prepared so that it could be accessed for bombardment, microinjection etc. For this, the outer leaves are removed entirely. The images were taken one day after the bombardment with a laser scanning microscope. The vector used for the bombardment was a fluorescent protein-coding expression vector. Figure 11(A) shows a microscope image with a side view of the prepared meristem. Figure 11(B) shows the fluorescence detected in this side view (white dots). The embryo layers have been dyed with a suitable dye.

Figure 12 A-C (Fig. 12 A-C) show the vertical bombardment of the exposed meristems in older maize plants (5-10 day old seedlings) in accordance with example 3. Since the meristem in older plants is completely surrounded by leaves (as it is in seedlings), it had to be prepared so that it could be accessed for bombardment, microinjection etc. For this, the outer leaves are removed entirely. The images were taken one day after the bombardment with a laser scanning microscope. The vector used for the bombardment was a fluorescent protein-coding expression vector. Figure 12 (A) shows a microscope image with a top down view of the prepared meristem. Figure 12 (B) shows the fluorescence detected in this view (white dots). Figure 12 (C) shows the region where fluorescence has been detected, enlarged by a factor of 2. The embryo layers have been dyed with a suitable dye.

Figure 13 A and B (Fig. 13 A and B) show a germinating embryo with transient fluorescence in the meristematic regions. The embryonic target structure is shown in Figure 13 (A). The same target structure is shown in Figure 13 (B), with fluorescence visualised by means of fluorescence microscopy. The fluorescence was brought about through the application of a fluorescence marker. In the black and white image, the white regions in Figure 13 (B) correspond to the regions in which fluorescence was detected.

Figure 14 shows an exemplary vector map of the plasmid pJET1.2-hmg-exon3-5 in accordance with example 1.

Figure 15 shows an exemplary vector map of the plasmid pJET1.2-hmg-3′part/ in accordance with example 1.

Figure 16 shows an exemplary vector map of the plasmid pEn Chimera-hmg-gRNA14, in accordance with example 1.

Figure 17 A and B (Fig. 17 A and B) show the 2-gRNA strategy used for the methods disclosed herein. Figure 17 A shows the use of two gRNAs, gRNA-1 and gRNA-2, which activate a region of the genomic DNA with the goal of excising the region between them from a genomic DNA region by means of a CRISPR nuclease, e.g. a Cas nuclease or any other CRISPR nuclease. (RE: restriction enzyme). Figure 17B shows the results of the analysis of an editing event after applying the 2-gRNA strategy to the genome of a maize plant. For this, the genomic DNA is isolated from maize plants, and the target gene (hmg13 (HMG transcription factor 13; GRMZM2G066528)), is amplified with PCR. Figure 17 B shows the results of a separation in a 1% gel with the default parameter of 100 V as visualized using the fluorescence of ethidium bromide. Band 5 contains the molecular weight marker (given in base pairs; GeneRuler 50 bp DNA Ladder (Thermo Fisher Scientific Inc., USA; SM0373) 1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 50 bp). Bands 1 and 2 each show the results for non-edited maize plants, while band 4 shows the results after editing. The PCR product is smaller because the region between the two gRNA target regions has been excised.

Figure 18 shows a portion of the *Nicotiana benthamiana* NbTTG1 gene. The target gRNA regions are indicated by the shaded arrows, while the primer bonding sites (fw: forward, re: reverse) are indicated by black arrows. Cleavage points are indicated for restriction enzymes, which are used when analysing the outcome of an edit.

Figure 19: TRV obtained in uninoculated distal *Nicotiana benthamiana* leaf tissue. Figure 19 shows the leaf inoculation of *N. benthamiana* with a red fluorescent protein construct (RFP) and a control construct using tobacco rattle virus (TRV)-mediated expression. The construct pZFN-tDT-nptII functioned as a control which exclusively allowed the expression of the RFP in the inoculated leaves and not in the distal leaves. In the black and white image shown in Figure 19, the light(er) regions correspond to the originally detected red fluorescence, while black regions indicate those regions in which no fluorescence could be detected.

Figure 20 A-H (Fig. 20 A-H) show fluorescence images of various floral structures that have been infected with TRV, which can express a red fluorescent protein. All of the figures A to H show a light-field image on the left, an image with a red filter in the middle, and an image with a green (GPF) filter on the right. The latter served as a control for autofluorescence. A floral meristem is shown in Fig. 20A (original image in black-and-white) and B (counterpart to A with adjusted contrast/exposure). Fig. 20C (original image in black-and-white) and D (counterpart to C, with adjusted contrast/exposure) show a flower bud. A pistil is shown in Fig. 20E (original in black-and-white) and F (counterpart to E, with adjusted contrast/exposure). A prepared pistil with exposed ovaries is shown in Fig. 20G (original in black-and-white) and H (counterpart to G, with adjusted contrast/exposure adjustment).

Figure 21 shows the quantification of TRV titres in *N. benthamiana* inoculated with pTRV1 (=Negative control), pTRV1+pTRV2-tDTco (= positive control) and pTRV1+pTRV2-Cas9 10 dpl.

Figure 22 shows the protein indication in Cas9 (160 kDa) expressed in and subsequently isolated from leaf material of transgenic maize plants. The PageRuler Prestained Protein Ladder (10-170 kDa; from top to bottom 170, 130, 100, 70, 55, 40, 35, 25, 15, 10 kDa) was used. The exposure time was 30 minutes. Band 1: pre-dyed protein marker; band 2: 10 µg protein from maize-expressing Cas9; band 3: 15 µg protein from maize-expressing Cas9; band 4: 20 µg Protein from maize-expressing Cas9.

Figure 23 shows an exemplary virus sequence (BMV). The various primer combinations used for the quantifying system of the gRNA are indicated by arrows. "Fw" indicates forward primers, and "re" indicates reverse primers, flanking a sequence of interest. A specific gRNA for the HMG transcription factor gene integrated in the construct of interest that is to be analysed is indicated by "hmg gRNA." The chimera RNA shown ("Chimera RNA Mali et al.") describes a chimeric, artificial RNA construct, supported by the disclosure of Mali et al., 2013 *supra,* wherein the gRNAs described therein have been specifically adapted for use in plant cells, as explained in the example.

Figure 24 shows a maize plant of the genotype A188 in the V7 stage (left-hand image), the same plant after insertion of an artificial window in the region protecting the tassel tissue

(middle image), and the subsequent injection of an *Agrobacterium* solution in the region of the exposed tassel (right-hand image).

Figure 25 A-C (Fig. A-C) shows immature embryos (Fig. 25A) of a maize plant, which was isolated and subsequently bombarded with a particle bombardment comprising a CRISPR/Cas 9 construct, and a red fluorescent protein-expressing plasmid. Fig. 25 B shows the fluorescence (white and light regions) on the first day after the bombardment. Fig. 25 C shows a mature maize plant that was obtained from the embryos bombarded in this manner and subsequently raised to maturity from Fig. 25 A and B.

Figure 26 shows an immature *Beta vulgaris* embryo, obtained according to the method described in detail below.

Figure 27 A and B (Fig. 27 A and B): Fig. 27 A: Immature grains of wheat after meristem transformation. Fig. 27 B: corresponds to the fluorescence image of Fig. 27A. The light regions correspond to the detected fluorescence (light/white regions in the black-and-white image). After the meristem transformation, germinating wheat plants could be obtained directly from the treated grains of wheat (A and B).

Figure 28 shows the location of the immature florescence in wheat in the left-hand image. The middle image and the right-hand image demonstrates the further development, from left to right, of the meristematic tissue that has been transformed as described below.

Detailed Description

Definitions

The term "plant" or "plant cell" as used herein refers to plant organisms, plant organs, differentiated and undifferentiated plant tissues, plant cells, seeds and their progeny or descendants. "Plant cells" includes, for example, cells of seeds, mature and immature embryos, meristematic tissues, seedlings, callus tissue, leaves, flowers, roots, plant buds, gametophytes, sporophytes, pollen and microspores, protoplasts, macroalgae and microalgae.

The term "fertile plants", as used herein, refers to a fertile plant capable of reproduction, i.e. a fertile plant is a plant that can produce viable male and female gametes. A male-sterile plant, accordingly, is a plant that cannot produce viable male gametes, but which may be female-

fertile. A female-sterile plant is a plant that cannot produce viable female gametes, but which may be male-fertile.

The term "plant material", as used herein, means any material which can be obtained from a plant at any stage of development. The term thus encompasses plant cells, tissue and organs as well as formed plant structures and sub-cellular components such as nucleic acids, polypeptides, as well as all chemical plant substances which are present inside a plant cell and/or can be produced by a plant cell.

The term "chromosomally or extrachromosomally integrated", as used herein, relates to the transient introduction and/or the formation of one or more recombinant constructs of the present invention and thus to the subsequent fate of the one or more recombinant constructs in a plant target structure, for example a cell, wherein both the one or more recombinant constructs and also the conditions for the introduction thereof are maintained in a manner such that the at least one recombinant construct is not integrated into the endogenous nucleic acid material of a plant target structure comprising the genome or extrachromosomal nucleic acid of the plant target structure, for example a cell, so that the at least one recombinant construct is not chromosomally or extrachromosomally integrated into the endogenous DNA/RNA of the target cell and thus not passed on to the descendants of the cells. The one or more recombinant construct(s) or its transcription or translation products are thus only temporarily active in the target cell, i.e. transient, constitutive or inducible, but cannot be inherited by the descendants of the target cells, i.e. they are also not actively present in the descendants of a target cell.

The term, "homologous recombination," as used herein, indicates a process that takes place in all organisms. This requires homologous, double-strand DNA sections. "Homologous" therefore means that there is a large similarity in the nucleotide sequences of two sequences. With naturally occurring double-strand breaks, damage can be repaired through homologous recombination, in that the information on the intact chromatid in the genome of an organism can be used as a template. If, in accordance with the present disclosure, a targeted and precise double-strand break is inserted in a nucleic acid target region of interest within the framework of genome editing, homologous recombination can also be used to repair the break, wherein, consequently, the targeted design of a DNA repair template may be used to obtain the targeted effect on the nucleic acid target regions of interest that are to be repaired. Different organism are differentiated with regard to the ratio of homologous to non-homologous recombinations,

as occurs in nature (see above, NHEJ). In general, the length of the homologous region affects the frequency of homologous recombination events, i.e. a longer homologous region results in a greater frequency. The length of the homologous region used to achieve homologous recombination is species-dependent. In some cases, it may be necessary to use at least five kilobases (kb) of homology, but homologous recombination has also been observed in a homologous region having only approximately 25 base pairs (bp).

"Homology directed repair" (HDR) refers to a cellular mechanism for repairing double-strand as well as single-strand DNA breaks. HDR thus comprises elements of homologous recombination, as well as single-strand annealing (SSA) (Lieber Michael et al., Annu.Rev.Biochem.79:181-211, 2010). The most frequent form of HDR in a cell is homologous recombination, wherein this type of repair also requires the highest sequence homology between donor and acceptor DNA. Other forms of HDR comprise single-strand annealing (SSA). SSA is non-conserving, occurs naturally between direct repetitions of >30 bp, and results in deletions. The HDR of nicks, i.e. single-strand breaks, involves a different mechanism to the mechanism involved in HDR for double-strand breaks (Davis und Maizels PNAS, 2014 E924-32). Because, in accordance with the present disclosure, CRISPR nucleases are proposed that induce both double-strand breaks as well as single-strand breaks, the term "HDR" or homologous recombination therefore refers to the repair of a single-strand break or a double-strand break that has been inserted in a targeted manner, with the use of a suitable repair template.

"Herbicide resistance" and "herbicide tolerance," as used herein, refer to the thw ability of a plant or a plant cell to resists or tolerate the effects of a herbicide or pesticide. This property is normally obtained through at least one protein or one RNA, which has been either artificially inserted in a plant cell, e.g. as a transgene, or acquired through the (targeted) modification of an endogenous gene. The term "progeny or descendants" as used herein means, in the context of a recombinant microorganism, a plant or a cell in accordance with the present disclosure, the descendants of such an organism or such a cell which derive from the original organism or the original cell on the basis of natural reproductive asexual cell division and differentiation processes. A person skilled in the art is aware that mutations in the genome of an organism can be introduced naturally during cell division and differentiation, causing the progeny or descendant to differ genomically from the parent organism, but still be assigned to the same (sub)species. Such progeny modified by natural processes which introduce modifications into

other DNA regions in addition to the specifically introduced modification are thus comprised in the term "progeny or descendants" in the present disclosure.

The term, "CRISPR nuclease," as used herein, refers in general to a nuclease as it occurs in a naturally occurring CRISPR system, as well as to modifications, mutations, and catalytically active fragments thereof. In a naturally occurring CRISPR locus, the CRISPR nuclease is the molecule that forms the effector molecule, and can recognize and/or cleave a nucleic acid target structure through interaction with a crRNA and, optionally, a tracrRNA, or together with an artificial gRNA. CRISPR nucleases therefore comprise Cas nucleases, Cpf1 nucleases, or other CRISPR effector domains and/or nuclease domains, comprising Csf1 and combinations and variations thereof. Moreover, this term also comprises nucleases that have been modified in a targeted manner and converted to nickases, for obtaining single-strand breaks, or nuclease-null variants, for bonding and recognition purposes, but not for obtaining a double-strand break. Because the term "CRISPR/Cas" has become established as a synonym for all types of CRISPR systems in the literature, this term should be used for any CRISPR type I-V system, as well as the associated effector proteins, in the sense of the present disclosure, unless specifically indicated otherwise.

The term "vector" or "vector system" as used herein, means a transport means which can introduce a recombinant construct, comprising nucleic acids or polypeptides as well as further sequences such as regulatory sequences or localization sequences directly or indirectly into a desired target cell or target plant structure, into the desired cellular compartment. Direct introduction is carried out directly into a target plant cell or plant target structure which contains nucleic acids to be specifically modified in accordance with the present disclosure. Indirect introduction encompasses introduction into a structure, e.g. cells of leaves or other plant organs and tissues, which do not directly comprise the target plant cells of interest, but which ensure the systematic propagation and transport of the vector, comprising a recombinant construct in accordance with the present disclosure, into the plant target structure, e.g. meristematic tissues or cells or stem cells. The term "vector" or "vector system", as used herein in the context of transfection of amino acid sequences, encompasses suitable agents for peptide or protein transfection, e.g. ionic lipid mixtures or agents which are suitable for transfection of a nucleic acid, e.g. carrier materials via which nucleic acid and amino acid sequences can be introduced into a cell by means of particle bombardment, e.g. using gold and tungsten particles. Furthermore, in particular when applying the method and constructs disclosed herein, this term

also encompasses viral vectors, i.e. modified viruses, e.g. those which derive from one of the following viruses: Maize Streak Virus (MSV), Barley Stripe Mosaic Virus (BSMV), Brome Mosaic virus (BMV, access numbers: RNA1: X58456; RNA2: X58457; RNA3: X58458), Maize stripe virus (MSpV), Maize rayado fino virus (MYDV), Maize yellow dwarf virus (MYDV), Maize dwarf mosaic virus (MDMV), positive-strand RNA viruses of the *Benyviridae,* family, e.g. *Beet necrotic yellow vein virus* (access numbers: RNA1: NC_003514; RNA2: NC_003515; RNA3: NC_003516; RNA4: NC_003517) or the *Bromoviridae* family, e.g. viruses of the *Alfalfa mosaic virus genus* (access numbers: RNA1: NC_001495; RNA2: NC_002024; RNA3: NC_002025) or the *Bromovirus* genus, e.g. BMV (see above), or the *Cucumovirus* genus, e.g. *Cucumber mosaic virus* (access numbers: RNA1: NC_002034; RNA2: NC_002035; RNA3: NC_001440), or the *Oleavirus* genus, dsDNA viruses of the *Caulimoviridae* family, in particular the *Badnavirus* or *Caulimovirus* families, e.g. various *Banana* streak viruses (see, e.g., access numbers: NC_007002, NC_015507, NC_006955 or NC_003381) or *Cauliflower mosaic virus* (access number: NC_001497), or viruses of the *Cavemovirus, Petuvirus, Rosadnavirus, Solendovirus, Soymovirus* or *Tungrovirus* genus, positive strand RNA viruses of the *Closteroviridae* family, e.g. the genus *Ampelovirus, Crinivirus, e.g. Lettuce infectious yellows virus* (access numbers: RNA1: NC_003617; RNA2: NC_003618) or *Tomato chlorosis virus* (access number: RNA1: NC_007340; RNA2: NC_007341), *Closterovirus, e.g. Beet yellows virus* (access number: NC_001598), or *Velarivirus*, single-strand DNA (+/−) viruses of the *Geminiviridae* family, e.g. viruses of the *Becurtovirus, Begomovirus family, e.g. Bean golden yellow mosaic virus, Tobacco curly shoot virus, Tobacco mottle leaf curl virus, Tomato chlorotic mottle virus, Tomato dwarf leaf virus, Tomato golden mosaic virus, Tomato leaf curl virus, Tomato mottle virus*, or *Tomato yellow spot virus*, or *Geminiviridae* of the *Curtovirus* genus, e.g. *Beet curly top virus*, or *Geminiviridae* of the genus Topocuvirus, Turncurtvirus or *Mastrevirus*, e.g. *Maize streak virus* (see above), *Tobacco yellow dwarf virus, Wheat dwarf virus*, positive strand RNA viruses of the *Luteoviridae* family, e.g. the genus *Luteovirus*, e.g. *Barley yellow dwarf virus-PA V* (access number: NC_004750), or the genus *Polerovirus*, e.g. *Potato leafroll virus* (access number: NC_001747), single-strand DNA viruses of the *Nanoviridae* family, comprising the genuses *Nanovirus* or *Babuvirus*, double-strand RNA viruses of the *Partitiviridae* family, comprising *inter alia* the families *Alphapartitivirus, Betapartitivirus* or *Deltapartitivirus*, viroids of the *Pospiviroidae* family, positive strand RNA viruses of the *Potyviridae* family, e.g. comprising the genuses *Brambyvirus, Bymovirus, Ipomovirus, Macluravirus, Poacevirus*, e.g. *Triticum mosaic virus* (access number: NC_012799), or *Potyviridae* of the genus *Potyvirus*, e.g. *Beet*

mosaic virus (access number: NC_005304), *Maize dwarf mosaic virus* (access number: NC_003377), *Potato virus Y* (access number: NC_001616), or Zea *mosaic virus* (access number: NC_018833), or *Potyviridae* of the genus *Tritimovirus*, e.g. *Brome streak mosaic virus* (access number: NC_003501) or *Wheat streak mosaic virus* (access number: NC_001886), single-strand RNA viruses of the *Pseudoviridae* family, e.g. the genuses *Pseudovirus*, or *Sirevirus*, double-strand RNA viruses of the *Reoviridae* family, e.g. Rice dwarf virus (access numbers: RNA1: NC_003773; RNA2: NC_003774; RNA3: NC_003772; RNA4: NC_003761; RNA5: NC_003762; RNA6: NC_003763; RNA7: NC_003760; RNA8: NC_003764; RNA9: NC_003765; RNA10: NC_003766; RNA11: NC_003767; RNA12: NC_003768), positive strand RNA viruses of the *Tombusviridae* family, e.g. comprising the genuses *Alphanecrovirus, Aureusvirus, Betanecrovirus, Carmovirus, Dianthovirus, Gallantivirus, Macanavirus, Machlomovirus, Panicovirus, Tombusvirus, Umbravirus* or *Zeavirus*, e.g. *Maize necrotic streak virus* (access number: NC_007729), or positive strand RNA viruses of the *Virgaviridae* family, e.g. viruses of the genus *Furovirus, Hordeivirus*, e.g. *Barley stripe mosaic virus* (access numbers: RNA1: NC_003469; RNA2: NC_003481; RNA3: NC_003478), or the genus *Pecluvirus, Pomovirus, Tobamovirus* or *Tobravirus*, e.g. *Tobacco rattle virus* (access numbers: RNA1: NC_003805; RNA2: NC_003811), as well as negative strand RNA viruses of the order *Mononegavirales*, in particular the *Rhabdoviridae* family, e.g. *Barley yellow striate mosaic virus* (access number: KM213865) or *Lettuce necrotic yellows virus* (access number/specimen: NC_007642/AJ867584), positive strand RNA viruses of the order *Picornavirales*, in particular the *Secoviridae* family, e.g. the genuses *Comovirus, Fabavirus, Nepovirus, Cheravirus, Sadwavirus, Sequivirus, Torradovirus*, or *Waikavirus*, positive strand RNA viruses of the order *Tymovirales*, in particular the *Alphaflexiviridae* family, e.g. viruses of the genus *Allexivirus, Lolavirus, Mandarivirus*, or *Potexvirus, Tymovirales*, in particular of the *Betaflexiviridae* family, e.g. viruses of the genus *Capillovirus, Carlavirus, Citrivirus, Foveavirus, Tepovirus*, or *Vitivirus*, positive strand RNA viruses of the order *Tymovirales*, in particular the *Tymoviridae* family, e.g. viruses of the genus *Maculavirus, Marafivirus*, or *Tymovirus*, and bacterial vectors such as *Agrobacterium* spp., for example, with *Agrobacterium tumefaciens* being an example. Finally, the term also encompasses suitable transport means for introducing linear nucleic acids (single-stranded or double-stranded) into a target cell. Knowing the constructs disclosed herein, the person skilled in the art will be aware of all further sequences which a vector must contain in order to be functional in a desired target cell. The person skilled in the art will also be aware of the conventional production, processing and use of vectors of this type.

The term "vector system" as used herein, denotes a system which consists of or contains at least one or more vector(s). Thus, a vector system may comprise a vector which contains/codes for two different recombinant constructs comprising nucleic acid and/or amino acid sequences. Furthermore, a vector system can also contain several vectors which in turn contain/code for at least one nucleic acid or amino acid sequence in accordance with the present disclosure.

The terms, "quantitative trait locus" or "QTL," as used herein, refer to a DNA region that is associated with the differential expression of a quantitative phenotypic trait in at least one defined genetic background, e.g. in at least one plant population. The QTL region comprises, or is closely linked to, the gene or genes that affect the trait in question. An allele of a QTL can therefore comprise numerous genes or other genetic factors within a coherent genomic region, or a linkage group, e.g. a haplotype. An allele of a QTL can designate a haplotype within a defined window, wherein this window represents a coherent genomic region, which can be defined and tracked with a set of one or more polymorphic markings. A haplotype can be defined by the unique fingerprints of alleles for each marker within the defined window.

As will be explained in greater detail below, a number of methods are available to the person skilled in the art for identifying those plant target structures, comprising at least one meristematic cell, or an entire plant or a plant material or a plant cell thereof, which contribute to a targeted modification in their genomic DNA in or close to a target region of a nucleic acid, without the use of marker phenotypes that can be checked. Such methods are based on the direct analysis of a target region of a nucleic acid or target sequence of interest in order to detect an arbitrary modification in this nucleic acid region or sequence, and comprise, but are not limited to, PCR processes, sequencing processes, nuclease digestion, southern blots, northern blots, and any arbitrary combination thereof.

The term "nucleic acid" or "nucleic acid sequence" as used herein refers to both natural and synthetic deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), which may also contain synthetic nucleotide analogues. The nucleic acids which are used in accordance with the present invention for the synthesis of a desired product such as protein or RNA or for the specific control thereof, for example a CRISPR nuclease, including, inter alia, a Cas nuclease or a Cpf1 nuclease, or a gRNA, may be "adapted for use in a plant target structure". In one embodiment, said sequences may be codon-optimized, i.e. where the codon use of a gene or a

RNA is specifically adapted to the target cell/target organism. The person skilled in the art is aware that a desired target gene which codes for a protein of interest can be modified without changing the translated protein sequence in order to account for the specific species-dependent codon use. Thus, the nucleic acids of the present invention may specifically be adapted to or are adapted to the codon use of *Hordeum vulgare, Sorghum bicolor, Secale cereale*, Triticale, *Saccharum officinarium, Zea mays, Setaria italic, Oryza sativa, Oryza minuta, Oryza australiensis, Oryza alta, Triticum aestivum, Triticum durum, Hordeum bulbosum, Brachypodium distachyon, Hordeum marinum, Aegilops tauschii, Malus domestica, Beta vulgaris, Helianthus annuus, Daucus glochidiatus, Daucus pusillus, Daucus muricatus, Daucus carota, Eucalyptus grandis, Erythranthe guttata, Genlisea aurea, Nicotiana sylvestris, Nicotiana tabacum, Nicotiana tomentosiformis, Solanum lycopersicum, Solanum tuberosum*, Coffea *canephora, Vitis vinifera, Cucumis sativus, Morus notabilis, Arabidopsis thaliana, Arabidopsis lyrata, Arabidopsis arenosa, Crucihimalaya himalaica, Crucihimalaya wallichii, Cardamine flexuosa, Lepidium virginicum, Capsella bursa-pastoris, Olmarabidopsis pumila, Arabis hirsuta, Brassica napus, Brassica oleracea, Brassica rapa, Brassica juncacea, Brassica nigra, Raphanus sativus, Eruca vesicaria sativa, Citrus sinensis, Jatropha curcas, Glycine max, Gossypium ssp.* or *Populus trichocarpa*. Furthermore, in accordance with the present disclosure, the sequence of the gRNA or the sequence coding for the gRNA must be adapted to the target region of a nucleic acid within a plant target structure. In a further embodiment, the gRNA or the sequence coding for the gRNA must additionally be adapted in the region which is responsible for interaction or coupling with a Cas nuclease and/or an effector domain.

The term "sequences", as used herein, refers to nucleic acid sequences as well as to amino acid sequences, wherein the respective sequence may also contain synthetic analogues or synthetic linkages as elements in addition to natural nucleotides and amino acids.

The terms "polypeptide", "polypeptide sequence", "protein sequence" and "amino acid sequence" are used interchangeably herein.

The term, "catalytically active fragment", as used herein, in particular in reference to CRISPR nucleases or variations thereof, refers to an amino acid core sequence, derived from a given amino acid template sequence, provided that the resulting catalytically active fragment comprises all or part of the active centre of the template sequence, and therefore still fulfils the same enzymatic function as the template sequence. These modifications, i.e. truncations, are

well known to the person skilled in the art, and are particularly useful when working with sterically demanding enzymes for creating multifaceted and more stable truncated enzymes comprising the catalytically active fragment.

The term "regulatory sequence", as used herein, refers to a nucleic acid or a protein sequence which can control cis or trans transcription and/or translation of a disclosed nucleic acid sequence.

The term "construct" or "recombinant construct" (used interchangeably herein), as used herein, refers to a construct comprising, inter alia, plasmids or plasmid vectors, cosmids, yeast artificial chromosomes or bacterial artificial chromosomes (YACs and BACs), phagemids, bacteriophage vectors, an expression cassette, single-stranded or linear nucleic acid sequences or amino acid sequences, and viral vectors, i.e. modified viruses, which can be introduced into a target cell in accordance with the present disclosure. A recombinant construct may comprise CRISPR/Cas tools or parts thereof comprising at least one gRNA or at least one CRISPR nuclease variant and/or at least one further effector domain, either in the form of a nucleic acid or an amino acid sequence. Furthermore, the recombinant construct may comprise regulatory sequences and/or localization sequences. The recombinant construct may be integrated into a plasmid vector and/or be isolated from a plasmid vector, e.g. in the form of a polypeptide sequence or as a single-stranded or double-stranded nucleic acid not linked into a plasmid vector. After introduction, the construct is preferably extrachromosomal and not integrated into the genome and usually in the form of a double-stranded or single-stranded DNA, a double-stranded or single-stranded RNA or a polypeptide. The term "plasmid vector", as used herein, relates to a construct that was originally obtained from a plasmid. These are normally circular, autonomous, replicating, extrachromosomal elements in the form of a double-stranded nucleic acid sequence. In genetic engineering, these original plasmids are modified in a targeted manner, in that, inter alia, resistance genes, target nucleic acids, localization sequences, regulating sequences, etc. are inserted, while maintaining the structural components of the original plasmid, such as the replication source. Numerous plasmid vectors for use in a target cell of interest are commercially available are known to the person skilled in the art, in addition to the modification thereof for specific cloning strategies. Such known plasmid vectors are also referred to as standard vectors herein in order to imply that the basis vector is commercially available and can be readily adapted to suit the respective experiment by a person skilled in the art.

The term, "enhancer" or "enhancer element," refers to a base/nucleotide sequence that has a characteristic sequence. An enhancer is a cis-regulatory element and can affect how a transcription complex binds to a promoter, and thus the transcription activity of a gene. A promoter, in turn, is a DNA sequence that can regulate the expression of a coding sequence or a functional RNA. The promoter sequence is composed of both proximal as well as distal elements in relation to a regulated sequence, wherein the latter are frequently referred to as enhancers. Promoters may have a broad activity spectrum, and may also be active, or activatable on a tissue-specific or development-specific basis, e.g. in the cells of roots, seeds, meristematic cells, etc. Promoters may be constitutive or inducible, wherein the induction can be stimulated through a number of environmental factors. A promoter can be "strong", i.e. capable of activating a high level of transcription of the regulated sequence, or "weak". Promoters are frequently strongly regulated. A promoter in the sense of the present disclosure can be an endogenous/native promoter, or an artificial (synthetic/chimeric) or transgenic promoter, which has either been obtained from another species, or which is artificial or synthetic/chimeric, i.e. not present in this form in nature, or is comprised of various promoter elements.

The terms "3′ non-coding sequence", "transcription terminator", "terminator", or "termination sequence", as used herein, refer to DNA sequences that are located downstream, i.e. in the 3′ direction of a coding sequence, and comprise polyadenylation recognition sequences and other regulating signal-coding sequences which are capable of affecting mRNA processing and/or gene expression. The polyadenylation signal is normally characterized in that it causes poly-A-nucleotides to be added to the 3′ end of an mRNA precursor.

The term, "functionally linked", as used herein, refers to the bonding of nucleic acid sequences to a single nucleic acid fragment, such that the individual fragments of genes or regulating sequences, or other regions, are physically joined, and such that individual sequences or segments can regulate, hybridize, or affect one another. A promoter is then functionally linked to a coding sequence, provided it is capable of regulating the expression of this coding sequence, i.e., the coding sequence is then subject to the transcriptional regulation of the promoter in question. Moreover, coding sequences can be functionally linked to regulating sequences, in either a clockwise or counter-clockwise orientation. Complementary RNA regions can be linked, directly or indirectly, at 5′ with the target mRNA, or 3′ with the target

mRNA, or inside the target mRNA, or a first region of the complementarity is functionally linked at 5′ and its complement is functionally linked at 3′ to the target mRNA.

The terms, "stable transformation" or "stable integration", as used herein, relate to the insertion of a nucleic acid sequence of interest, e.g. in the form of a DNA repair template or a portion thereof, or of a suitable vector, into the genome of a plant target structure of interest, wherein the genome comprises both the nucleic as well as the extra-nucleic genome, such as the genome of organelles, resulting in a genetically stable and thus inheritable modification of the genome. By contrast, the terms "transient transformation" or "transient insertion" or "transient integration", as used herein, relate to the insertion of a nucleic acid sequence of interest into a plant compartment of interest, comprising the nucleus, organelles or the cytoplasm, or a further compartment inside a plant cell, by means of which, either the transcription and/or translation, or, in the case of a direct-effector molecule (DNA, RNA, or protein), the inserted molecules or complexes, can deploy their effect inside the plant cell, but the corresponding sequences and/or effector molecules are not stably integrated into the genome of the cell, and thus not inherited.

The term, "genome," as used herein, relates to the totality of the genetic engineering material, comprising genes and non-coding sequences present in a cell of an organism or a virus or an organelle, and therefore comprises both the nucleic (if present) as well as the extra-nucleic (if present) genome. Furthermore, the term "genome", as used herein, relates to the entire set of chromosomes that are inherited as a (haploid) unit of an ancestor organism.

One incentive for developing new molecular markers in plant species is the potential for increasing the efficiency of targeted plant cultivation through marker assisted selection (MAS). Genetische Marker-Allele, oder alternativ die oben geschilderten quantitativen Merkmals-Loci (QTL)Allele werden dafür verwendet, um Pflanzen oder Pflanzenmaterial oder eine pflanzliche Zelle zu identifizieren, die einen gewünschten Genotyp an einem oder mehreren Locus/Loci enthalten, und von denen angenommen wird, dass sie den erwünschten Genotyp zusammen mit einem gewünschten Phänotyp an ihre Nachkommen weitergeben können. Gene technology marker-alleles (or QTL alleles) can also be used for identifying plants which contain a desired genotype at a locus, or at numerous, unlinked or linked, loci, e.g. a haplotype, from which it can be assumed that they can pass on the desired genotype, together with a desired phenotype, to their descendants. With respect to the marker assisted selection, the term "marker", as used herein, can therefore mean both marker and QTL loci. As soon as it has been determined for a

desired phenotype and a polymorphic chromosomal locus, e.g. a marker locus or a QTL, that they segregate collectively, it is possible to use these polymorphic loci to select alleles that correspond to the desired phenotype. This approach is referred to as marker assisted selection (MAS). To this end, a nucleic acid sequence corresponding to the marker nucleic acid is detected in a biological sample from a plant to be analysed. This detection can be performed in the form of a hybridization of a nucleic acid probe on a marker, e.g. using allele-specific hybridization, southern blot analysis, northern blot analysis, *in situ* hybridization, hybridization of primers followed by PCR amplification of a region of the marker, or the like, or through any arbitrary combination thereof. Numerous methods for detecting markers are known to the person skilled in the art. After the presence or absence of a specific marker has been confirmed in the biological sample of interest, comprising at least one plant cell, preferably a meristematic cell, the corresponding plant is selected, and can be used subsequently for obtaining descendant plants through selective breeding.

Likewise, the method according to the present invention can be used for analysing a meristematic cell, modified *in planta* in a targeted manner, for the presence or absence of a specific marker. Preferably either female or male gametes or germ cells can be obtained from these meristematic cells, wherein in particular, the pollen of a plant modified *in planta* in this manner can be used directly for subsequent selective cultivation. Because there is demand within traditional plant cultivation to insert traits of interest into a target plant which code for a high yield and/or other desirable traits, in order to develop improved plant cultivars, there is a significant demand for marker assisted selection in order to reduce the time needed for the elaborate and expensive testing of a large number of samples.

In accordance with the method of the present disclosure, phenotype markers can also be inserted into a plant target structure of interest in a targeted manner. A "phenotype marker," as used herein, refers to a marker that can be selected, which facilitates the analysis and detectability of a plant cell or target structure of interest. Phenotype markers comprise, in general, either positive or negative selectable markers such as visible markers or (antibiotics) resistance genes. Any plant marker that can be used in a plant target structure of interest, in particular a meristematic cell, can be used. Selectable or detectable markers normally comprise DNA segments that allow a cell, or a molecule marked with a "tag" inside a cell of interest, to be identified, frequently under specific conditions. Such markers can code an activity selected from, but not limited to, the production of RNA, peptides, or proteins, or the markers can

provide a bonding site for RNA, peptides, proteins, inorganic and organic compounds or composites and the like. By way of example, selectable markers comprise, without being limited thereto, DNA segments comprising restriction enzyme cleavage points, DNA segments comprising a fluorescent probe, DNA segments that code products that provide resistance to otherwise toxic compounds, comprising antibiotics, e.g. spectinomycin, ampicillin, kanamycin, tetracycline, BASTA, neomycin-phosphotransferase II (NEO) and hygromycin-phosphotransferase (HPT), DNA segments that code products that would naturally not be present in a plant target cell of interest, e.g. tRNA genes, auxotrophic markers and the like, DNA segments that code products that can be readily identified, in particular visibly observable markers, e.g. phenotype markers such as β-galactosidases, GUS, fluorescent proteins, e.g. green fluorescent protein (GFP) and other fluorescent proteins, e.g. blue (CFP), yellow (YFP) or red (RFP) fluorescent proteins, and surface proteins, wherein those fluorescent proteins that exhibit a high fluorescence intensity are of particular interest, because these proteins can also be identified in deeper tissue layers if, instead of a single cell, a complex plant target structure or a plant material or a plant comprising numerous types of tissues or cells is to be analysed, new primer sites for PCR, the recording of DNA sequences that cannot be modified in accordance with the present disclosure by restriction endonucleases or other DNA-modifying enzymes or effector domains, DNA sequences that are used for specific modifications, e.g. epigenetic modifications, e.g. methylations, and DNA sequences with a PAM motif, which can be identified by a suitable CRISPR system in accordance with the present disclosure, in addition to DNA sequences without a PAM motif, such as are naturally present in an endogenous plant genome sequence.

The methods according to the present invention can be used specifically for the cultivation of plants, in order to introduce one or more transgenic traits into a plant, or the at least one plant target structure of interest, comprising at least one meristematic cell. Currently, transgenic traits are inserted randomly into the plant genome through transformation systems, wherein this takes place with physical/mechanical methods, or biologically, for example through the biolistic bombardment of plant material or transformation with *Agrobacterium* and/or viral vectors. Over the last few years, specific protocols for the targeted insertion of transgenes into the genomes of plant cells have become increasingly more common. One important technology is site-specific integration (SSI), which allows for the targeted insertion of a transgene at the same site in a chromosome where a transgene has already been inserted. Moreover, over the last few years, an increasing number of intentionally target-specific nuclease systems have become

established to facilitate the cleaving of a chromosomal target point through nucleases. Nucleases which are currently frequently used for genome editing in eukaryotic genomes comprise, inter alia, mega-nucleases, zinc finger meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and a constantly growing family of CRISPR nucleases, as well as variations that have been modified in a targeted manner, and catalytically active fragments thereof. CRISPR-based nuclease systems have proven to be extremely useful for high precision target-specific and programmable modification of nucleic acid target regions of interest. The CRISPR system is guided by a, frequently chimeric, gRNA, and does not allow for purely protein-based targeting and target selection, providing a high level of reliability and a reduction in undesired off-target effects. Moreover, the present disclosure offers a further advantage of the CRISPR systems intrinsically composed of two components, specifically in that either a gRNA and/or a CRISPR nuclease, or a variant or catalytically active fragment thereof, can be provided with a further effector domain in a targeted manner, by means of which the variability and the field of application of the CRISPR system can be significantly expanded. Through a reprogramming of a CRISPR nuclease, a nuclease-null variant can be created which has lost its catalytic activity in terms of the cleavage of DNA, but retained its DNA identification function. Through the combination of a molecule modified in this manner with an effector domain, in particular an effector domain that allows the epigenetic modulation of the genome of a target cell of interest, targeted epigenetic modifications, e.g. methylations, demethylations, acetylations, de-acetylations, phosphorylations, de-phosphorylations, or ubiquitinations, can be integrated into a histone protein, or another arbitrary protein inside a nucleosome in the cell nucleus of an eukaryotic cell of interest through the transient insertion of a CRISPR system, comprising at least one gRNA, at least one CRISPR nuclease, and at least one effector domain. As a result, targeted structural adaptations to chromosomal regions can be effected to achieve modified activation states, even when the CRISPR systems used for this purpose are only integrated in a transient manner into a plant target structure of interest, and thus cannot be inherited, wherein these structural adaptations can then potentially be inherited.

The CRISPR systems disclosed herein and the disclosed methods for the targeted modification of at least one meristematic cell, are suited in particular for genome editing of plant cells or organisms, because off-target cleavage, which is frequently lethal for the target cells, or leads to undesired side effects, can be avoided through the high level of precision.

In one embodiment, the CRISPR nuclease components of the CRISPR system, or a variant, comprising nickases or nuclease-null variants, or an active fragment thereof, can be stably integrated in a plant genome. The expression of the CRISPR nuclease can be regulated by a plant-specific promoter, wherein the promoter can be a constitutive promoter, a tissue-specific promoter, or an inducible promoter, e.g. a promoter which can be induced by temperature, stress, development stage, or chemically induced. In the absence of a further essential component of the CRISPR system, i.e. a synthetic gRNA or a crRNA, the Cas nuclease is not capable of cleaving and/or identifying DNA, such that the mere presence of the Cas nuclease has little or no effect on the plant cell of interest and its metabolism. It is therefore an advantage of the disclosed method in the field of plant cultivation and development, that cell lines or transgenic plant cells can be produced and propagated, which can express a Cas nuclease in a constitutive or inducible manner, or a variant or a catalytically active fragment thereof, without having negative consequences on cell integrity or viability. In order to acquire the activity of a CRISPR nuclease, whether it is stably integrated or provided in a transient manner, as described above, the presence of a gRNA or a crRNA is always necessary as a further reliability mechanism, which can be integrated into a plant target structure comprising at least one meristematic cell of interest in a stable or transient manner through a number of methods. The gRNA can be integrated into the cell as a transcribable DNA construct in the form of a genetic construct, such as a vector, wherein the gRNA transcribes under the control of an adequate promoter, in either a constitutive or inducible manner, and can thus be provided in a functional manner. Alternatively, the gRNA can also be directly integrated into a plant target structure of interest as RNA. CRISPR nucleases and gRNA can thus be integrated simultaneously or at different times, wherein the gRNA and CRISPR nuclease are preferably provided at different times or locations, such that the less stable RNA and the protein CRISPR nuclease can interact in the cell compartment of interest in a stoichiometrically ideal composition. If the target of the targeted modification is an RNA, then the compartment of interest is the cytoplasm of a target cell. If the nucleic acid target region of interest is genomic DNA or the nucleosome, then the compartment of interest is the cell nucleus of a plant target structure comprising at least one meristematic cell. In this embodiment, it may be necessary that the gRNA and/or CRISPR nuclease are functionally linked to suitable nuclear localization sequences, in order that the CRISPR molecules or the CRISPR complex composed of gRNA and CRISPR nucleases, as well as optional effector domains associated therewith, can reach their site of action. In another embodiment, if the nucleic acid target region is located in an organelle, in particular plastids, the presence of plastid localization sequences, e.g. mitochondrial localization sequences or

chloroplast localization sequences, may be necessary for conducting the CRISPR tools to the site of action in accordance with the present disclosure.

A gRNA in accordance with the present disclosure can be a single molecule, or it may be used or present in the form of two separate RNAs, corresponding to crRNA and/or tracrRNA.

The term "recombinant", as used herein, means a series of nucleic acids or amino acids, in particular not occurring naturally as a totality. Furthermore, the term, "recombinant" also comprises those series of nucleic acids or amino acids that occur naturally with regard to their nucleic acid or amino acid sequences, but can also be obtained through a targeted modification or synthesis, e.g. nucleic acid or amino acid sequences obtained synthetically, or through bio-engineering, e.g. nucleic acid or amino acid sequences that are obtained through a fermentative process, which may exist in nature, but can also be produced in a targeted manner in an organism other than the source organism.

The term, "epigenetics" or "epigenetic", as used herein, describes the structural adaptation of chromosomal regions in order to code, signal, conserve, and to potentially pass onto the descendants of a cell, modified states of the activation. Accordingly, structural and potentially inheritable modifications are obtained via modifications that are not coded in the genomic DNA itself.

When the present disclosure refers to the "sequence homologies" or "sequence identities" of nucleic acid sequences or protein sequences in the form of percentages, these values can be calculated using EMBOSS Water Pairwise Sequence Alignments (Nucleotide) www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html) for nucleic acid sequences, or EMBOSS Water Pairwise Sequence Alignments (Protein) (www.ebi.ac.uk/Tools/psa/emboss_water) for amino acid sequences. Tools for local sequence alignments available from the European Molecular Biology Laboratory (EMBL) European Bioinformatics Institute (EBI) use a modified Smith-Waterman algorithm (see http://www.ebi.ac.uk/Tools/psa/ and Smith, T.F. & Waterman, M.S. "Identification of common molecular subsequences" Journal of Molecular Biology, 1981 147 (1):195-197). Furthermore, the respective paired alignments of two sequences using the modified Smith-Waterman algorithm are carried out using the default parameters currently available from EMBL-EBI. These are as follows: (i) for amino acid sequences: matrix = BLOSUM62, Gap

open penalty = 10 and Gap extend penalty = 0.5 and (ii) for nucleic acid sequences: matrix = DNAfull, Gap open penalty = 10 and Gap extend penalty = 0.5.

In the context of the present invention, the term "homologous sequences" or "homologues" or similar terms should be understood to be a reference to nucleic acid sequences which have the same phylogenetic origin. Preferably, proteins which are coded by these nucleic acid sequences have the same function. Homologous nucleic acid sequences exhibit at least 70%, preferably at least 75%, at least 80%, at least 85% or at least 90%, particularly preferably at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity.

The term "nucleic acid target region", as used herein, relates to any genomic as well as extrachromosomal DNA or RNA, in particular mRNA, of a target organism or a target cell which is to be modified and which can be modified by the method and constructs disclosed herein and is purposefully not limited to gene regions, i.e. regions which carry the information for the transcription of an mRNA region. These target regions are thus natural or endogenous target regions, wherein the terms, "endogenous" and "natural" are used interchangeably in this context. Moreover, the term, "nucleic acid target region," is not limited to an endogenous sequence. If an artificial nucleic acid target region has been previously inserted into a target cell of a target structure of interest, the term, "nucleic acid target region," can thus refer to an artificially inserted nucleic acid target region.

The term "complementary" or "complementarity", as used herein, describes the relationship between two DNA or RNA nucleic acid regions, the nucleobases of which fit together on the basis of a lock and key principle and form hydrogen bonds between each other (hybridize). In this regard, Watson-Crick base pairing of the bases adenine and thymine/uracil or guanine and cytosine are considered to be complementary. Other non-Watson-Crick pairings, such as reverse Watson-Crick, Hoogsteen, reverse Hoogsteen and wobble pairings are encompassed by the term "complementary", provided the corresponding base pairs form hydrogen bonds together, i.e. two different nucleic acid strands can hybridize together on the basis of their complementarity.

The term "hybridize" or "hybridization" should be understood to mean a procedure during which a single-stranded nucleic acid molecule is added to a maximally complementary nucleic acid strand, i.e. undergoes base pairing. Examples of standard methods for hybridization are

described in Sambrook et al, 2001. This should be understood to mean preferably at least 60%, still more preferably at least 65%, 70%, 75%, 80% or 85%, and particularly preferably 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the bases of the nucleic acid sequence undergo base pairing with the maximally complementary nucleic acid strand. The possibility of such an addition depends on the stringency of the hybridization conditions. The term "stringency" relates to the hybridization conditions. Stringency is considered to be high when base pairing is difficult and low when base pairing is facilitated. The stringency of the hybridization conditions depends on conditions including salt concentration or ionic strength and the temperature. In general, stringency can be increased by increasing the temperature and/or by reducing the salt content. The term "stringent hybridization conditions" should be understood to mean those conditions in which a hybridization primarily only occurs between homologous nucleic acid sequences. In this case, the term "hybridization conditions" relates not only to the conditions prevailing during the actual addition of the nucleic acids, but also to the conditions prevailing during the subsequent washing steps. Examples of stringent hybridization conditions are conditions in which overwhelmingly only those nucleic acids hybridize which exhibit at least 70%, preferably at least 75%, at least 80%, at least 85% or at least 90%, particularly preferably at least 95%, at least 96%, at least 97%, at least 98% or at least 99% sequence identity. Examples of stringent hybridization conditions include: hybridizing in 4×SSC at 65° C and multiple subsequent washes in 0.1×SSC at 65° C for a total of approximately 1 hour. The term "stringent hybridization conditions", used herein, can also mean: hybridization at 68° C in 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA and 1% BSA for 16 hours and two subsequent washes with 2×SSC and 0.1% SDS at 68° C. Preferably, the hybridization is carried out under stringent conditions.

Detailed Description

The present invention relates to a method for the production of a maize plant as defined in Claim 1.

Meristematic cells belong to a tissue type in a plant which is described as the meristem or formation tissue. In the manner of stem cells in animal organisms, meristematic plant cells, because they are undifferentiated cells, have the ability (depending on environmental factors) of differentiating into any specialized cell type.

In all embodiments, the individual components can be introduced simultaneously or in sequence on the same or on different constructs. In some embodiments involving transient introduction, it can be beneficial to first introduce the construct carrying one or more protein components of the system, i.e. CRISPR nuclease or a variant or catalytically active fragment thereof, and optionally, an effector domain. Optionally, if this is a DNA construct, this at least one construct can then be first translated by the cell. The constructs that carry the gRNA and the optional further DNA repair templates and/or effector domains can then be introduced in a temporally offset manner. As a result, it can be ensured that the less stable gRNA can interact directly with the CRISPR nuclease of interest, and that decomposition of the gRNA will not prevent an effective DNA editing.

In accordance with the present invention, a special method is provided which can either directly or indirectly specifically control the small population of meristematic cells in a plant as a target plant structure. The at least one meristematic target cell may be controlled directly or indirectly, i.e. at least one recombinant construct in accordance with the present disclosure may be introduced directly into the at least one meristematic target cell or the at least one recombinant construct may be introduced into any plant cell or any plant tissue with the aid of a suitable vector, wherein the at least one recombinant construct can then be transported to the plant target structure. This is accomplished by means of the systemic propagation of at least one recombinant construct introduced into a plant cell or into a plant tissue by means of a vector.

The term “plant target structure”, as used herein, encompasses at least one meristematic plant cell which may be present as tissue, plant material, as a whole plant or as an isolated cell, wherein the meristematic plant cell also contains at least one nucleic acid target region. The at least one target nucleic acid region contained in the plant target structure comprises DNA and RNA sequences and may be present in the target structure chromosomally or extrachromosomally. The targeted CRISPR-based methods for modifying a target nucleic acid region of interest can therefore be used in the modification of genomic DNA, comprising the epigenetic modification of genomic DNA, or the modification of plastid or mitochondrial DNA, as well as in the modification of RNA (e.g. silencing).

In one aspect of the present invention, which concerns the introduction of a specific nucleic acid modification into a non-chromosomal target structure, the term “plant target structure”, as used herein, encompasses at least one plant cell which may be present as tissue, plant material,

as a whole plant or as an isolated cell, wherein the plant cell additionally contains at least one target nucleic acid region comprising DNA and RNA.

In accordance with the present invention, at least one target nucleic acid region in a meristematic plant cell as the target structure is modified by transiently introduced CRISPR/Cas tools and/or further effector domains if appropriate. Since the at least one meristematic cell modified in this manner can directly and immediately pass on the specific modification in the target nucleic acid region to its descendants by means of subsequent cell division and differentiation, the method of the present invention does not require any additional crossing and selection steps in order to provide a plant, plant material or a plant cell with the desired target modification. Moreover, plant organisms or plant target structures which carry the specifically introduced modification may be obtained from embryonal or secondary meristems such as pollen or ovaries, optionally with self-fertilization or cross-fertilization,

The method of the present invention has the further advantage that the CRISPR/Cas tools and/or any further effector domains are introduced into the plant target structure, i.e. a meristematic cell or a meristematic tissue, in only a transient manner, so that no stable integration of the CRISPR/Cas tools such as CRISPR nuclease, gRNA and any regulatory sequences as well as any additional effector domains occurs into the endogenous chromosomal or endogenous extrachromosomal nucleic acids of the target plant structure.

In accordance with the present disclosure, it has been found that, by exploiting the mechanism of action of RNA-directed DNA modification of the CRISPR/Cas tools, further effector domains, in accordance with the method provided herein, can be introduced, allowing the spectrum of specific genome editing to be broadened. Either the CRISPR nuclease variant or the catalytically active fragment thereof or the gRNA or both may be linked with an effector domain.

An “effector domain”, as used herein, encompasses DNA-modified or RNA-modified or DNA-binding or RNA-binding polypeptides or nucleic acids, encompassing all types of monomeric, dimeric or multimeric nucleases, such as TALE nucleases, meganucleases, zinc finger nucleases, ribonucleases, deoxyribonucleases, exonucleases, endonucleases and restriction endonucleases of type I, II, III or IV and the like, and including nickases, transcription activators and suppressors, phosphatases, glycosylases or enzymes which can effect epigenetic

modifications, such as acetylases, methylases, methyl transferases, proteins which can bind methylated DNA, or histone deacetylases, aptamers, comprising single-stranded DNA or RNA sequences as well as peptides, fluorescent proteins, marker nucleic acid sequences or marker amino acid sequences and the like, and combinations thereof. With respect to enzymes or polypeptides in general, the term "effector domain" also encompasses a catalytic domain or nuclear domain of the respective enzyme or polypeptide, for example a binder protein, wherein the catalytic domain or nuclear domain is still capable of carrying out the enzymatic or binding function of the respective native enzyme or polypeptide. The design of such truncated domains and their adaptation to the desired function is known to the person skilled in the art.

Methods and constructs are provided herein, by means of which gRNA and/or CRISPR nuclease or the catalytically active fragment thereof already linked to a further effector domain is/are provided on a recombinant construct. The gRNA and/or the CRISPR nuclease, comprising at least one effector domain, are then introduced on at least one recombinant construct into a target structure, in order to form a functional complex there, following transcription and, optionally, translation.

In a further embodiment, a method is provided in which the at least one gRNA or the at least one CRISPR nuclease or the catalytically active fragment thereof and/or at least one further effector domain is provided separately on different recombinant constructs. In accordance with this method, the gRNA component may be provided as DNA or RNA, the CRISPR nuclease or variant thereof or the catalytically active fragment thereof may be provided as DNA or RNA or as a polypeptide sequence and the effector domain may be provided as DNA or RNA or as a polypeptide sequence. The gRNA and the CRISPR nuclease, optionally comprising at least one effector domain, can thus be pre-assembled *in vitro*, and then introduced into a target structure.

According to one embodiment of the present invention, which comprises the simultaneous introduction of at least one gRNA and at least one CRISPR nuclease variant, or a catalytically active fragment thereof, together with at least one effector domain, the effector domain can be linked to the gRNA, or the CRISPR nuclease variant or the catalytically active fragment thereof, by a nucleic acid or amino acid linker, in order to ensure an ideal arrangement of the domains in relation to one another, and as a result, ensure their functionality through the adequate flexibility of the domains in relation to one another. According to one embodiment,

it is preferred that, in order to produce a plant, plant material, or a plant cell, optimized in a targeted manner, a DNA repair template is also provided, in addition to a gRNA and a CRISPR nuclease, e.g. a Cas or a Cpf1 nuclease, which can comprise effector domains independently. This embodiment is particularly preferred if a CRISPR nuclease, or a catalytically active fragment thereof, is used, which is capable of catalysing the introduction of a targeted DNA double-strand break in a nucleic acid target region of interest. The additional provision of a DNA repair template, either in the form of single-strand or double-strand DNA, can out-complete the natural and error-prone NHEJ repair mechanism of a plant cell, in order to bring about an even greater precision in the genome editing, in addition to providing the possibility of targeted introductions of insertions, mutations or deletions. The DNA repair template can be provided in the form of a recombinant construct, either separately or on the same construct that is used for the introduction of the gRNA and/or the CRISPR nuclease. Alternatively, the DNA repair template can be introduced directly into a target cell or target structure of interest through transfection or transformation. Normally, a DNA repair template is designed such that it comprises left and right homology arms, which flank the position that is cleaved by a CRISPR nuclease. The two homology arms may have a length of numerous hundreds of base pairs (bp), e.g. at least 100, at least 200, at least 300, at least 400, or at least 500 base pairs (bp), up to 1 kilobase (kb) or more. A homologous region, i.e. a region of sequence homology, can comprise at least 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5- 50, 5-55, 5-60, 5-65, 5- 70, 5-75, 5-80, 5-85, 5-90, 5-95, 5-100, 5-200, 5-300, 5-400, 5-500, 5-600, 5-700, 5-800, 5-900, 5-1000, 5-1 100, 5-1200, 5-1300, 5-1400, 5-1500, 5-1600, 5-1700, 5-1800, 5-1900, 5-2000, 5-2100, 5-2200, 5-2300, 5-2400, 5-2500, 5-2600, 5-2700, 5-2800, 5-2900, 5-3000, 5-3100 or more bases so that the homologous region has a sufficient level of homology to facilitate homologous recombination with the corresponding genomic region. A "sufficient level of homology" in this context means that two polynucleotides exhibit sufficient structural similarity, and thus can serve as a substrate for a homologous recombination. Accordingly, the level of homology between the respective homology arm of a DNA repair template and the corresponding target nucleic acid region can vary. In general, with shorter homologous regions, a higher level of homology is needed in order to obtain an adequate accumulation of complementary nucleic acid sequences. Accordingly, the level of homology, i.e. the sequence identity, can be 50%, 55%, 60%, 65%, 70%, 71 %, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81 %, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. In addition, the DNA repair template comprises a central construct, which carries a sequence, such as a transgene, that is to be introduced, or a modification that is to be

introduced. The degree of success of the introduction of a targeted modification into a targeted nucleic acid structure can be checked subsequently by means of (quantitative) PCR methods. In a PCR method, such a construct can be amplified using primers that are specifically for the two homology arms, the sequences of which can thus then be determined in order to establish whether the repair was performed by the NHEJ machinery of the cell, or by homologous recombination, assisted by the DNA repair template.

According to a further embodiment, first, at least one first plant, plant material, or plant cell is provided, comprising at least one CRISPR nuclease, preferably a Cas nuclease or a Cpf1 nuclease, wherein the CRISPR nuclease is integrated in a transient manner. This embodiment is particularly advantageous if this at least one first plant, plant material, or plant cell is later to be crossbred with at least one second plant, wherein the second plant, or at least one plant meristematic cell thereof, comprises a gRNA, with interacts with the Cas nuclease of the first plant, and can thus cause a targeted genome editing. The successful introduction of a targeted modification into a target nucleic acid region of interest in accordance with the present invention can be readily verified by a person skilled in the art using methods such as polymerase chain reaction and the like, especially if the target nucleic acid region of interest, and thus the region where the potential PCR primers can accumulate, is known, and is relevant for the design of a gRNA and/or a DNA repair template.

Activators and suppressors which may be used in accordance with the present invention preferably comprise SEQ ID NOs: 1-4 as well as sequences with at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology with these sequences, which, despite modification, still carry out the same function as the sequences with the corresponding SEQ ID NOs.

A targeted modification according to the present disclosure can therefore comprise: (i) the exchange of at least one nucleotide of a target nucleic acid region; (ii) the deletion of at least one nucleotide in a target nucleic acid region; (iii) the insertion of at least one nucleotide in a target nucleic acid region; (iv) the targeted epigenetic modification of the region that regulates at least one target nucleic acid region; (v) the bonding and/or visualization to/of at least one nucleotide in a target nucleic acid region; or (vi) the interaction with and/or the cleavage of at least one RNA target nucleic acid region, or any combination of (i) through (vi). The methods

according to the present invention can be used, in particular, for the precise and rapid development of traits in a plant, plant material, or a plant cell.

In another embodiment of the first aspect of the present invention, a method is provided for producing a plant, plant material, or a plant cell, in which at least one plant target structure, comprising at least one meristematic cell, at least one gRNA, at least one CRISPR nuclease, or a catalytically active fragment thereof, and/or an effector domain, as well as at least one DNA repair template, is provided, wherein the targeted modification of the target nucleic acid region of interest involves the introduction of at least one heterologous sequence, i.e. non-endogenous sequence, which comprises a gene selected from the group composed of a reporter gene, a selection marker, a gene that provides immunity to a disease, a herbicide resistance gene, a gene providing resistance to insects or nematodes, a gene involved in carbohydrate metabolism, a gene involved in fatty acid metabolism, a gene involved in amino acid metabolism, a gene involved in plant development, a gene involved in the regulation of plant growth, a gene involved in improving the yield of a plant material of interest, a gene involved in providing resistance to drought, a gene involved in providing heat resistance, a gene involved in providing resistance to a salt or salts, or a gene that is coded by a functional RNA, wherein the functional RNA is selected from the group composed of an miRNA, a siRNA, or another RNA that can form an inverted repeat structure, e.g. a ddRNAi construct, that codes both a sense and antisense strand, as well as a hairpin loop connecting the sense and antisense strand, into the genome of a plant target structure of interest, comprising at least one meristematic cell.

Moreover, the methods according to the present disclosure are suitable for the formation of a complex trait locus. A complex trait locus is a chromosomal segment that has at least two modified nucleic acid regions and can be integrated into a target nucleic acid region according to the present disclosure in a single step, or sequentially, wherein the at least two modified nucleic acid regions are genetically linked to one another, wherein the at least two modified nucleic acid regions both come from an endogenous plant locus, or the modification involves a mutation or deletion of chromosomal DNA, or the at least two modified nucleic acid regions are transgenic sequences, or a combination thereof. Because the DNA repair template according to the present disclosure may have a central construct with a length of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 1000, 2000, 3000, 4000 bp or more, the methods according to the present disclosure are suitable for introducing a complex trait locus into a plant target structure, comprising at least one

meristematic cell, and introducing a complex trait directly into a plant target structure with greater precision and efficiency, through the use of a CRISPR system and a DNA repair template. Because at least one plant, at least one plant material, or at least one plant cell can be obtained directly according to the methods of the present invention, there is the possibility of obtaining plant cells, plant material, or plants in a short period of time that have been modified in a targeted manner and are suitable for further crossbreeding, breeding, or further targeted modifications. In one embodiment, a first fertile plant with a first targeted modification in its genome can be crossed with a second plant with a second targeted modification in its genome, such that the targeted modifications can be physically linked, i.e. become part of the same nucleic acid molecule, wherein at least the first or the second, or both, plants are obtained according to the methods of the present disclosure. Artificial recognition sites in the form of a modified target nucleic acid region can be introduced in a locus through the intrinsic properties of the CRISPR system and knowledge of PAM motifs, as well as the interaction of an artificial gRNA with a CRISPR nuclease, in order to subsequently generate a nested complex locus, comprising more than one targeted modification.

In another embodiment, a complex trait locus can also be directly introduced in a single step into a plant target structure comprising at least one meristematic cell. Because the CRISPR system according to the present disclosure can be scaled, in that it comprises multiple CRISPR nucleases, multiple gRNAs, optionally aligned with the CRISPR nucleases and/or a target nucleic acid region of interest, as well as at least one DNA repair template comprising at least one trait of interest that is to be integrated in the genome of a plant target structure, in addition to suitable homology arms, it is therefore not the case that the methods disclosed herein are limited to affecting just one trait in a targeted manner. Instead, it is possible to introduce a complex genotypic trait in a plant target structure of interest in a stable manner that results in at least one phenotypic trait.

In one embodiment according to the methods in the present disclosure, a process for producing a complex trait locus in a plant, plant material, or plant cell is therefore disclosed, wherein the method comprises the following steps: (a) selection of a genomic target nucleic acid region of interest in a plant, wherein the genomic target nucleic acid region comprises at least one first target nucleic acid sequence and one second target nucleic acid sequence; (b) bringing of at least one plant target structure, comprising at least one meristematic cell in contact with at least one first gRNA, one second gRNA, and optionally, at least one DNA repair template, and at

lest one CRISPR nuclease or a catalytically active fragment thereof, wherein the first and the second gRNAs, and the at least one CRISPR nuclease or the catalytically active fragment thereof, can form a complex, which allows at least one CRISPR nuclease to introduce a double strand break, or a single strand break in the case of a nickase, in at least one first and one second nucleic acid target region, wherein, optionally, the at least one gRNA or the at least one CRISPR nuclease also comprises an effector domain, or can be associated with at least one effector domain; (c) identification of a cell from step (b), which comprises at least one first targeted modification on the first nucleic acid target sequence, and one second modification on a second nucleic acid target sequence; and optionally, (d) acquiring a first fertile plant from the at least one meristematic cell from step (c), wherein the fertile plant comprises the first targeted nucleic acid modification and the second targeted nucleic acid modification, wherein the first targeted nucleic acid modification and the second targeted nucleic acid modification are physically linked, i.e. located on the same nucleic acid strand.

In another embodiment, the method comprises a process for producing a complex trait locus in which at least two modified nucleic acid target sequences in a genomic target nucleic acid region of interest are modified in a plant, plant material, or plant cell, comprising the following steps: (a) selection of a genomic target region in a plant, plant material, or plant cell, comprising at least one meristematic cell, wherein the genomic target region comprises a first nucleic acid target sequence and a second nucleic acid target sequence; (b) bringing the at least one plant cell, comprising at least one meristematic cell, in contact with a first gRNA, a CRISPR nuclease, or a catalytically active fragment thereof, and optionally, a first donor DNA in the form of a DNA repair template, wherein the first gRNA, and the first CRISPR nuclease or the catalytically active fragment thereof, can form a complex that allows the CRISPR nuclease to insert a double-strand break in the first target nucleic acid region, wherein the gRNA and/or the CRISPR nuclease can optionally comprise an effector domain, or be associated with an effector domain; (c) identification of the at least one meristematic cell from (b), which comprises the first targeted modification in the first nucleic acid target sequence; (d) acquiring a first fertile plant from the cell from step (c), wherein the first fertile plant comprises the first targeted modification; (e) bringing at least one plant, plant material, or plant cell, comprising at least one meristematic cell, in contact with a second gRNA, a second CRISPR nuclease or a catalytically active fragment thereof, and optionally, a second donor DNA in the form of a DNA repair template; (f) identification of a cell from step (e), wherein the cell comprises at least one second targeted modification in a second nucleic acid target sequence; (g) acquiring

a second fertile plant from the cell from step (f), wherein the second fertile plant comprises the second targeted modification; and (h) obtaining fertile descendants from the second fertile plant from step (g), wherein the fertile descendant plants comprise both the first and the second targeted modification in a target nucleic acid region of interest, wherein the first targeted modification and the second targeted modification are physically linked.

The tools and methods disclosed herein are therefore valuable tools for targeted and efficient genome editing in higher plants through the use of CRISPR tools as well as targeted homologous recombination as a repair mechanism. In particular, through the method disclosed herein, it is possible to circumvent the natural and error-prone "non-homologous end joining" (NHEJ) DNA repair mechanism, which frequently leads to mutations or chromosomal deletions.

Selected traits, which, according to the present disclosure, are introduced into a plant, or can be effected in a plant in the form a targeted modification through genome editing, comprise, without being limited thereto, resistances, comprising resistances to herbicides and pests, comprising prokaryotic and eukaryotic pests and viruses, e.g. bacteria, fungi, protozoa, plant pathogenic viruses, nematodes, insects or other animal organisms, obtaining higher yields, wherein the yields can relate to any desired plant product, e.g. an increased seed, fruit, carbohydrate, protein, or fat yield, or other plant metabolism products, comprising further primary metabolites or secondary metabolites and the like. One target of genome editing can be the endogenous 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. EPSPS naturally catalyses the conversion of phosphoenolpyruvate and 3-phospho-shikimate into phosphate and 5-enolpyruvylshikimate-3-phosphate. Through the introduction of targeted mutations in this endogenous gene, a mutated EPSPS coding gene can be obtained, which provides resistance to the herbicide N-(Phosphonomethyl)glycine, or a salt thereof.

Moreover, the methods according to the present disclosure can be used to introduce traits into a plant or to modify undesired traits in a targeted manner. There is a significant demand for foodstuffs, or food products, that contain a low portion of acrylamide. Acrylamide, which has been categorized as carcinogenic, is an undesired by-product of the Maillard reaction of the amino acids asparagine and glutamine when they react with reduced sugars such as aldoses (e.g. glucose) or acyloines at temperatures above approx. 170° C. There is therefore a significant level of interest in reducing the content of educts that potentially form acrylamides,

in starchy plant products such as potato products, such as asparagine, in order to create safe foodstuffs. The method disclosed herein is capable of producing a potato plant which can be modified in a targeted manner such that the gene is affected in a targeted manner with respect to asparagine metabolism, by influencing an asparagine synthetase gene or another gene that is involved in asparagine metabolism.

Another endogenous target gene of interest in the genome of a plant cell which can be modified to provide herbicide tolerance or resistance, is the acetohydroxy acid synthase (AHAS) gene. AHAS inhibitor herbicides are important herbicides used in agriculture throughout the world. By modifying at least one allele of an endogenous AHAS gene with the method disclosed herein, a herbicide-tolerant or herbicide-resistant plant cell can be created, from which a fertile plant, plant material, or plant cell can be obtained within a short period of time on the basis of the approach disclosed herein for targeting a meristematic cell.

Plants that are resistant to various environmental factors, in particular crop plants for energy and food production, have also become increasingly important. These environmental factors include heat, drought, cold, the condition of the ground and the nutrient supply, and salinity. Consequently, there is a high interest in creating plants that can thrive in changing, and frequently less than optimal environmental conditions. These traits also include properties, which can be introduced into a plant, in particular a crop plant, in a targeted manner according to the methods of the present invention, or can be induced in at least one plant target structure of interest through the targeted modification of at least one target nucleic acid region.

Specifically with regard to the optimization of traits that are agronomically relevant in a plant, it may also be of interest to modify the endogenous sequences, thus controlling the sequences that function as regulatory sequences, comprising promoters, in a targeted manner.

In one embodiment according to the present invention, a method is therefore proposed for producing a plant, plant material, or a plant cell, which comprises at least one targeted modification in at least one meristematic cell of interest, wherein the targeted modification is the modification of an endogenous promoter. The targeted modification of the promoter can comprise the replacement of the promoter, or a fragment thereof, with another promoter, or a fragment thereof, wherein the promoter exchange results in any combination of the following: increased promoter activity, increased promoter tissue specificity, increased pathogen-

inducible promoter activity, reduced promoter activity, reduced promoter tissue specificity, reduced pathogen-inducible promoter activity, new promoter activity, an inducible promoter activity, a pathogen-inducible promoter activity, an expanded spectrum of possible gene expression, which is regulated by the promoter, a modification of the temporal, spatial, or developmental stage-dependent gene expression of a target nucleic acid region, in this case a plant gene of interest, by means of which active promoters that are only active in a specific development stage can likewise be active in another development stage, or, regarded spatially, can be active in another tissue, or a mutation of DNA bonding elements, and/or the deletion or addition of DNA bonding elements. The promoter, or the fragment thereof, which is to be modified according to the method of the present disclosure, can be a promoter or fragment thereof, which is endogenous in the plant cell of interest, but can also be an artificial promoter or a transgenic promoter that is present in a plant target structure of interest, which comprises at least one meristematic cell. Preferably, the promoter or fragment thereof that is to be modified is integrated into the chromosomal or extrachromosomal genome of a plant target structure of interest comprising at least one meristematic cell. The promoter that is to be modified, or the fragment thereof, may also be present on an extrachromosomal, not genomically integrated construct, e.g. a plasmid.

Such endogenous genes exhibit further interesting traits that code relevant metabolism, information, and/or signal transduction proteins, e.g. kinases, transcription factors, zinc finger proteins or heat shock proteins. A targeted modification of these genes, and thus the coded proteins, makes it possible to intervene in numerous physiological processes, and thus provides the possibility of controlling metabolic processes in a targeted manner. Furthermore, these genes, and the associated regulatory DNA elements and the regions that code regulatory proteins, are target nucleic acid regions of interest that are responsible for the fertility and/or sterility of a plant.

Further biotic and abiotic factors, the reaction possibilities, on the basis of which a target plant of interest can be modified according to the method of the present disclosure through targeted modification, comprise nutrient shortages, reactions to exposure to toxic metals, trace elements, quality, in particular the quality of the seeds or grain, optimized nutrient content, starch quality and quantity, the size of the seeds or grains, the overall carbohydrate content, comprising starch, sucrose, and other monosaccharides, diaccharides and polysaccharides, nitrogen fixation and use, fatty acid and/or oil content and/or the composition of the fat/oil, comprising

saturated and unsaturated fats, an increase in the lysine content, or other amino acids, or sulphur in a plant product, or a combination of the aforementioned traits. Examples of genes that can increase grain yield include ammonia-induced glutamate-dehydrogenases. Examples of genes that affect amino acid biosynthesis include anthranilate synthases (EC 4.1.3.27).

In another embodiment, the target nucleic acid region that is to be modified in a targeted manner can be a promoter, wherein the targeted modification comprises the replacement of a native EPSPS1 promoter with a plant ubiquitin promoter.

In a further embodiment, the target nucleic acid region that is to be used in a targeted manner can be a promoter, wherein the targeted modification of the promoter comprises the replacement of an endogenous NPK1 promoter from maize with a stress-inducible RAB17 maize promoter.

In an embodiment according to the present disclosure, the nucleic acid target region of interest can be a promoter, whereby the promoter to be targeted for modification is selected from the group comprising *Zea mays* PEPC1 promoter (Kausch et al, Plant Molecular Biology, 45:1-15, 2001), a *Zea mays* Ubiquitin promoter (UBI1ZM PRO, see Christensen et al, Plant Molecular Biology 18:675 - 689, 1992), a *Zea mays* Rootmet 2 promoter, an Actin promoter from rice (US-ACTIN PRO, McElroy et al, The Plant Cell, edition 2, 163 - 171, February 1990), a Hirse RCC3 promoter, a *Zea mays* GOS2 promoter, a *Zea mays* ACO2 promoter, or an Oleosin promoter from *Zea mays.*

Because the methods disclosed herein, as outlined above, are also suitable for introducing targeted insertions into a target nucleic acid region of interest through the combination of a CRISPR system, in particular the combination of at least one specific CRISPR nuclease, or a variant or active fragment thereof, with a specific gRNA and a DNA repair template, and a further embodiment of the present disclosure pertains to the creation of a method for inserting a promoter or a promoter element in a genomic nucleic acid target region of interest in a plant target structure, comprising at least one meristematic cell, wherein the promoter insertion may result in any of the following phenotype modifications: increased promoter activity, i.e., increased promoter strength, increased promoter tissue specificity, a new promoter activity, an inducible promoter activity, an expanded spectrum of gene expression for the gene that is regulated by the promoter, or that is set through the introduction of an exogenous promoter

under the control of this newly introduced promoter, a modification of the temporal, spatial or developmental stage gene expression, a mutation of DNA bonding elements, and/or the addition of DNA bonding elements. Selected promoter elements that can be introduced according to the methods of the present invention into a plant target structure comprising at least one meristematic cell of interest, comprise, without being limited thereto, promoter nuclear elements, e.g. a CAAT box, a CCAAT box, a Pribnow box, a bonding element that promotes pathogen inducibility, such as a W-box, S-box, or D-box, and/or a TATA box, regulatory sequences that can affect translation, and/or a repressor system for obtaining an inducible expression, e.g. a tet operator/repressor/inducer element, or a sulfonylurea repressor/operator/inducer element. Further regulatable promoter/operator systems which can be introduced into a plant target structure of interest for the purposes of the present disclosure, are known to the person skilled in the art. Examples of promoters which can be introduced as an exogenous promoter into a plant target structure of interest include the DRE promoter. This promoter was originally described by Yamaguchi-Shinozaki und Shinozaki (1994), Plant Cell 6, 251 - 264 , as a cis-operating promoter element in promoters of the drought resistance gene *rd29A*which contains a conserved nuclear sequence comprising new base pairs, TACCGACAT. The introduction of a DRE promoter into an endogenous promoter of an arbitrary plant gene can therefore produce the inducible expression of the gene regulated by this promoter following a drought/aridity stimulus. A further example comprises ABA responsive elements, which contain a (c/T) ACGTGGC consensus sequence, and are found in numerous ABA and/or stress-regulated genes (Busk & Pages (1998), Plant Mol. Biol. 37:425 - 435). The insertion of 35 S enhancer or MMV enhancer into an endogenous promoter region in a plant cell can likewise increase the expression of the regulated gene. Therefore, through the targeted and precise modification of a promoter, or a portion thereof, in accordance with the present disclosure, the expression of a gene regulated by the promoter can be affected in a targeted manner, and the targeted modification introduced in this manner can be passed down directly to descendants, because the primary target cell is a meristematic cell, in accordance with the present invention, such that a fertile plant or plant material, or a plant cell therefrom, can be obtained, which has the desired promoter modification in its genome, and furthermore has a desired phenotypic trait, which is the result of the gene regulated by the promoter through the modified expression.

In another embodiment according to the present disclosure, a method is provided, which relates to the targeted modification of a terminator, using the methods disclosed herein. Accordingly,

the target nucleic acid region of interest in a plant target structure comprising at least one meristematic cell can be a terminator, wherein the modification comprises the replacement of the terminator, or a fragment thereof, with another terminator, or a fragment thereof, wherein the terminator exchange may involve one or more of the following phenotypic trait modifications: increased terminator activity, increased tissue specificity of the terminator, reduced terminator activity, reduced tissue specificity of the terminator, a mutation of DNA bonding elements and/or a deletion or addition of DNA bonding elements. The terminator (or fragment thereof) that is to be modified in a targeted manner can be a terminator (or fragment thereof) of an endogenous gene, but it can likewise be an artificial, or chimeric or synthetic terminator, or a transgenic terminator. Likewise, the replacement terminator, i.e. the terminator, or fragment thereof, that is to be introduced through the method disclosed herein into the genome of a plant target structure of interest, can also be an endogenous terminator, an artificial terminator comprising a chimeric terminator, or a transgenic terminator. Exemplary terminators can be selected from the group comprising a maize ARGOS 8 or SRTF18 terminator, a tomato PIN-II terminator, a millet-actin terminator, a millet SB-GKAF terminator, a rice T28 terminator, an AT-T9 terminator, or a GZ-W64-A terminator. According to a preferred embodiment of the present invention, the terminator element that is to be replaced is used by the combination of at least one gRNA, adapted to at least one CRISPR nuclease and a target nucleic acid region of interest, together with a DNA repair template, wherein the central element of the DNA repair template serves as a donor sequence for the insertion of a terminator or terminator element of interest into a genomic target nucleic acid region of a plant target structure comprising at least one meristematic cell.

In another embodiment, the gRNA/CRISPR nuclease/DNA repair template system disclosed herein is used in a meristematic cell to specifically delete a terminator, or a terminator element, that is genomically anchored in a plant target structure of interest.

In addition to promoters and terminators, other regulatory sequences exist in the genomes of eukaryotic cells that are important for regulating gene or functional RNA transcription. In one embodiment according to the present disclosure, the CRISPR system disclosed herein is used to modify or replace these regulatory sequences in a targeted manner in order to anchor these targeted modifications or replacements in a stable manner in the genome of a plant target structure of interest, to pass them on to the descendants via the initially modified meristematic cell, and thus be able to observe a targeted phenotype modification in the plant material or plant

cells thereof obtained in this manner. Exemplary regulatory sequences according to the present disclosure comprise, without being limited thereto, 3′UTR (not translated) regions, 5′UTR regions, transcriptional activators, transcriptional enhancers or suppressors, translation repressors, splicing factors, miRNAs, siRNAs, artificial miRNAs, incRNAs, promoter elements, CaMV 35S enhancers, MMV enhancer elements, SECIS elements, polyadenylation signals, and polyubiquitination sites.

In some embodiments, the genome editing, i.e. the targeted modification of a target nucleic acid region, comprises the targeted modification or replacement of regulatory elements, resulting in one or more of the following effects and/or phenotypic expressions: modified protein translation, RNA cleavage, RNA splicing, and transcription or post-translational modifications. In one embodiment, the target nucleic acid region of interest, which is to be modified in a meristematic cell in a targeted manner, is a polyubiquitination site, wherein the targeted modification of the polyubiquitination site results in a modified protein degradation rate for a target protein of interest. The ubiquitin tag marks proteins, so that these can subsequently be degraded in proteasome or through a process called autophagy. Proteasome inhibitors are known for possibly causing protein overproduction. The targeted modification of a target nucleic target region of interest that codes a protein of interest can therefore also lead to at least one amino acid modification of the protein of interest, wherein the modification allows for the subsequent polyubiquitination of the protein, i.e. a post-translational modification, which leads to a modification of the protein degradation, or the rate of protein degradation in the protein of interest.

In one embodiment, the genomic sequence of interest that is to be modified is a polyubiquitination site in a maize EPSPS gene, wherein the targeted modification of the polyubiquitination site results in an increased protein content, because the relevant protein is broken down at a lower rate.

In another embodiment, the genomic target nucleic acid sequence inside a meristematic cell that is to be modified in a targeted manner according to the methods of the present invention is an intron site, wherein the targeted modification comprises the introduction of an intron-promoting motif into the intron, resulting in a modulation (increase/decrease) of the transcription activity of the gene comprising this intron.

In another embodiment, the target nucleic acid region of interest inside the genome of a plant target structure that is to be modified in a targeted manner is an intron site, wherein the targeted modification comprises the replacement of a specific intron, e.g. a soya bean EPSP synthase 1 intron, with another intron, e.g. a soya bean ubiquitin intron 1.

In one embodiment according to the present disclosure, the target nucleic acid sequence of interest that is to be modified in the genome of a meristematic cell of a plant of interest in a targeted manner is an intron or UTR site, wherein the targeted modification comprises the insertion of at least one micro RNA into this intron or UTR site, by means of which the expression of the gene that comprises the intron or UTR site also leads to the expression of this inserted micro RNA, which leads in turn to the ability for each target gene of interest to be "silenced" by the micro RNA that has been transcribed in this manner, whether it is an endogenous plant gene or the gene of a plant pest, without affecting the gene expression of the gene that carries the intron. Gene silencing is a process in which the expression of a gene is reduced or deactivated. In such cases, gene regulation comprises the inhibition of the transfer of genetic information from the DNA to the mRNA, or the subsequent translation of the information stored on the mRNA into a protein. The processes that first take place after the transcription of the genetic information from the DNA to the transferring mRNA are referred to as post-transcriptional gene silencing. These phenomena are frequently referred to as RNA interference or RNAi, which are regulatory processes involving specific RNA molecules, such as micro RNAs and siRNA or artificial ddRNAi hairpin constructs. Post-transcriptional gene silencing can result in a more intensive degradation of a target mRNA of interest, impairing the formation of the gene product (protein). As a result, both endogenous as well as exogenous products can be silenced or translated at a significantly lower frequency by means of a process called host-induced gene silencing (HIGS).

In one embodiment, the method disclosed herein is used for the targeted modification of a target nucleic acid region inside a plant target structure comprising at least one meristematic cell, using the combination of a CRISPR nuclease and a gRNA, and, optionally, at least one effector domain, for the targeted modification of a transcription factor, i.e. to mutate or delete a transcription factor, or to insert a transcription factor into a target nucleic acid region of interest, using a suitable donor construct in the form of a DNA repair template. Examples of transcription factors include zinc finger transcription factor or the *tapetal development and function* factor (TDF; DE 10 2015 004 187 A1). The insertion of a single base pair into the

transcription factor-coding sequence can result in a frameshift mutation, which in turn produces a new protein, which maintains its DNA bonding activity, but has lost its transcription activation capacity. Accordingly, the mutated zinc finger transcription factor protein, for example, competes to bind to cytokinin-oxidase gene promoters, and blocks the expression of cytokinin-oxidase. The reduction of cytokinin-oxidase expression can increase the cytokinin level in rice plants, and promote panicle growth, increase ear growth in maize and increase the yield of a plant product of interest in numerous plants. The mutated TDF, on the other hand, can lead to male sterility in wheat, which can be implemented to provie an advantage when cultivating hybrid wheat plants.

In another embodiment, the methods according to the present disclosure can be used for the targeted modification of splices in a genomic target nucleic acid region of interest in a plant target structure comprising at least one meristematic cell, or alternatively, to introduce splices into the genomic target nucleic acid region of interest. In eukaryotic cells, mRNA that is obtained from pre-mRNA molecules and subsequently subjected to a maturation process is used for the synthesis or expression of proteins. The pre-mRNA molecules are capped, spliced and subsequently stabilized by the addition of a poly-A strand. Eukaryotic cells have developed a complex process for splicing, which results in alternative variants of an original pre-mRNA molecule. In maize cells, the splicing process can be affected by splicing sites at the exon-intron bonding sites. One example of a canonical splicing site is AGGT. Gene coding sequences may contain numerous alternative splicing sites, which can affect the overall efficiency of the pre-mRNA maturation process, and thus decisively limit the accumulation of proteins in cells. gRNA/CRISPR nuclease pairs disclosed herein can be used, together with effector domains and a DNA repair template, which can be used to introduce a specific modification template into a plant target structure of interest, to modify a genomic target nucleic acid region of interest, such that a canonical splicing site is inserted or created at a specific position with high precision. In one embodiment, a plant EPSPS gene can be affected, for example, wherein the targeted modification of the gene comprises the modification of alternative splicing sites such that this targeted genome editing results in an increased production of functional gene transcriptions and gene products.

If the method disclosed herein has an endogenous plant gene as the nucleic acid target region, one or more of the following effects can be obtained through the targeted modification: increased protein/enzyme activity, increased functionality of a protein of interest, reduced

protein activity, reduced protein functionality, a site-directed mutation, the replacement of a protein domain, a protein knock-out, a new protein functionality, or a modified protein functionality.

In one embodiment, the protein knock-out can comprise the introduction of a stop codon into the coding sequence of interest.

In another embodiment, the protein knock-out can comprise the deletion of a start codon in a coding sequence of interest.

In a further embodiment according to the present disclosure, the methods disclosed herein can be used for the targeted silencing of a gene of interest.

In one embodiment, the aim is gene silencing of an endogenous plant gene, and in another embodiment, the target gene in which the expression is to be modified is not an endogenous plant gene, but instead, the gene of a plant pathogen, comprising a bacterial gene, a eukaryotic gene, comprising genes from protozoa, nematodes, fungi, insects, or other animal predators or plant pathogens, or a viral gene. The process for silencing genes referred to as RNAi takes place in the cytoplasm of a target structure of interest, as this is where the necessary proteins and protein complexes are present in their functional form. The methods disclosed herein can thus be used in two different embodiments: (1) inverted gene fragments can be inserted into a target nucleic acid region of interest in a targeted manner through the methods disclosed herein. These gene fragments can be subsequently transcribed, resulting in a double-strand RNA structure, e.g. an RNA hairpin structure, which can subsequently silence an endogenous or exogenous gene. Alternatively, in accordance with this first embodiment, as stated above, a nucleic acid sequence can also be introduced, in a targeted manner, into a genomic target nucleic acid region which codes as functional RNA for an miRNA or a siRNA, wherein the siRNA or miRNA construct subsequently mediates the gene silencing. (2) In a second embodiment, the CRISPR nucleases disclosed herein and the associated gRNAs can be modified such that the artificial CRISPR system is specific to RNA as a target nucleic acid structure. To this end, further effector domains can be associated with either the gRNA and/or the modified CRISPR nucleases of interest. This approach is particularly advantageous, when, instead of the targeted modification of a genomic target region, RNA is to be modified directly in the framework of a *gene silencing* approach.

In another embodiment, the methods disclosed herein are suitable for facilitating trait mapping in the context of plant cultivation. As regards qualitative traits, the methods disclosed herein can be used for the targeted elimination of candidate genes in the identified chromosomal region, in order to determine, on this basis, whether or not the deletion of a gene has an effect on the expression of a trait of interest. As regards quantitative traits, the expression of a trait of interest is controlled by multiple *quantitative trait loci* (QTL) which differ and vary significantly in terms of size, complexity and statistical significance, which can also be located over numerous chromosomes in the genome of a plant. A QTL is therefore a portion of a chromosome, or a portion of numerous chromosomes, that has an effect on the expression of a specific quantitative phenotype trait of interest. In differing from discrete traits, e.g. blossom colours in plants that are present in numerous different, differentiated states, quantitative or consistent traits can be measured without gradation on a continuous scale. In the case of a negative effect on QTL regions that define a complex trait, the methods described herein can thus be used in one embodiment in order to eliminate entire chromosomal regions inside a plant target structure comprising at least one meristematic cell of interest through marker-assisted mapping, in order to mark specific regions for selective deletion, or redistribution.

In another embodiment of the present disclosure, the methods disclosed herein can be used to modify a genomic region of interest, which is flanked by two different target nucleic acid regions, according to the present disclosure, by means of two independent gRNA/CRISPR nuclease pairs, optionally using a DNA repair template. This modification can be performed simultaneously or in sequence order. The removal preferably takes place simultaneously, and the resulting deletion can be subsequently repaired, optionally using a DNA repair template, by linking the two chromosomal ends without the target deleted nucleic acid region of interest.

In an alternative embodiment, a target region of interest can be modified through inversions, mutations in the cleavage sites, or duplication of a region of interest.

Exemplary herbicide-resistant proteins or genes according to the present disclosure include acetolactate synthase (ALS) inhibitors, in particular if the herbicide is of a sulfonylurea type, herbicide resistance-coding genes that inhibit the effects of glutamine synthases, e.g. phosphinothricin or BASTA, glyphosate, e.g. EPSPS genes and GAT genes, HPPD inhibitors, e.g. HPPD genes and the like. By way of example, the bar gene codes resistances to the

herbicide BASTA, whereas the *nptII* gene provides resistances to the antibiotics kanamycin and geneticin (G418) and ALS gene mutants code or provide resistance to the herbicide chlorsulfuron.

Exemplary genes according to the present disclosure that provide resistance to diseases or plant pathogens can provide resistance to plant pests such as the corn rootworm, Bromius obscurus, or the larva thereof, the European corn borer, and the like. Disease resistance genes and/or insect resistance genes include lysozymes or cecropins for protecting against microorganisms, or proteins such as defensins, glucanases or chitinases, for protecting against fungi pathogens, or *Bacillus thuringiensis* endotoxins, protease inhibitors, collagenases, lectins or glycosidases for controlling nematodes or insects.

Moreover, the methods according to the present disclosure, as indicated above, can be used for generating male-sterile or female-sterile plants. The creation of male-sterile maize plants is highly advantageous because such a plant does not require the manual or mechanical removal of the tassel, i.e. the male inflorescence that produces pollen, which can be time consuming and expensive. Exemplary male sterility genes are, e.g. MS26, MS45, or MSCA1. Maize plants can be cultivated through both self-pollination as well as cross-pollination techniques. The maize plant has male flowers located on the tassel, and female flowers located on the ear, wherein the same plant has both male and female flowers. As a result, a maize plant can be reproduced through both self-pollination and cross-pollination. Breeding programs combine desirable traits of two or more strains, or from different sources in so-called breeding pools, from which new inbreeding strains or DH (double-haploid) strains are obtained, which are developed through self-fertilization and subsequent selection of desired phenotypes. A hybrid maize type is a cross between two such inbreeding or DH strains, wherein each of the two parental inbreeding or DH strains carries one or more desirable characteristics that are lacking in the other parental strain, or that can complement the other strain. The new inbreeds or DHs are then crossed with other inbred or DH strains, and the hybrids thereof are examined in order to identify those plants of potential economical and agronomical interest. The hybrid descendants of the first generation (as well as descendants of the first generation in general) are labelled F1. The F1 hybrid is stronger and more robust than its parents. This effect, also referred to as heterosis, can express itself in a variety of ways, such as an increased vegetative growth or an increased yield. Hybrid maize seeds can be generated using a male-sterilization system for manual or mechanical tassel removal. By removing the male tassel, the female

flowers of an inbreeding strain can only be pollinated with pollen from a male inbreeding strain of interest. The resulting seeds are therefore hybrids (F1) and produce hybrid plants. It is, however, frequently difficult to prevent self-pollination in female plants, particularly in field tests. As a result, seeds of a female inbreeding strain are then harvested together with hybrid seeds. As explained above, the seeds of a female inbreeding strain or DH strain are not as economically interesting as the F1 seeds, because no heterosis effect occurs. As a result, there is a high demand in plant breeding for male-sterile plants, which can be produced for the production of hybrid seeds for plants of agronomic interest, e.g. maize or wheat, which can be obtained ideally with low labour and production costs. Mutations that cause male-sterility in maize plants or wheat, for example, were obtained in the prior art through numerous methods, e.g. using X-rays or UV radiation, chemical treatment, or through the insertion of transposable elements (Chaubal et al, 2000 Am. J. Bot. 87: 1193-1201). There is nevertheless still a strong demand for new genes that affect male fertility in a plant of interest, and reliable methods for introducing this gene, or a targeted modification of interest, into the genome of a plant of interest with precision. Exemplary genes that are responsible for male sterility include the aborted microspores (AMS) gene from Arabidopsis, the Arabidopsis MS1 gene, the NEF1 gene, the Arabidopsis AtGPAT1 gene, the Arabidopsis dde2-2-mutation, the Arabidopsis faceless pollen-1 gene (flp1), the Arabidopsis male meiocyte death 1 gene, the tapetum-specific zinc finger gene (TAZ1), the tapetum determinant 1 gene, and the *tapetal development and function* (TDF) gene.

Because the methods disclosed herein are suitable for both stable as well as transient integration of a targeted modification in a target nucleic acid region of a plant target structure comprising at least one meristematic cell, a male-sterile or female-sterile plant or plant material can be obtained directly, for example, because the targeted modification, which can be introduced in accordance with the method of the present invention into a meristematic cell, is passed on directly to the descendants of this cell. Using the technologies disclosed herein *in vivo*, a male-sterile or female-sterile plant, in particular a maize plant, can therefore be obtained without further crossbreeding.

In one embodiment according to the present disclosure, a process is provided that is suitable for selecting or defining a plant, plant material, or a plant cell that comprises the at least one targeted modification in a nucleic acid target region, comprising a genomic target region or an RNA target region, wherein the method comprises the following steps:

a) Obtaining a first plant that comprises at least one CRISPR nuclease, or a variant or catalytically active fragment thereof, in at least one meristematic cell, wherein the CRISPR nuclease is capable of inserting a double-strand or single-strand break in a genomic target region, or RNA nucleic acid target region, of interest;
b) Obtaining a second plant that comprises at least one gRNA, which is capable of forming a complex with the CRISPR nuclease, the variant, or the catalytically active fragment thereof, from step a);
c) Crossbreeding the first plant, from step a), with the second plant, from step b);
d) Checking the descendants from step c), or the cells thereof, for modifications in a target nucleic acid region of interest; and
e) Selection of a descendant plant, a plant material or a plant cell, which comprises the desired targeted modification in at least one target nucleic acid region of interest.

In a further embodiment of this selection process according to the present disclosure, the gRNA and/or the CRISPR nuclease also comprise at least one effector domain, which is associated with, or can be associated with, the gRNA and/or the CRISPR nuclease, and/or, if the gRNA and/or the CRISPR nuclease, as well as the at least one effector domain, are provided on a recombinant construct, a coding sequence for an effector domain. The effector domain can be associated or linked to the gRNA and/or the CRISPR nuclease in a covalent or non-covalent manner.

In another embodiment according to the present disclosure, a process for selecting a plant, plant material, or a plant cell of interest is provided, which comprises a target nucleic acid region that has been modified in a targeted manner, either in its genome or in its transcriptome, i.e. the entirety of all of the transcribed genes or functional RNAs in a cell at a specific point in time, wherein the process comprises the following steps:
(a) Obtaining a first plant that comprises at least one CRISPR nuclease, or a variant or catalytically active fragment thereof, that is capable of causing a double-strand break, single-strand break, and/or specific DNA bind in a target nucleic acid region;
(b) Obtaining a second plant that comprises a gRNA, wherein the gRNA is capable of forming a complex with the CRISPR nuclease, or the variant or catalytically active fragment thereof, wherein the Cas nuclease, the variant, or the catalytically active fragment thereof, as well as the gRNA, are provided directly, or in the form of at least one recombinant construct, and wherein the gRNA and/or the CRISPR nuclease, or the variant or catalytically active fragment

thereof, are associated with or can be associated with at least one effector domain or one coding sequence for an effector domain; and wherein the first plant, from (a), or the second plant, from (b), also comprises a DNA repair template, which comprises at least one donor DNA as a central component thereof, wherein the DNA repair template is introduced directly, through transformation or transfection, or in a recombinant manner, in the form of at least one recombinant construct, into the first or second plant, the plant material, or the plant cell;
(c) Crossbreeding the first plant, from step (a), with the second plant, from step (b) and optionally providing at least one gRNA and/or one DNA repair template, provided this was not stably integrated in the genome of the first and/or second plant;
(d) Assessment of the descendants of the plant from step (c), or the plant cells thereof, with regard to whether a targeted modification can be observed in the at least one target nucleic acid target region of interest;
(e) Selection of a descendant plant, or a plant material or plant cell thereof, which comprises the desired insertion introduced into the at least one target nucleic acid region of interest, wherein the insertion is introduced via the donor DNA as part of the DNA repair template.

The methods disclosed herein are therefore suitable for high precision gene targeting of a transgene of interest, and/or also producing complex transgenic trait loci, because, as explained above, according to the method of the present disclosure, multiple transgenes can also be inserted, either simultaneously or successively, into a plant target structure of interest comprising at least one meristematic cell. A complex transgenic trait locus is a genomic locus that carries numerous transgenes that are genetically linked to one another. By inserting independent transgenes within 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, or up to 5 centimorgans (cM) of one another, the transgenes can be configured as individual genetic loci (see, e.g., US 2013/0263324 A1). A centimorgan indicates the distance between two linked genes, markers, target nucleic acid regions, or loci, or an arbitrary pair thereof, wherein 1% of the meiosis products are recombinant. Therefore, 1 centimorgan is equivalent to a distance corresponding to 1% of the mean recombination frequency between the two linked genes, markers, target nucleic acid regions, loci, or an arbitrary pair thereof. After selecting the plant, plant material, or plant cell of interest, which comprises the transgene of interest, those plants that contain at least one transgene can then be crossbred, in order to produce an F1 plant, plant material, or plant cell, that contains both transgenes. One in five hundred of the descendants of these F1 plants would then comprise the two different transgenes, recombined on the same chromosome. The complex locus can then be used for further breeding as the only genetic locus having both

transgenic traits. The process can be repeated as often as desired in accordance with the method disclosed herein, in order to collect as many traits as possible, or desired, in a complex locus. Subsequently, the chromosomal intervals that correlate with a phenotype or trait of interest can be identified. There are numerous methods available to the person skilled in the art for identifying chromosomal intervals. The boundaries of such chromosomal intervals are drawn such that they comprise markers that are linked to the gene that controls the trait of interest. In other words, the chromosomal intervals are drawn or defined such that each arbitrary marker lying within an interval, including the terminal markers that define the boundaries of the interval, can be used as a marker. In one embodiment, the chromosomal interval can comprise at least one QTL or more than one QTL. A pronounced proximity of multiple QTLs in the same interval can however obscure the correlation of a specific marker with a specific QTL in the diagnostics, because a marker may indicate a link with more than one QTL. Conversely, if two markers that are in close proximity display a segregation from the desired phenotype trait, it may not be clear whether each of these markers identifies the same QTL, or two different QTLs. Furthermore, a plant, plant material, or a plant cell is disclosed, which is or can be obtained in accordance with one of the methods described above, in accordance with the first aspect of the present invention.

Methods for breeding and cultivating microorganisms and viruses which can be used in accordance with the present disclosure as vectors, are known to the person skilled in the art.

In one embodiment, the recombinant construct according to the present disclosure is introduced into the plant target structure with the aid of at least one vector or vector system.

In another embodiment, the recombinant construct according to the present disclosure is introduced into the target cell directly without an additional vector, preferably by mechanical methods, by transfection or by using endocytosis. One embodiment of the present invention also proposes the introduction of at least one recombinant construct into a plant target structure. In the present disclosure, vectors and vector systems encompass those which are selected from the group consisting of SEQ ID NOs:12-15 and 25-38, as well as sequences with at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences which, despite modification, still carry out the same function as the respective unmodified vector or vector system with the

corresponding SEQ ID NO. The specified vectors and vector systems may include codon-optimized or truncated recombinant constructs, or they may contain specific point mutations in order to ensure their activity in different target cells. The sequence of SEQ ID NO: 31 is a hybrid sequence in which the region between the *BcII* and the *BssHII* cleavage sites of the Fescue segment RNA3 of the Brom Mosaic Virus (see NCBI: DQ530425) is replaced with the corresponding section of the R_BMV_RNA3_SI13′A/G (Hema & Kao 2004, Journal of Virology) fragment. Further, in accordance with the present disclosure, an *Agrobacterium spp.* is proposed as a vector and can be used alone or in combination with other introduction means or vectors. In accordance with one embodiment, the aforementioned vectors and vector systems with SEQ ID NOs:12-15 and 25-38 or sequences with the aforementioned sequence homology may be used for this purpose as a framework structure in order to introduce the recombinant constructs comprising at least one gRNA as well as at least one CRISPR nuclease and/or at least one effector domain into a plant target structure. The molecular biological methods and procedures required in this regard are familiar to the person skilled in the art.

Recombinant constructs in accordance with the present disclosure comprise those selected from the group, consisting of SEQ ID nos: 23 and 24 and sequences having at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences which, despite modification, still perform the same function as the corresponding non-modified recombinant construct with the corresponding SEQ ID NO. The specified recombinant constructs may include codon-optimized or truncated sequences, or they may contain specific point mutations in order to ensure or modify their activity or binding capability in different target cells. In SEQ ID NO:23, the positions 16239-16258 correspond to the position for the relevant gRNA of interest which can and must be modified dependent on the target nucleic acid sequence. In SEQ ID NO:24, the positions 16645-16664 correspond to the position for the relevant gRNA of interest which can and must be modified dependent on the target nucleic acid sequence.

In one aspect, the present disclosure relates to methods for producing a plant, a plant material or a plant cell, whereby the recombinant construct is selected from SEQ ID NOs: 23 and 24, and sequences having at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences.

In a further aspect, the present disclosure relates to a plant, a plant material or a plant cell, obtainable or obtained by a method comprising the integration of a recombinant construct in accordance with SEQ ID NOs: 23 and 24, and sequences having at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences, into a plant target structure comprising at least one meristematic cell.

In a further aspect, the present invention concerns the use of at least one recombinant construct in accordance with SEQ ID NOs: 23 and 24, and sequences having at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences for the targeted modification of at least one target nucleic acid region in a plant cell.

All Cas-polypeptide sequences as well as Cas-coding nucleic acid sequences which have been specially optimized for use in a plant cell or for which their coding constructs carry suitable regulatory sequences which can effect adequate transcription and/or translation in a plant cell in the cellular compartment provided therefor, including the cell nucleus, the cytosol, a mitochondrion, a plastid, including a chloroplast, are suitable for use in the present invention. Furthermore, in one embodiment, the respective CRISPR nucleases must have their intrinsic nuclease function. Therefore, a catalytically active fragment derived from a native CRISPR nuclease can also be used as the CRISPR nuclease according to the present disclosure, provided the catalytically active fragment still fulfils the same enzymatic catalytic function as the native enzyme from which it is derived.

Alternatively, according to one aspect of the present disclosure, Cas nickases or catalytically active fragments thereof may be used, i.e. Cas polypeptides which are modified such that they only cleave one DNA strand instead of producing a DNA double-strand break in the manner of a native CRISPR nuclease. This provides the possibility of increased specificity, since two recombinant constructs including a Cas nickase have to be used in order to produce a double-strand break. It also provides the possibility of introducing a targeted offset double-strand break instead of a “blunt” cut.

Finally, in accordance with one aspect of the present disclosure, Cas-zero nucleases or catalytically active fragments thereof, i.e. variants which no longer have any nuclease activity, may also be used. In this regard, it provides the possibility of using the CRISPR nuclease together with a further effector domain in accordance with the present disclosure, e.g. a further DNA-modifying or RNA-modifying or DNA-binding or RNA-binding polypeptide or nucleic acids in accordance with the present invention, broadening the spectrum for introducing targeted modifications into a plant target structure.

The person skilled in the art is aware of the possibility of introducing specific mutations into the catalytic domains of a CRISPR nuclease in order to "reprogram" these into a nickase or an endonuclease-null variant.

Examples of CRISPR nucleases or catalytically active fragments thereof or sequences coding CRISPR nucleases or catalytically active fragments thereof for use in the present disclosure are disclosed in SEQ ID NOs 16-22 and in UniProtKB/Swiss-Prot database access no Q99ZW2.1 (SEQ ID NO: 39) and also comprise those sequences with at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences which, despite modification, still carry out the same function as the respective unmodified sequences with the corresponding SEQ ID NO or which are accessible under the said database accession number.

A further aspect of the present invention uses the mechanism of activity of RNA-controlled DNA modification underlying the CRISPR/Cas system such that an effector domain is directed by a specific adapted gRNA to the desired position of a target nucleic acid region in a target plant structure instead of or together with the CRISPR nuclease, so that the effector domain can be precisely placed in order to carry out the desired nucleic acid edit.

In one embodiment of this aspect, the target nucleic acid region is a genomic DNA.

In a further embodiment of this aspect, the target nucleic acid region is a mitochondrial or plastid DNA, wherein the recombinant construct comprises a localization sequence which

comprises the localization of the recombinant construct in the corresponding target compartment, for example in a mitochondrion or a chloroplast.

In one embodiment, the CRISPR nuclease or catalytically active fragment thereof or the sequence coding for the CRISPR nuclease or catalytically active fragment thereof, and/or the effector domain or the sequence coding for the effector domain additionally comprises a sequence selected from a nuclear localization sequence, a plastid localization sequence, preferably a mitochondrial localization sequence and a chloroplast localization sequence. A nuclear localization process can be selected from SEQ ID NO: 49-58, which disclose the following sequences: Simian virus 40 (SV40) monopartite: MAPKKKRKV; A. *tumefaciens* VirD2 (pTiA6): KRPRDRHDGELGGRKRAR; *A. tumefaciens* VirD2 (pTiC58): KRPREDDDGEPSERKRER; *A. tumefaciens* VirE2 (pTiA6) #1: KLRPEDRYVQTERYGRR; A. *tumefaciens* VirE2 (pTiC58) #1: KLRPEDRYIQTEKYGRR; *A. tumefaciens* VirE2 (pTiA6) #2: KRRYGGETEIKLKSK; *A. tumefaciens VirE2* (pTiC58) #2: KTKYGSDTEIKLKSK;A. *rhizogenes* GALLS (pRi1724): KRKRAAAKEEIDSRKKMARH; *A. rhizogenes* GALLS (pRiA4): KRKRVATKEEIEPHKKMARR;A. *rhizogenes VirD2* (pRiA4): KRPRVEDDGEPSERKRAR.

One or more nuclear localization sequences can be combined with at least one effector domain, which are preferably united on a plasmid vector.

In a further embodiment, the gRNA or the gRNA-coding sequence coding additionally comprises a sequence selected from a nuclear localization sequence, a plastid localization sequence, preferably a mitochondrial localization sequence, and a chloroplast localization sequence.

In a further embodiment of this aspect, the target nucleic acid region is a ribonucleic acid (RNA) in any plant compartment, for example the cytosol. In accordance with this embodiment, an gRNA which has been modified in a targeted manner may be provided which is capable of interacting with a target RNA structure. The gRNA may in addition comprise a further effector domain, for example an aptamer.

The person skilled in the art will be aware that the design of the corresponding at least one gRNA which is used together with at least one CRISPR nuclease and/or with at least one effector domain, is dependent on the specificity and in particular the binding and recognition properties of the CRISPR nuclease and/or the effector domain as well as the target nucleic acid region which is to be specifically modified.

Wild type CRISPR nucleases, in particular of type Cas9, produce a "blunt" double-strand break in the target DNA sequence, i.e. without a single-stranded DNA overhang. Moreover, these nucleases can also leave single nucleotide overhangs behind due to, inter alia, offset cleavage of the two individual strands of a DNA double-strand. This activates the endogenous DNA repair mechanisms of the target cell, comprising the so-called non-homologous end joining, NHEJ. This mechanism is prone to errors, however, in particular since it can cause insertions and deletions (INDELs) to be introduced at the location of the double strand break, leading to mutations at the sites where the individual DNA strands are re-joined. By means of NHEJ, single or multiple gene knock-outs may be mediated, wherein after the targeted DNA break, the DNA strands are brought together with a modified sequence that was obtained in a frameshift or another mutation, which can prevent the functional expression of one or more genes of interest, again by endogenous mechanisms. A further repair mechanism is homology-directed repair (HDR) or homologous recombination (HR). These mechanisms use homologous DNA as a template or matrix, from which the sequence information can be copied in order to repair a DNA break. At least one precise edit, insertion, or gene exchange can take place through the targeted provision of a DNA repair template, which is homologous over a specific length relative to a genomic DNA region in which a DNA break is to be induced inside a target cell of interest. The precise modifications obtained in this manner comprise no undesired or uncontrollable mutations, as is always desirable in any gene editing approach. Both repair mechanisms, NHEJ and HDR/HR, constitute naturally occurring mechanisms for DNA repair which are present in every cell disclosed herein.

In one embodiment according to all of the aspects of the present disclosure, a DNA repair matrix or a repair template, is provided, which repairs a single-strand or double-strand break in a site-oriented and precise manner, which was previously inserted by a CRISPR nuclease, or a variation or catalytically active fragment thereof, and/or an effector domain in a nucleic acid region of interest.

A decisive factor in the site-directed introduction of the modification of a target nucleic acid region is, in accordance with the above mechanism of the type II CRISPR/Cas system, the targeted selection and the targeted design of the gRNA sequences in order to avoid cleavage of off-target regions other than the target region. The identification of suitable PAM motifs depending on the CRISPR/Cas tools used and optional further effector domains and the use of this information for the design of suitable recombinant constructs is familiar to the person skilled in the art.

In accordance with one embodiment of the present disclosure, the genome or the extrachromosomal target nucleic acid region of a cell is thus initially investigated for suitable PAM sequences in order to be able to design a suitable gRNA in a targeted manner.

The term "guide RNA" or "gRNA", as used herein, denotes a single-stranded or double-stranded or partially double-stranded nucleic acid molecule which may consist of natural or synthetic RNA and/or of natural or synthetic DNA and has the function of being capable of building a complex with a CRISPR nuclease or a catalytically active fragment thereof, whereupon the CRISPR nuclease or the catalytically active fragment thereof is rendered capable of recognizing a target nucleic acid region. Furthermore, in addition to the CRISPR nuclease interaction domain, a gRNA functions as a recognition domain for specific hybridization to a complementary target nucleic acid molecule of interest. Therefore, a gRNA comprises a crRNA and optionally, a tracrRNA, as explained above. The gRNA can therefore be a synthetic dual molecule that unites numerous functionalities, or the gRNA can comprise only one functionality. The crRNA and/or the tracRNA can have a length of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more nucleotides. Consequently, gRNAs may form an intrinsic hairpin region by complementary base pairing, whereupon the natural tracrRNA/crRNA hairpin structure is imitated (see Jinek et al, 2012, above) and in addition, depending on the desired target structure, and comprise a suitable recognition domain. If a Cpf1 nuclease is selected as a CRISPR nuclease, the gRNA can consist of a crRNA that does not comprise a structure used by tracrRNA (see Zetsche *et al.* 2015, above). Accordingly, a gRNA according to the present disclosure can comprise one or more domains. The gRNA can also comprise one or more spacer regions, which do not interact with a bonding partner or target molecule, but instead are used for the correct folding and orientation of the gRNA or for the linking of a crRNA and a tracrRNA. This spacer can consist of DNA and/or RNA and can have a length of

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 nucleotides. A gRNA according to the present invention comprises at least one sub-region composed of RNA, wherein the RNA can consist of natural or synthetic nucleotides. If desired, a gRNA of interest, preferably at its 5′ or 3′ ends, may carry a modification, wherein the modification is selected from the addition of a group composed of acridine, amine, biotin, Cascade Blue, cholesterol, Cy3 @, Cy5 @, Cy5.5 @ daboyl, digoxigenin, dinitrophenyl, EDANS, 6-FAM, fluorescein, or derivatives therefrom, 3′-glyceryl, HEX, IRD-700, IRD-800, JOE, phosphate, psoralen, rhodamine, ROX, Thiol (SH), spacers, TAMRA, TET, AMCA-S″, SE, BODIPY<0>, Marina Blue®, Pacific Blue®, Oregon Green®, Rhodamine Green®, Rhodamine Red®, Rhodol Green® and Texas Red®, a locked nucleic acid (LNA), 5-methyl dC, 2,6-Diaminopurin, 2′-Fluor A, 2′-Fluor U, 2′-O-Methyl RNA, one or more phosphorothioate(s) as a spine, a polyethylene-glycol link, or a covalent 5′-3′ link, resulting in the circularization, or combinations thereof. For specific embodiments, it may be of interest to reduce the length of the gRNA in a targeted manner, in order to design gRNAs comprising fewer than 100 nucleotides, fewer than 95 nucleotides, fewer than 90 nucleotides, fewer than 85 nucleotides, fewer than 80 nucleotides, fewer than 75 nucleotides, fewer than 70 nucleotides, fewer than 65 nucleotides, fewer than 60 nucleotides, fewer than 55 nucleotides, fewer than 50 nucleotides, fewer than 45 nucleotides, fewer than 40 nucleotides, fewer than 35 nucleotides, or fewer than 30 nucleotides, in order to obtain a higher specificity of the CRISPR nuclease by reducing the length. In other embodiments, the gRNA according to the present invention can comprise at least one effector domain, e.g. an aptamer, or a DNA-modifying or RNA-modifying molecule, or a binding site for a protein or peptide, in order to thus expand the functionality of the gRNA molecule.

In another embodiment according to the present disclosure, the at least one gRNA can also be associated with at least one nucleic acid molecule, *in vitro* or *in vivo*, for the purpose of specific DNA repair after a double-strand break has been induced by a CRISPR nuclease. This (DNA) repair template, or HDR template, can be inserted, as a single-stranded and/or double-stranded DNA, directly, or in the form of a recombinant construct, into a target structure of interest. The repair template thus facilitates targeted homologous recombination, by means of which the

specificity, as well as the range of application, of the genome editing can be significantly expanded.

In accordance with the present disclosure, gRNAs may be used which are specially adapted for use in a plant cell.

In accordance with the present invention, in addition, any gRNA as described herein may additionally be introduced to at least one effector domain, such as an aptamer, coupled with or together with the effector domain so that the functionality of the gRNA is broadened. The recombinant construct comprising a gRNA and at least one effector domain may be introduced into the plant target structure as a DNA construct or RNA construct using a suitable recombinant construct and/or vector. The effector domain can consist of a nucleic acid, but can also be a polypeptide or a sequence coding for same.

In one embodiment, the gRNA coupled with the CRISPR nuclease or the catalytically active fragment thereof and/or the effector domain, for example the DNA-modifying or RNA-modifying or the DNA-binding or RNA-binding polypeptide or nucleic acid, is introduced into the plant target structure.

In a further embodiment, the gRNA is introduced into the plant target structure as a separate recombinant construct independently of the CRISPR nuclease and/or the effector domain, for example the DNA-modifying or RNA-modifying or the DNA-binding or RNA-binding polypeptide or nucleic acid.

The gRNA may be introduced into the plant target structure as a DNA molecule or RNA molecule. In one embodiment, the gRNAs may be introduced directly in the form of a synthetic nucleic acid, for example as RNA, optionally also in a complex with a CRISPR nuclease or a catalytically active fragment thereof, or in another embodiment, in the form of an activatable and transcribable recombinant DNA construct, into the target cell. Furthermore, in accordance with the present disclosure, an individual gRNA may be used or dual or multiple gRNAs in one or more recombinant construct(s) may be introduced into a cell simultaneously, wherein the gRNAs have the same or individual regulatory sequence(s). Suitable gRNAs for insertion into a target cell can be selected according to the aspect of the present invention explained in

greater detail below, wherein this aspect provides an *in vitro* screening method for identifying a gRNA or a gRNA coding sequence.

Since the interaction domain of a conventional CRISPR/Cas gRNA always interacts with the same CRISPR nuclease, individual gRNAs which carry a different recognition sequence as a further component, may be used in a multiplexing strategy, i.e. the specific modification of several target regions in a single approach. In this regard, it may always be necessary for a PAM sequence to be located adjacent to or within the target region. The design of a suitable gRNA may be determined *in silico* by a person skilled in the art who is aware of the CRISPR nuclease used, the target nucleic acid region, the nature of the desired nucleic acid modification, selected from mutation, insertion or deletion, as well as the desired target cell. The effectiveness of these gRNAs *in vivo* as well as possible off-target effects must, however, be evaluated separately for each gRNA. In addition, for unestablished systems, such as the transient transformation of meristematic plant cells, suitable vectors and methods have to be established for introducing at least one gRNA together with at least one suitable CRISPR nuclease and/or at least one effector domain, for example a DNA-modifying or RNA-modifying or the DNA-binding or RNA-binding polypeptide or nucleic acid, so that the concerted activity of both molecules in the target cell can be ensured. A further difficulty, in addition to the pure designing and synthesis or provision of the gRNA, is the fact that plant genomes are very complex, and to date no reliable method exists for pre-testing that would allow for a conclusion to be drawn regarding whether a selected gRNA, interacting with the desired CRISPR nuclease or the catalytically active fragment thereof, can actually effectively modify a target nucleic acid region in a plant cell. In one embodiment of the present invention, the methods according to the invention and thus the plants, plant materials or cells produced thereby are based on the naturally occurring DNA repair mechanism in the target cell.

In another embodiment according to the aspects of the present disclosure, the repair of a single-strand or double-strand DNA break which was previously mediated by a CRISPR nuclease or a catalytically active fragment thereof and/or a further effector domain, is repaired by one or more HDR templates in the form of a DNA repair template which is/are not naturally present but has/have been introduced into the target cell.

In one embodiment in the context of the present disclosure, a DNA repair template is disclosed which can optionally be introduced into a target cell together with or at a separate time to the

CRISPR constructs and/or a further at least one effector domain, in order to induce a specific HDR mechanism and thereby introduce specific nucleic acid sequences at the site of the double-strand break. In this regard, targeted genome editing comprising both knock-ins and the specific repair of the DNA lesion may be carried out to prevent an unwanted mutation at the site of the DNA break. A knock-in can mean the specific insertion of at least one nucleotide, at least 2 nucleotides, at least 3 nucleotides, at least 4 nucleotides, at least 5 nucleotides, at least 6 nucleotides, at least 7 nucleotides, at least 8 nucleotides, at least 9 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 500 nucleotides or at least 1,000 nucleotides into the target nucleic acid in the plant cell. A knock-in can also mean the introduction of an entire gene expression cassette, which may comprise up to 10,000 nucleotides. Genome editing can also mean the targeted replacement of at least one nucleotide with another nucleotide. A knock-in may also be achieved by implementing two, three, four or more exchanges or a combination of insertions and exchanges. Insertions means the specific insertion of at least one nucleotide, at least 2 nucleotides, at least 3 nucleotides, at least 4 nucleotides, at least 5 nucleotides, at least 6 nucleotides, at least 7 nucleotides, at least 8 nucleotides, at least 9 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 500 nucleotides, at least 1000 nucleotides, at least 2000 nucleotides, at least 5000 nucleotides, at least 10000 nucleotides or at least 15000 nucleotides into the target nucleic acid of the plant cell. A nucleic acid sequence as an insertion may be a sequence, or a part thereof, of a transcription factor binding site, a regulatory sequence, a polypeptide-coding sequence, an intron sequence, a sequence coding for a non-coding RNA (for example IncRNA), an expression cassette, a non-coding sequence, e.g. for use as a marker, in particular a selectable marker, e.g. a marker for marker-assisted selection in the context of cultivation, or an RNAi construct. Furthermore, a knock-in may also be brought about by the deletion of sequence sections which interfere with the function of a gene (for example the deletion of transposon insertions). The DNA repair template may be introduced into the plant target structure of interest as a single-strand or as a double-strand nucleic acid.

In one embodiment, a target nucleic acid in a plant cell is specifically deactivated (knock-out), i.e. the transcription and (if applicable) translation of the nucleic acid is inhibited. This may be carried out by the targeted insertion, mutation or deletion of a regulatory sequence such as a promoter or terminator sequence of a target nucleic acid or by the specific mutation or deletion

of the target nucleic acid itself or parts thereof. Furthermore, a knock-out can be implemented using targeted mutations or deletions which change the reading frame of a target nucleic acid or the targeted mutation or deletion of potential splice signals. In one embodiment, this knock-out is carried out without the insertion of an HDR template via the NHEJ pathway; in another embodiment, in addition to the CRISPR constructs and/or a further effector domain, an HDR template or DNA repair template is introduced into the target cell. Examples of targeted mutations include an exchange of at least one nucleotide for another nucleotide, preferably with the consequence that the codon concerned then codes for a different amino acid. A specific knocked-out target nucleic acid in a plant cell has at least one targeted mutation or deletion, but may also comprise two, three, four or more targeted mutations and/or deletions.

In embodiments according to which a Cas or a Cpf1 gene, or another effector nuclease in the form of DNA, can be introduced on a corresponding construct into a target cell of interest, the nuclease-coding gene can comprise a suitable promoter that is functionally linked to the nuclease-coding sequence in order to improve its transcription. The promoters can be constitutively active, or they can be inducible promoters, which are first activated in response to an appropriate stimulus (chemical or physical, comprising light, temperature, etc.). Likewise, a construct that codes a gRNA can comprise a suitable promoter. Suitable promoters for plant cells, in accordance with the present disclosure, can be selected from a group composed of: a maize-ubi-intron promoter (SEQ ID NO: 7), a maize U3 promoter (SEQ ID NO: 10), a plant U6 polymerase III promoter, e.g. a wheat U6 promoter (SEQ ID NO: 8), a U6 promoter derived from rice (see Mikami et al., Plant Mol. Biol. 2015, 88(6), 561-572), or a U6-26 promoter derived from *Arabidopsis thaliana*, a rice U3 promoter (SEQ ID NO: 9) and a Brachypodium EF1 promoter (SEQ ID NO: 40), or a simple or double 35S promoter derived from the cauliflower mosaic virus, comprising, inter alia, a 35SPPDK promoter (see Yoo et al. Nature Protocols 2, 1565-1572 (2007), but the promoters are not limited thereto, because the promoters are selected based on the respective plant cell of interest.

In another embodiment, the natural NHEJ mechanism of a plant cell can be deliberately suppressed by adding an appropriate inhibitor or by a targeted knock-out or knock-down of an endogenous nucleic acid sequence involved in the NHEJ process. This facilitates the introduction of a targeted modification into the desired nucleic acid sequence as it allows the NHEJ mechanism of a cell to be suppressed. In one embodiment of the present invention, in which the plant target structure is an isolated meristematic cell of a seedling/plant or a plant

embryo or exposed meristematic cells of a plant in a later stage of development, the target plant structures comprising meristematic cells before, during and after introduction of the at least one recombinant construct in accordance with the present disclosure are cultivated in a manner such that an oxidation of the isolated structures is prevented. In one embodiment, this involves the addition of an antioxidant.

Table 1 below contains a list of suitable media for the cultivation of different plant target structures comprising meristematic cells. A person skilled in the art and aware of a method disclosed herein and constructs in accordance with any aspect of the present disclosure can readily identify additional suitable reaction conditions, e.g. buffers, additives, temperature conditions and pH conditions, in addition to any further necessary additives.

Table 1: Medium compositions for culturing various target plant structures with meristematic cells (MS Medium = Murashige Skoog medium; MS Salt = Murashige Skoog salt (Toshio Murashige, Folke Skoog: *A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures.* In: *Physiologia Plantarum*, Jg. 15 (1962), Volume 3, p. 473-497, ISSN 0031-9317, doi:10.1111/j.1399-3054.1962.tb08052.x).

Embryo	Embryo	Embryo Bombardment	Embryo Bombardment	Agro-Embryo	Agro-Embryo	Plant meristem exposure
MM1 (MM=Maturation Medium) - maturation medium 1	MM2 - maturation medium 2	MM1OSM (osmotic agent)	MM1MOD (MOD = modified)	MM1Tim (Tim= Timentin)	MM1ACE	Meristem peeling - antioxidant
MS salts	MS medium	MS salts	MS salts	MS salts	MS salts	MS salts
30.8 g/l saccharose	3.4 g/l saccharose	30.8 g/l saccharose	30.8 g/l saccharose	30.8 g/l saccharose	30.8 g/l saccharose	95 mg/L cysteine
		36.4 g/l sorbitol	95 mg/l L-cysteine	150mg/l Timentin	19.62g/l ACE	100 mg/L ascorbic acid
		36.4 g/l Mannitol	4.25mg/l silver nitrate			
		95 mg/l L-cysteine				
		4.25mg/l L-silver nitrate				

The transient introduction of the construct disclosed herein into meristematic cells or tissue has the advantage that they develop from these reproductive tissues, via which the specific

modification can then be stably passed on to the next generation, while the next generation is free from the constructs which had previously been introduced. The methods and constructs disclosed herein also allow for seeds to be harvested directly from the plant which has been modified in this manner which carry the stable DNA modification without requiring an intermediate step of cell culture in the form of callus production and regeneration. This also removes the need for the selection and regeneration steps and the media and additives required for cell culture.

In one embodiment, the nucleic acid sequence which is used for the targeted modification of a target nucleic acid region comprises at least one or more regulatory sequences.

In one embodiment, the nucleic acid sequence used for specific modification of a target nucleic acid region comprises, as a regulatory sequence, at least one or more promoter(s), optionally a plant-specific and tissue-specific, a phenotypic, a constitutive or inducible promoter, which is suitable for inducing transcription in a desired target cell. A promoter is a nucleic acid region which is involved in the recognition and also binding of RNA polymerases as well as other proteins in order to regulate transcription. Suitable promoters for either the gRNAs or the CRISPR nucleases or the sequence coding the catalytically active fragment thereof are well known to the person skilled in the art. An inducible promoter can be induced by stimuli such as temperature, chemicals, pH, light, endogenous plant signals, for example those which are emitted after the plant is inured, and the like. Exemplary promoters are selected from the group consisting of SEQ ID NOs:5-11, and also include such sequences with at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences which, despite modification, still perform the same function as the corresponding non-modified sequence with the corresponding SEQ ID NO. Further advantageous promoters are selected from the group consisting of promoters of the Wall-Associated Kinases (WAKs) 1 and 2 (see, for example, Wagner TA, Kohorn BD. Wall-Associated Kinases Are Expressed throughout Plant Development and Are Required for Cell Expansion. The Plant Cell. 2001;13(2):303-318 and e.g. NCBI entry: NCBI Reference Sequence: NC_003070.9), a promoter of the SCARECROW1 (*scr*1) gene (see e. g. Tissue Specificity and Evolution of Meristematic WOX3 Function; Rena Shimizu, Jiabing Ji, Eric Kelsey, Kazuhiro Ohtsu, Patrick S. Schnable and Michael J. Scanlon Plant Physiology February 2009 vol. 149 no. 2 841-850 and e.g. NCBI entry: NCBI Reference Sequence:

NC_003070.9), a promoter of the FAF2 and FAF4 genes (see e.g. The FANTASTIC FOUR proteins influence shoot meristem size in Arabidopsis thaliana Vanessa Wahl, Luise H Brand, Ya-Long Guo, Markus Schmid Wahl et al. BMC Plant Biology 2010, 10:285 http://www.biomedcentral.com/1471-2229/10/285 and e.g. NCBI entry: NCBI Reference Sequence: NC_003070.9), a promoter of the OSH1 gene (see e.g. Sato et al. (1996) Proc.Natl. Acad. Sci. USA, 93: 8117-8122 and e.g. GenBank entry: GenBank: CP002688.1 or GenBank: AP008209.2) or a promotor of a metalloprotein gene, e.g. from rice (e.g. GenBank: BAD87835.1). The promoters of the present invention may be naturally occurring, synthetic or chimeric promoters or a combination thereof. A preferred promoter in accordance with the present disclosure is a promoter which is active in a meristematic plant cell or a promoter which is active in plastids of a plant cell. In one embodiment, the nucleic acid sequence which is used for the targeted modification of a target nucleic acid region also comprises at least one terminator as a regulatory sequence.

In one embodiment, the nucleic acid or amino acid sequence which is used for the targeted modification of a target nucleic acid region, comprising a gRNA and a CRISPR nuclease or a catalytically active fragment thereof, or a sequence coding therefor, comprises at least one or more nuclear localization sequence(s) (NLS), which brings about nuclear localization of the nucleic acid and polypeptides used for the targeted modification of a target nucleic acid region.

In one embodiment, the recombinant construct comprising a nucleic acid or amino acid sequence which is used for the targeted modification of a target nucleic acid region, a gRNA and a CRISPR nuclease or a catalytically active fragment thereof or a sequence coding therefor, comprises one or more plastid localization sequence(s) (PLS), for example a mitochondrial or chloroplast localization sequence (MLS or CLS), which brings about the localization of the nucleic acids and polypeptides used for the targeted modification of a target nucleic acid region in the corresponding plant plastids.

In one embodiment, the nucleic acid sequence which codes for a CRISPR nuclease or a catalytically active fragment thereof disclosed herein, or a CRISPR nuclease or a catalytically active fragment thereof disclosed herein, also contains a tag sequence. A tag sequence is part of a nucleic acid or protein which may be located upstream and/or downstream and/or within the sequence with respect to the CRISPR nuclease or the gRNA or the nucleic acid sequence coding for the CRISPR nuclease or the gRNA, to, inter alia, optionally facilitate its localization

and visualization within a target cell. Particular preference is given to tag sequences from the following list: polyhistidine(His)-tag, glutathione-S-transferase (GST) tag, thioredoxin tag, FLAG tag, a tag with fluorescent properties, selected from a green fluorescing protein (GFP) tag, a DsRed tag, an mCherry tag and the like, a streptavidin or strep tag, maltose binder protein (MBP) tag, chloroplast transit peptide, mitochondrial transit peptide, a snap tag and/or a secretion tag.

In another embodiment, fusion constructs are proposed that may be used in the method according to the present invention. These fusion constructs comprise fusion proteins as well as fusion nucleic acids. Fusion proteins can be composed of a CRISPR nuclease, a variant or catalytically active fragment thereof, or the sequence that codes the CRISPR nuclease or the variant or catalytically active fragment thereof, as an element, as well as, optionally, one of the aforementioned tags and an effector domain, or an effector domain-coding nucleic acid sequence. As a result, it is possible to introduce the effector domain disclosed herein and/or one or more identical or different CRISPR nucleases, or variants or catalytically active fragments thereof, as a physically linked unit into a plant target structure of interest, or to express them in a plant target structure of interest. Preferably, the effector domain, optionally comprising a left-handed amino acid sequence, is fused to the N-terminus or C-terminus of the CRISPR nuclease, or the variant or catalytically active fragment thereof. The optionally present left-handed amino acid sequence, or the nucleic acid sequence that codes this left-handed sequence, allows both the CRISPR nuclease as well as the effector domain to be positioned ideally, without affecting one another sterically, such that both the effector domain as well as the CRISPR nuclease can deploy their activities. A left-handed amino acid sequence can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more, up to 50 or 100 amino acids, and in some cases, it can be even longer. Moreover, fusion nucleic acids are also disclosed, wherein a gRNA of interest is linked in a covalent manner to an effector domain of interest. Moreover, non-covalent fusion nucleic acids are also disclosed, which comprise a gRNA as well as an effector domain and/or DNA repair template, wherein the non-covalent linking can take place in an association of the respective components through hybridization, i.e. through base pairing of complementary regions. Moreover, fusion constructs are disclosed, in which gRNA and/or a CRISPR nuclease of interest are chemically linked, *in vitro*/ex *vivo*, to an effector domain of interest, and subsequently introduced into a plant target structure of interest comprising at least one meristematic cell, whereby these effector molecules are already linked, thus increasing the availability in a plant cell of interest, by means of which the

efficiency of the method disclosed herein can be increased. Moreover, the association of a gRNA with an effector molecule and/or a DNA repair template can lead to not only the effector domain and/or the DNA repair template fulfilling their function in the context of genome editing, but also an increase in the stability of a gRNA. This can significantly increase the breakdown of a gRNA by cellular RNAs, before it can exercise its function, particularly in embodiments in which the gRNA is inserted directly into a plant target structure of interest comprising at least one meristematic target cell through transformation or transfection.

As with the gRNA and/or the CRISPR nuclease, comprising, inter alia, a Cas nuclease or a Cpf1 nuclease, the fusion constructs described above, comprising fusion proteins and fusion nucleic acids, and/or mixed fusion proteins, comprising nucleic acids and proteins, can be introduced into a plant target structure of interest in a stable or transient manner, as recombinant constructs, or at least one of the molecules can be directly introduced, as RNA, DNA, or protein, into a plant target structure of interest, comprising at least one meristematic cell. By way of example, a gene that codes for a CRISPR nuclease of interest can first be codon-optimized. This gene can then be introduced, in a stable or transient manner, in the form of a recombinant construct, into a plant target structure of interest. Alternatively, the CRISPR nuclease can also be translated *in vitro* and subsequently introduced directly into a plant target structure of interest as a protein. In one embodiment, a gRNA can then be inserted directly into a plant target structure of interest as RNA. This can be implemented, as described in greater detail below, through particle bombardment or other transfection processes that are familiar to the person skilled in the art. Likewise, the function constructs described above are consequently inserted as either a recombinant construct or inserted directly into a target nucleic acid region of interest.

In one embodiment according to the methods of the present invention, a transient expression of the CRISPR system disclosed herein, comprising at least one gRNA, one CRISPR nuclease, preferably also at least one effector domain, and optionally one DNA repair template, can be introduced in a transient manner, through particle bombardment, into a plant target structure of interest comprising at least one meristematic cell. This can involve, gold or tungsten particles being coated with polyethylenimine (PEI). For this, the gold particles are first washed, and re-suspended in ethanol after centrifuging and optional washing, and stored at −20° C. In order to coat the particles with PEI (Sigma #P3143), the washed mixture of gold particles is centrifuged in ethanol, and the ethanol is discarded. The particles are then washed once in dd$H2_2O$ in order

to remove alcohol residue, and then added to 250 µl of a 0.25 mM PEI solution, followed by a pulsed ultrasonic treatment, in order to suspend the particles. The sealed containers are then snap-frozen in a dry ice/ethanol mixture, and the suspension is then lyophilised overnight. At this point, the dried, coated gold particles can be stored for at least three weeks at 80° C. Prior to further use, the particles are rinsed three times, in each case with 250 µl of 2.5 mM HEPES buffer, pH 7.1, followed by a pulsed ultrasonic treatment, and then briefly vortexed, before being centrifuged. The particles are then suspended in a final volume of 250 µl HEPES buffer. A 25 µl aliquot of particles is transferred into a clean reaction vessel, before the DNA binding takes place. In order to bind uncoated DNA to the gold particles, the particles are subjected to a pulsed ultrasonic treatment, before one microgram of DNA (in 5 µl nuclease-free water) is added and the mixture is carefully pipetted a few times before incubation for 10 minutes at room temperature. The particles are centrifuged briefly, normally for 10 seconds, the supernatant is removed, and 60 µl fresh ethanol is added. The particles with PEI-precipitated DNA are washed twice in 60 µl ethanol. The particles are then centrifuged and the supernatant is discarded, after which the particles are re-suspended in 45 µl water. To bind a second DNA (DNA-2) thereto, a precipitation is used with a water-soluble cationic lipid transfection reagent. 45 µl of the particle-DNA suspension, corresponding to the gold particles to which the first DNA was binded, are briefly subjected to an ultrasonic treatment, and 5 µl of a 10 nanogram/microliter DNA-2, and 2.5 µl of the water-soluble cationic lipid transfection reagent are then added. The solution is incubated on an orbital shaker for 10 minutes, and subsequently centrifuged at 10,000 g for one minute. Subsequently, the supernatant is removed, and the particles are re-suspended in 60 µl ethanol. The solution can then be transferred to macro-carriers, and the gold particles, to which the first and second DNA were sequentially binded, are then transfected into a meristematic cell of interest, using a standard protocol for particle bombardment with a PDS-1000 apparatus. Standard protocols for a PDS-1000 apparatus can be obtained from the manufacturer (Bio-Rad).

In one embodiment in accordance with the present disclosure, the method for the production of a plant, a plant material or a plant cell also comprises a screening step. In this step, by carrying out a process for the analysis of the nucleic acid sequence of a target region, for example by means of a polymerase chain reaction or probes, a check is performed to determine whether the insertion, activation and subsequent reaction of the at least one recombinant construct according to the present disclosure has resulted in the desired targeted modification of a target nucleic acid region. Procedures for carrying out this screening are known to the

person skilled in the art in respect of any and all plant target structures and also target nucleic acid regions. However, there are currently no standard procedures that would facilitate checking the effective interaction of a gRNA, a CRISPR nuclease, or a catalytically active fragment thereof, and a target nucleic acid region of interest in terms of the actual efficacy of a gRNA of interest for a specific target nucleic acid region, in particular a target nucleic acid region within a plant cell, in an *in vitro* screening process, in order to thereby monitor the time expenditures and costs related to the use of CRISPR/Cas constructs, particularly in a high throughput process. In addition, most of the available *in silico* tools (see www.dna20.com/eCommerce/cas9/input, for example), are specialised for *E. Coli*, yeast, or animal genomes or model plants, but not for important monocotyledons like dicotyledonous crop plants, which frequently differ significantly from model plants, specifically with regard to the PAM distribution in genomic regions.

In one aspect of the present invention, an *in vitro* screening method is therefore provided for identifying a gRNA or a gRNA coding sequence in an *in vitro* assay for identifying a gRNA or a gRNA coding sequence, that, together with a CRISPR nuclease or a catalytically active fragment thereof, is suitable for the targeted modification of a target nucleic acid region in a plant cell, comprising the following steps: (i) provision of one or more target nucleic acid region(s) of a plant, plant material, or a plant cell; (ii) insertion of the one or more target nucleic acid region(s) in at least one vector; (iii) provision of at least one gRNA; (iv) provision of at least one CRISPR nuclease or a catalytically active fragment thereof; (v) bringing the at least one CRISPR nuclease or a catalytically active fragment thereof in contact with the at least one vector *in vitro*, under suitable reaction conditions, which allows the interaction of a gRNA with a CRISPR nuclease and thereby the catalytic activity of the CRISPR nuclease or the catalytically active fragment thereof, wherein the at least one vector is brought into contact, in each case, with exactly one gRNA and exactly one CRISPR nuclease or a catalytically active fragment thereof, in a separate reaction preparation; (vi) analysis of the reaction products from step (v); and (vii) identification of a gRNA or a gRNA coding sequence which is capable, together with a specific CRISPR nuclease or a catalytically active fragment thereof, of the targeted modification of a target nucleic acid region in a plant cell.

According to this aspect of the present invention, the term *in vitro* is to be understood such that the at least one target nucleic acid region is not in its natural environment, i.e. a plant cell, but instead is first transferred into a suitable vector for the purpose of the *in vitro* screenings.

Numerous results can then be generated from this pre-screening within a short time, which indicate the suitability of at least one gRNA, in interaction with the corresponding CRISPR nuclease or catalytically active fragment thereof, for the targeted modification of a target nucleic acid region in an intact plant cell. The candidates that have been established in this manner can then be used with a significantly higher success rate, both *in vitro* as well as *in vivo*, comprising *in planta*.

In one embodiment, the PCR amplifier of the target nucleic acid region is derived in accordance with this aspect from genomic DNA, wherein the genomic DNA comprises, in addition to the nuclear genome, the genomes of plastids, such as mitochondria and chloroplasts. In another embodiment, the PCR amplifier of the target nucleic acid region is derived in accordance with this aspect from plant RNA.

The at least one vector according to this aspect of the present invention is preferably a plasmid vector, although any of the other vectors disclosed herein that are suitable for the cloning and stable preservation of a PCR amplifier of a target nucleic acid region of interest can also be used. The cloning of one or more target nucleic acid region(s) in at least one vector is familiar to the person skilled in the art. The vector can ideally comprise more than one target region of interest, such that multiple target genes of interest can be analysed. Alternatively, multiple vectors that comprise at least one nucleic acid target region of interest could also be provided.

The gRNA for use according to this aspect must be applied in an active ribonucleic acid form, i.e. the gRNA can be created synthetically, and optionally also modified. Alternatively, the gRNA can also be produced in a recombinant manner, i.e. through *in vitro* or *in vivo* transcription, and optionally through a purification step.

The CRISPR nuclease or the catalytically active fragment thereof is provided as an amino acid sequence. A commercially available CRISPR nuclease or a variant thereof can be used for this purpose. Alternatively, in another embodiment, the CRISPR nuclease or the active fragment thereof can be produced in a recombinant manner, and optionally isolated and/or purified, before it is used in the *in vitro* screening process according to the present disclosure.

In another embodiment, the CRISPR nuclease or the active fragment thereof that has been provided is coupled to at least one effector domain. The corresponding earlier statements in

this disclosure apply accordingly as regards possible effector domains and their potential advantages and fields of application, Including the effector domains/Cas constructs in an *in vitro* screening process provides the advantage that possible undesired positive or synergistic effects of the respective effector domains, which have a steric as well as chemical/physical effect on the Cas or Cpf1 constructs, can already be analysed in the pre-testing phase. In particular, this relates to one possible effect of the at least one effector domain on the gRNA-Cas interaction, as well as the subsequent binding to and modification of the target nucleic acid region of interest, in addition, or alternatively, to the actual field of application for the respective effector domain.

The at least one gRNA and the at least one CRISPR nuclease, or the catalytically active fragment thereof, is brought into contact with the at least one vector *in vitro* under suitable reaction conditions. Suitable reaction conditions in this context are to be understood as those that allow both the binding of the gRNA to the respective CRISPR nuclease or the catalytically active fragment thereof, as well as the interaction of the gRNA/Cas complex with the target region of interest and the catalytic activity of the CRISPR nuclease, or catalytically active fragment thereof. A person skilled in the art and aware of a method and constructs disclosed herein in accordance with any aspect of the present disclosure can readily identify additional suitable reaction conditions, e.g. buffers, additives, special cofactors that are needed, temperature conditions and pH conditions, in addition to any further necessary additives.

According to one embodiment, the reaction products are analysed in a qualitative manner in accordance with the *in vitro* screening process. According to another embodiment, the reaction products are analysed in a quantitative manner in accordance with the *in vitro* screening process. According to a further embodiment, the reaction products are analysed in both a qualitative as well as quantitative manner in accordance with the *in vitro* screening process.

In one embodiment, the *in vitro* screening process is a high output process, i.e. numerous gRNAs and/or numerous CRISPR nucleases or catalytically active fragments thereof, and/or numerous target nucleic acid regions on one or more vectors in separate reaction vessels can be tested simultaneously. This upscaling is of particular advantage for quickly acquiring a variety of data regarding suitable gRNA/Cas candidate pairs for the respective at least one target nucleic acid region of interest. Alternatively, the question of which gRNA/Cas candidate pairs interact particularly efficiently can be analysed as a variable, particularly when

investigating the use of new CRISPR nucleases or catalytically active fragments thereof. It is also possible to investigate whether the addition of an effector domain to a CRISPR nuclease or catalytically active fragment thereof has an effect on the gRNA/Cas interaction, or the subsequent catalytic activity of the CRISPR nuclease or catalytically active fragment thereof, as part of a high output process.

In accordance with the present disclosure, the vectors and/or recombinant constructs may be used for the targeted modification of a target nucleic acid region in a plant cell by mechanical methods, including particle bombardment, microinjection and electroporation, or by induced endocytosis, suitable vectors, direct transfection and the like. In one embodiment of the present disclosure, the vectors and/or the recombinant constructs are introduced into the target cell or target plant structure by particle bombardment. To this end, the vectors are initially precipitated, e.g. onto gold or tungsten particles, and the target cell/target plant structure is then bombarded with the particles obtained thereby or with further processed particles using suitable equipment.

In a further embodiment of the present disclosure, the vectors and/or recombinant constructs are introduced directly or indirectly into the target cell or target plant structure by microinjection.

In another embodiment of the present disclosure, the vectors and/or recombinant constructs are introduced by spraying with subsequent take-up, for example during a viral infection, or infiltration into the target cell or target plant structure.

In accordance with one embodiment, the vectors and/or recombinant constructs are introduced into a meristematic cell by particle bombardment. This method is suitable both for introducing recombinant constructs comprising double-stranded plasmid DNA, linear single-stranded or double-stranded DNA, single-stranded or double-stranded RNA and polypeptides, as well as combinations thereof in all types of plant meristems in different stages of the development of a plant. Gold and tungsten, inter alia, can be used as the carrier material for the recombinant constructs. In a further embodiment of the present disclosure, the vectors and/or the recombinant constructs are introduced directly into the target cell or the desired compartment of a target cell by microinjection.

In accordance with this further embodiment, the vectors and/or recombinant constructs are introduced into a meristematic cell by microinjection. This type of introduction is suitable for all types of meristems (see Example 2 below). In addition, introduction in accordance with this embodiment is suitable both for introducing recombinant constructs comprising double-stranded plasmid DNA, linear single-stranded or double-stranded DNA, single-stranded or double-stranded RNA and polypeptides, as well as combinations thereof.

In a further embodiment of the present disclosure, the vectors are introduced by means of electroporation using high voltage pulses.

In a further embodiment of the present disclosure, the vectors are introduced by endocytosis, i.e. an endogenous mechanism by means of which exogenous material can be taken up into the cell.

In one embodiment, the vector is a viral vector which comprises the at least one recombinant construct. Introduction using a viral vector facilitates the propagation of the vector and the at least one recombinant construct it comprises. Suitable viral vectors which may be used or modified for application in accordance with the present disclosure are selected from the group comprising but not limited to SEQ ID NOs: 12-15 and 25-38 and also include sequences with at least 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence homology relative to these sequences. The person skilled in the art is aware that the sequence of a naturally occurring virus to be used as a viral vector must be adapted to the desired recombinant construct to be introduced, as well as the target plant structure of interest. In a further embodiment, the recombinant construct is introduced by *Agrobacterium spp.*-mediated transformation, in particular *Agrobacterium tumefaciens*- or *Agrobacterium rhizogenes*-mediated transformation, into a target plant structure. This type of introduction is well known to the person skilled in the art for various target plants as well as various target plant structures thereof.

The person skilled in the art is aware that the choice of introduction type can depend on the target plant cell as well as on the construct to be introduced, among other factors; as a result, different introduction types may be necessary depending on the target cell.

In a further embodiment of the present disclosure, the vectors and/or recombinant constructs are introduced by means of a combination of the aforementioned introduction methods. By way of example, a viral vector which contains the at least one recombinant construct of interest may be introduced into the target plant structure by means of *Agrobacterium tumefaciens* as the additional vector, or indeed by particle bombardment or microinjection.

In one aspect of the present disclosure, special methods for introducing vectors and/or recombinant constructs in accordance with the present disclosure into meristematic plant cells and tissues are disclosed. The accessibility of cells and tissue plays a decisive role in the transformation or transfection of meristematic cells and tissue. The accessibility of the various plant meristem types in the various stages of development in a plant varies significantly.

In one embodiment in accordance with the present disclosure, a method is disclosed for providing a target plant structure, comprising at least one meristematic cell, wherein at least one recombinant construct may be introduced into the target plant structure in accordance with the present disclosure. The method of this embodiment comprises, as a vital step, ensuring that a meristematic structure of interest which does not yet comprise differentiated meristematic cells is rendered accessible. If the target plant structure is a meristem in embryo, then it is essential to select a plant embryo of the right size and to direct the at least one recombinant construct in accordance with the present disclosure towards the deeper, i.e. inwardmost-lying meristematic cells as a target for transformation or transfection therewith, since the meristematic cells in the outer layers may already have reached a certain degree of differentiation and thus are no longer in accordance with the present invention. Preferably, the plant embryos are selected for their size such that they are provided with an exposed meristem. For maize embryos, this means that embryos of less than a 1.5 mm maximum diameter, preferably less than 1 mm as a maximum diameter, particularly preferably less than 0.7 mm as a maximum diameter and most preferably less than 0.5 mm as a maximum diameter may advantageously be used in accordance with the invention. A meristematic cell in the context of the present invention is thus a meristematic cell, the degree of differentiation of which still allows it to produce from the cells, after specific modification of a nucleic acid region of interest, all desired types of plant cells, in particular those types from which a fertile plant can be regenerated either directly or indirectly.

In a further embodiment, a method for providing a target plant structure comprising at least one meristematic cell is disclosed, into which at least one recombinant construct in accordance with the present disclosure may be introduced, wherein the meristematic cell is a cell of a seedling or an older plant.

In accordance with this embodiment, the meristem must be completely or almost completely exposed. In addition, care must be taken that the deeper lying, i.e. innermost-lying meristematic cells are targeted for a transformation or transfection with at least one recombinant construct in accordance with the present disclosure, since the meristematic cells in the outer layers may already have reached a certain degree of differentiation and thus are no longer suitable for use in the present invention. In accordance with this embodiment, the exposed meristems undergo an oxidation. In order to avoid damage to the meristematic cells, then, preferably, suitable antioxidizing protective measures are employed such as, for example, the use of an anti-oxidation agent or further protective measures to ensure further development of the target plant structure comprising at least one meristematic cell.

Sequence Listing—Free Text

The following details provide the translation into German of the details provided in the sequence listing as free text (numerical identification <223>) for the corresponding sequence identification number. All sequences contain under the numerical identification <213> the indication of an 'artificial sequence'.

SEQ ID NO:1
<223> VP16 activator-comprising sequence
SEQ ID NO:2
<223> VP16 activator
SEQ ID NO:3
<223> VP64 activator with glycine serine spacers
SEQ ID NO:23
<223> Vector1_TaU6
SEQ ID NO:24
<223> Vector1_ZmU3
SEQ ID NOs: 41 to 48

Protospacer region guide RNA 14, protospacer region guide RNA 16, protospacer region guide RNA 37, protospacer region guide RNA 38, protospacer region guide RNA 39, protospacer region guide RNA 43, protospacer region guide RNA 18 or protospacer region guide RNA 52.

Examples

The present invention will now be explained in more detail with the aid of the following nonlimiting examples.

Example 1: Production of CRISPR/Cas Constructs

The constructs were produced on the basis of Mali et al, 2013. The promoters were used against specific plant promoters and adapted to the gRNA of the respective target genes.

Constructs for monocotyledonous plants:

The promoters used were, inter alia, the maize-Ubi-Intron promoter (SEQ ID NO:7), the maize U3 promoter (SEQ ID NO:10), the wheat U6 promoter (SEQ ID NO:8), the rice U3 promoter (SEQ ID NO: 9) and a Brachypodium EF1 promoter (SEQ ID NO: 40). An exemplary construct had SEQ ID NO: 23 (vector1_TaU6 standard).

Constructs for dicotyledonous plants (not inventive)

Here, a parsley-Ubi4 (SEQ ID NO:5) and an Arabidopsis U6 promoter (SEQ ID NO:6) were used. An exemplary construct had SEQ ID NO:24 (Vector 1_ZmU3 standard).

The various gRNAs were prepared specifically for the respective target genes and cloned into the corresponding position in the vectors mentioned above. The position of the gRNA sequence corresponded to the nucleotides denoted "n" in SEQ ID NOs: 23 and 24.

In order to reduce the number of gRNAs that must be introduced into the plant for genome editing, an *in vitro* assay was established for testing the candidate gRNAs so that only the most suitable candidates are inserted in the plants.

For this, potentially suitable gRNAs were first defined by *in silico* analysis. As explained in the introductory part of the description, this definition depends on the target nucleic acid region of interest due to the dual function of the gRNA, particularly a PAM motif, as well as the desired CRISPR nuclease or the catalytically active fragment thereof, which are to be used.

In order to test the different gRNAs for different genes, in a first step, the target nucleic acid region of interest, or sub-regions thereof, are amplified by means of PCR and cloned in standard vectors. "Standard vectors" in this sense are commercially available vectors or vector systems, which can be readily adapted to the requirements of the desired assay through means known to the person skilled in the art, in particular in that they function as a backbone for the cloning of nucleic acids of interest. Exemplary vectors can be selected from: pJet (Thermo Fisher Scientific, Inc., USA), pGEM-T (Promega BioSciences, LLC, USA) or pBluescript (Addgene, USA). These vectors serve as a substrate in the newly developed *in vitro* assay. In a second step, the various gRNAs were produced by means of *in vitro* transcription (Invitrogen MAXIscript T7 Kit; Cat. no. AM1312M).

The gRNAs were subsequently tested in an *in vitro* assay, and the potentially best candidates were selected and used for the further *in planta* tests. For this, a maize plant A188 *hmg13* gene, inter alia, was used as an exemplary target nucleic acid region (HMG transcription factor 13; see gene GRMZM2G066528 from EnsemblePlants or the maize genome data base). These were amplified by means of PCR, and cloned in the multiple cloning site from pJET1.2 accordingly, firstly the part comprising Exon 3-5 (hmg-fw4 and hmg-re2, see FIG. 14), and secondly the hmg-3′part (hmg-fw3 and hmg-re1, see FIG. 15). After this, the plasmids were linearised through digestion with PvuI, and the vector backbone was dephosphorylated in order to prevent recirculation, The resulting product was applied to a preparative gel. The product was then extracted and purified after the gel run. Subsequently, the concentration of the resulting, linearised vectors was measured. For a typical assay, approx. 3 µl of a 30 nM vector were used as a substrate for Cas digestion, carried out in at least triplicate in each case. The gRNA variants that were to be tested were cloned into the vector pEn-chimera (see, by way of example, FIG. 16 regarding gRNA14). The cloning into this vector was carried out in accordance with standard methods in molecular biology, as shall be explained below by way of example. The sequence from pEn-chimera was located in SEQ ID NO: 59. An RNA chimera was located thereon, which could be specified relatively easily via BbsI+Oligo. Subsequently, it can be transferred into the pDe-CAS9 via a gateway LR reaction. The RNA chimera is controlled by the promoter AtU6-26. The resulting vector was then digested with NcoI and *Xba*I, wherein the resulting fragment comprises the gRNA of interest. The desired fragment comprising the gRNA was gel-separated, extracted and cleaned by typical methods. For each assays, approx. 1 µg of the resulting fragment was used as a template for the *in vitro*

transcription (Invitrogen MAXIscript T7 Kit; Cat. no. AM1312M). An exemplary preparation comprises: 10 µl template (1 µg); 2 µl 10× transcription factor (Invitrogen); 1 µl 10 mM ATP; 1 µl 10 mM CTP; 1 µl 10 mM GTP; 1 µl 10 mM UTP; 2 µl T7-RNA polymerase (Invitrogen); 4 µl H_2O. The preparation was normally incubated for 2 hours at 37° C. 1 µl TURBO DNAse was added and the preparation was incubated for a further 15 minutes at 37° C. H_2O was added to obtain a volume of 100 µl, and the RNA obtained was purified according to the manufacturer's instructions (Qiagen RNeasy Kit). Following elution (two times with 50 µl H_2O), the exact volume and the concentration of the obtained RNA was determined. For the further assays, approx. 15 ng/µl of the *in vitro* transcription of a gRNA was used, so that with an RNA that is 140 nucleotides long, 300 nM were needed. Subsequently, an *in vitro* digestion was carried out as follows: the reaction preparation typically had a volume of 30 µl. In order to ensure an optimal cleavage efficiency, it is important to maintain a molar ratio of Cas9 and gRNA to the respective target nucleic acid region of 10:10:1 or higher. First, 300 nM of the respective gRNA to be tested was provided. 30 nM of substrate DNA, comprising in each case a single target nucleic acid region, was also provided. The reaction preparation was combined in the following order:

Component	30 µl preparation
Nuclease-free water	20 µl
10X Cas9 nuclease reaction buffer (NEB)	3 µl
300nM gRNA (~15 ng/µl)	3 µl (30nM final)
1 µM Cas9 nuclease	1 µl (~30nM final)
Reaction volume	27 µl
Pre-incubation for 10 min, at 37° C 30 nM Substrate DNA	3 µl (3nM final)
Total reaction volume	30 µl

This was then carefully mixed and briefly spun before the preparation was incubated for another hour at 37° C. Optionally, a treatment with proteinase K was performed by adding 1 µl enzyme and incubating for 15-30 minutes at 37° C. At this point, the fragment analysis could be performed (see Figure 8).

The results for 10 selected gRNAs, here, by way of example, in interaction with a Cas9 nuclease, are listed in Figure 8. It is clear from these results that there are qualitative as well as

quantitative differences in the efficiencies of the respective gRNAs and partner CRISPR nucleases regarding the cleavage efficiency of a target nucleic acid region of interest.

Further experiments (data not shown) were carried out with CRISPR nucleases from sources other than S. *pyogenes*, and with Cas nucleases that carry at least one point mutation, e.g.: a Cas nickase, in order to test *in vitro* the efficiency of these other Cas nucleases on a plant target nucleic acid region of interest in interaction with the respective gRNA. Moreover, initial successful *in vitro* experiments were carried out, which showed that Cas nuclease - gRNA pairs of interest that have been identified in a pre-screening step are also suitable for the targeted modification of an RNA as well as plant mitochondrial or plastid DNA, as is proven by the broad application spectrum of the present assay.

In order to test the broad applicability of the method for further important crop plants, the aforementioned novel *in vitro* screening process was likewise carried out using target nucleic acid regions derived from other crop plants, such as *Beta vulgaris*, *Brassica napus* and *Sorghum bicolor*.

Due to the specific features of the plant genome, it was necessary to carry out new *in silico* analyses in order to be able to define suitable target regions and thus suitable gRNAs. In addition, during the development of the process, other Cas nucleases, Cas nickases, Cpf endonucleases and enzymatically active fragments derived therefrom were employed, which additionally carry an effector domain. Furthermore, alternative Cas proteins, or Cas proteins with point mutations, for example, could also be used in the assay in order to test the efficiencies of the different Cas proteins, in particular also in direct interaction with the tested gRNAs.

This was done with the purpose of resolving a number of questions: (1) which gRNAs have a particularly high level of activity?; (2) what are the effects of the modifications in the gRNA, such as different lengths of protospacers or mismatches?; (3) which Cas proteins interact best with which gRNAs?; (4) which CRISPR nucleases, i.e. Cas nucleases or Cpf1 nuclease, or which mutations in a CRISPR nuclease, have an effect on the enzymatic effects of the enzyme?; and (5) does the coupling of an effector domain, and thus the creation of a sterically more demanding Cas construct, affect the interaction with the gRNA in question and thus the efficiency of the targeted modification of a target nucleic acid region of interest? In the course

of this further series of experiments, it has so far been determined that the reduction of a CRISPR nuclease to a catalytically active minimum fragment thereof is particularly advantageous in terms of the targeted cleavage efficiency. Moreover, it was discovered that it is possible for effector domains to bind to the CRISPR nuclease or the gRNA. Particularly in this case, the *in vitro* screening was indispensable, because the efficiency of these modified CRISPR nucleases or gRNAs was lower due to the larger steric strain caused by the effector domain, resulting in the interaction of the Cas and gRNA being more difficult for the pairs that were tested. Nevertheless, effective Cas-gRNA effector domain pairs were still identified.

Surprisingly, it was discovered that the results of the *in vitro* pre-testing, i.e. the screening stage, also correlate with their efficacy in subsequent tests.

In a further test, all of the gRNAs shown in Figure 8 were tested with regard to their efficiency in the actual, site-oriented modification in a plant meristem. As a result, the *in vitro* assay proved to be ideal for assessing the efficiency of the gRNA that was used, because not all of the gRNAs that were used resulted in an *in vivo* or *in vitro* cleavage in the template. Particularly for the Cas-gRNA pairs which have proven to be particularly efficient in *in vitro* screening assays, this efficiency was also confirmed in the subsequent testes in which plant material was employed, either *in vitro* or *in vivo*. A maize plant was used as the starting plant for these subsequent studies, and the target gene hmg13 was used as the specific target.

Example 2: Introduction of CRISPR/Cas Constructs

The constructs described above in Example 1 were introduced into the meristems using various methods. The prerequisite for doing so this was accessibility of the meristems; the material used determined the methods employed (see Example 4).

The following methods were employed:

- Particle bombardment:

Particle bombardment can be used for all of the meristems employed. Bombardment was carried out with dsplasmid DNA, linear dsDNA, RNA and protein as well as virus particles. Examples of carrier material include gold and tungsten. Test bombardments of embryo meristems (Fig. 5) and tassel meristems (Fig. 7) were carried out. With the aid of the red fluorescing protein, it was shown that it was possible to introduce DNA into these cells by means of particle bombardment. It is important to use suitable bombardment settings,

depending on the materials in question. Thus, a higher level of bombardment may lead to an increased level of transient transfection (see FIG. 9 for images in this regard), but, may also cause severe damage to the embryos, making germination and development impossible. Therefore, certain preliminary work was necessary, depending on the plant material of interest which served as the target structure, in order to adapt the suitable conditions of the particle bombardment to the respective requirements of the experiment.

Establishing suitable bombardment methods for the plant material used, as well as the desired effect (transient versus stable introduction) while minimising damage to the plant tissue and the destruction of the construct to be inserted, was therefore indispensable.

- Microinjection:

Microinjection can be carried out for all meristems, preferably using a microscope with a micromanipulator. Because of the size of certain meristem structures such as prepared tassel and ear meristems, the microinjection could also be carried out with microscopic monitoring. The injection can be carried out using various methods and, as discussed above for particle bombardment (Example 1), with different molecules. This can involve dsplasmid DNA, linear dsDNA, RNA and proteins in liquid solution being injected into the meristematic cells through a micro-cannula or nano-cannula, or applying dsplasmid DNA, linear dsDNA, RNA and protein, including virus particles, to micro needles or nano needles and transferring them to the meristematic cells by piercing with the needles.

An improvement to this technology involves the use of a combination of silicon carbide (SiC) whiskers (e.g. Silar® silicon carbide whisker) and microinjections. This involves precipitating ds-plasmid DNA, linear DNA, RNA, protein or virus particles onto the silicon carbide and injecting it into the meristems by means of microinjection cannulas.

This offers the advantage that there is the possibility of penetrating numerous cells in parallel through the distribution of the whiskers, instead of being limited to the transfection of a single meristematic cell. Because it is not necessary to penetrate into the cell with the cannula, and the whiskers are significantly smaller, there is less damage to the cells.

Vascular Puncture Infection/Inoculation (VPI):

Vascular puncture infection or inoculation, is a method described in Benavente, 2012 (Virus-Induced Gene Silencing in the Diverse Maize Lines Using the Brome Mosaic Virus-based

silencing vector) and Louie, 1995 (Louie R, 1995. Vascular puncture of maize kernels for the mechanical transmission of maize white line mosaic virus and other viruses of maize.

Phytopathology85: 139-143)which is used to introduce viruses, virus particles, agrobacteria, and naked DNA into intact maize kernels. This technique enables targeted introduction in the proximity of the embryo and the meristematic tissue. It offers the advantage that no preparatory steps are necessary, and the germinated seeds can be used immediately. This results in minimal damage to the tissue and only minor disruption to plant development. This method has been modified and implemented as follows: seeds containing a target nucleic acid region of interest were soaked in water for 4 hours at 30° C. The seeds were then incubated overnight in moist towels at room temperature. Subsequently, a plasmid or plasmid mixture, or a virus of interest, was pipetted onto the side of the seed kernel carrying the embryo. Normally, a 100 µl plasmid mixture is prepared in a concentration of 37.5 µg/100 µl, or 1.5 µg/4 µl for each plasmid. Using a notching tool, the inoculum is moved 1-2 mm into the scutellum along the embryo, toward the vascular bundle. Retaining pins at an angle of 45° to the surface of the kernel that is to be treated. Two inoculations are carried out at a distance of 1 mm from the embryo in order to avoid injuring the embryo. The drops are then left on the kernel.

Example 3: Transient Meristematic Transformation of Maize Seedlings and/or Embryos/Inventive Treatment of Meristem Tissue

The accessibility of the meristem varies widely in the individual stages. In the embryo (Fig. 1 and 2), the meristems are relatively easily accessible, provided that embryos of the right size are used. It is important that the deeper-located cells of the meristem are transformed, since the upper cells have already undergone a certain amount of differentiation and are no longer suitable. Figures 10 and 11 show two views of a maize embryo, as well as the locations of meristematic tissue, indicated by stars. This data was initially visualised with a fluorescence marker. It is clear from this that the targeting of plant meristematic cells and tissue is made possible through the provision of the novel method. This facilitates , a novel process for introducing nucleic acid structures, e.g. vectors, as well as, in particular, RNAs and amino acids, into a plant target cell. The spectrum of application comprises numerous possible constructs for the targeted genetic engineering modification of a plant cell, such as a CRISPR/Cas construct, viral vectors, RNAi constructs, etc., in order to obtain targeted knock-ins, knock-outs, or targeted point mutations in the target nucleic acid region of the plant cell.

Meristems in seedlings and older plants must be completely exposed since they are already surrounded by so many layers of tissue that they are not accessible to bombardment or a microinjection. Figures 3 and 4 show the prepared meristems which may be used for the transformation. As is the case for the embryo meristems, the upper cells have already undergone a certain amount of differentiation and are no longer suitable. As a result, the cells further inside in the meristems have to be transformed. The exposed meristems can be bombarded horizontally as well as vertically. In detailed studies it has been discovered that vertical bombardment significantly increases the hit rate in the suitable meristematic regions (see Fig. 11 and Fig. 12). This shows once again that, although particle bombardment is a known and established method, its effective application nevertheless requires optimization of various parameters (construct to be inserted, form and stage of the material to be transformed, pressure, orientation, etc.) for a specific task in the transformation of specific plant tissues.

Since the isolated meristems are free and thus are exposed to a great deal of oxidation and resulting dying off, they were treated with antioxidant in order to allow the seedling to develop into a plant.

In order to make the tassel meristems accessible, a method was developed which damaged the plant and meristems as little as possible. This involved cutting a window through the leaves at the level of the tassel meristems (Figure 6). This ensured that the leaves would not die off and that the plant could develop further completely normally and that the meristem would still be protected by the remaining leaves. In addition, the meristem very quickly (within a few hours/days) moved upwards so that it was once more completely protected. This reduced the probability of the meristem oxidizing and therefore dying off. It is possible with this method to ensure that the flower develops in an almost entirely standard manner, and to obtain pollen for self-fertilization or pollination. This in turn provides the advantage that reproductive cells modified in a targeted manner can be obtained from the plant, making time-consuming *in vitro* cultivation steps unnecessary.

The transfection then takes place using the methods described above (see Example 2). The embryos germinate and plants are cultivated to self-fertilization and harvest. Similar results were achieved with the seedlings and the adult plants, but without germination.

Example 4: Detection of Successful Targeted Genetic Modification

Detection is possible using various methods and at various times:

The presence of the desired targeted modification of a target nucleic acid region can be analysed in the early phases of the seedling, the developing plant and the pollen so that indications of successful mutations can be obtained. However, a clear result is only obtained when the descendants of the self-fertilization are analysed, as these provide the proof of an inherited mutation.

- Enrichment PCR:

This method is used when a restriction enzyme site is destroyed by the specific mutation. In this case, the isolated genomic or extrachromosomal DNA is digested with the enzyme which cleaves at this site so that wild type DNA is cleaved. Next, a PCR is carried out with primers which lie upstream or downstream of the restriction enzyme site on the genome. Ideally, only one product is obtained when a mutation has taken place and the DNA was not cleaved at this site. Since, the genomic or extrachromosomal DNA is usually not 100% digested, the PCR amplification material obtained is then digested anew with the enzyme in order to establish that a mutation has occurred and the restriction enzyme site has been mutated. The undigested fragments are then cloned and sequenced in order to carry out a precise analysis of the mutation. If the target nucleic acid region is an RNA, then it can first be transcribed into DNA using a method which is known to the person skilled in the art before an enrichment PCR process is performed.

- Sequencing:

If enrichment PCR is not possible, a Next Generation Sequencing (NGS) method is used to sequence the specific region and the sequences obtained are examined for mutations.

- Whole genome sequencing (WGS) to identify off-target effects:

In order to exclude the possibility of unwanted mutations, a WGS is carried out on the candidates with the desired mutations. Specific PCR and qPCR systems are also used to detect the absence of the constructs and viruses used.

Example 5: Viral vectors (not inventive)

Viruses offer the advantage that they can be introduced into a plant target structure as whole viral particles and also as DNA or RNA. The introduction of the viruses is achieved via the delivery methods listed in Example 2. By these means, a targeted introduction into the respective meristematic target regions of interest is achieved.

In addition, viruses offer the possibility of propagation in the cells, provided that this function has not been destroyed by a modification to their RNA/DNA sequence. The advantages provided by this approach are that the meristem does not have to be directly infected and it is sufficient to infect only a few cells in order for propagation into several cell or tissue types to occur.

With this application, there are other methods, in addition to the delivery methods described in Example 2, for introducing viruses or virus particles.

Virus particles, *in vitro* transcripts of the viruses, or agrobacteria that carry virus-coding T-DNA are inserted by rubbing them into the leaves, or via infiltration (with and without a vacuum), in order to generate a primary infection. The respective target cells and target tissues are then infected through systemic spreading.

In addition, plant sap that has a high titre of plant viruses is used for the infection. For this, either tobacco or spinach is infected with the viruses, after which, the plant sap containing the viruses is isolated and used for infecting the maize plants.

Aside from the broad spectrum of infection possibilities, and their spreading capabilities, DNA viruses offer the advantage of providing DNA templates for homologous recombination (HR). In this case, a large quantity of templates is provided by the replication of the virus inside one or more cells for homologous recombination after the double-strand break has been introduced. As a result, homologous recombination and incorporation of the template fragment occur with greater frequency.

In one series of tests, different BMVs (see SEQ ID NOS: 25-31 or DSMZ filing number: BMV Virus-Inoculum: PV-0945; reference for BMV plasmids (C13/F1+F2 & C13/F3-13m): Benavente et al., Maydica, Vol. 57, No. 3(2012): "Virus-Induced Gene Silencing in Diverse Maize Lines Using the Brome Mosaic Virus-based silencing vector.") and BSMVs (comprising at least one sequence selected from the SEQ ID NOS: 32-37 or DSMZ filing number: BSMV Virus-Inoculum: PV-0330; Reference for BSMV plasmids (pCaBS-α & pCaBS-β & pCa-γbLIC): Yuan, C., et al. (2011). PLoS One 6(10): e26468."A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots.") virus particles, plasmids, or plasmid mixtures, were therefore inserted into a plant or plant cell

of interest. *Nicotiana benthamiana*, maize A188, maize Va35, and *Spinacia oleracea*, inter alia, are infected with corresponding viruses, plasmids or a plasmid mixture. A rubbing inoculation, vascular puncture infection/inoculation, or *Agrobakterium*-mediated transformation were used.

For the rubbing inoculation, a DNA plasmid coating containing similar concentrations of different plasmids was prepared for the primary inoculation. By way of example, each plasmid was used in a concentration of 6 µg/µl. The different plasmids of the same concentration were then mixed in the same volume ratios. For each leaf, 6 µl plasmid mixture was applied in drops to the surface of the leaves, on which the carborundum had already been distributed. The plasmid mixture was then rubbed into the surface of the leaves with fingers. Alternatively, a plant sap infected with a virus can be used as the starting material. For the second inoculation, fresh or frozen plant leaves infected with a virus were ground in a homogeniser in this method, and the resulting powder/product was dissolved in a 3-4 ml inoculation buffer (0.2406 g KH_2PO_4 + 0.543 g Na_2HPO_4 in 500 ml deionized water). At this point, a small quantity of carborundum was added to the plasmid mixture or plant sap. The plasmid mixture or plant sap is introduced into the upper and lower surfaces of the leaf through rubbing, wherein this is achieved by submerging one or more fingers into the inoculum and then carefully applying the inoculum to at least one leaf by hand, wherein the leaf is preferably supported by the other hand. The rubbing inoculation can also be combined with a prior injury (incision) to a plant leaf, wherein an incision is first made in the leaf with a scalpel, and the rubbing inoculation then takes place directly into the injured leaf.

For the *Agrobakterium* (Ab)-mediated transformation, Ab cultures were first cultivated overnight at 28° C in 30 ml liquid Luria broth, comprising a suitable antibiotic, 10 mM MES, and 200 µm ACE. The next day, the overnight cultures were centrifuged at 4,400 rpm for 15 minutes. The supernatant was discarded, and the pellet was then centrifuged again at 4,400 rpm for 2 minutes. The remaining supernatant was discarded, and the pellet was re-suspended in a re-suspension medium (5 ml H_2O, 10 mM MES, 10 mM $MgCl_2$+20 µM ACE). The optical density OD_{600} of the suspension was adjusted to 1.5 using the re-suspension medium. The diluted Ab suspension was then incubated for 4 hours at room temperature. The infiltration of the Ab suspension then preferably takes place on the underside of a leaf of interest, e.g. a *Nicotiana benthamiana* leaf, wherein, normally, 2 leaves from each plant are inoculated.

The following Table 2 shows exemplary results for selected viruses and plant species, using different transformation methods:

Table 2: Overview of viral infection experiments (WpI: weeks post infection)

Virus material	**Infected plant species**	**Method**	**Result**
BMV virus particle DSMZ	*N. benthamiana*	Rub + carborundum	2 WpI: 2/2 plants with systemic BMV infection
BMV virus particle DSMZ	Maize A188	Rub + carborundum	2 WpI: 2/2 plants with local BMV infection
BMV tobacco juice infected with virus particles DSMZ	*N. benthamiana*	Rub + carborundum	2 WpI: 4/6 plants with systemic BMV infection
BMV tobacco juice infected with virus particles DSMZ	Maize A188	Rub + carborundum	2 WpI: 3/4 plants with local BMV infection
BMV tobacco juice infected with virus particles DSMZ	Maize Va35	Rub + carborundum	2 WpI: 1/6 plants with local BMV infection
BMV tobacco juice infected with virus particles DSMZ	Maize Va35	Leaf incision + rub + carborundum	2 WpI: 1/2 plants with systemic BMV infection
BMV plasmids C13/F1 +F2 and C13/F3-13m	*N. benthamiana*	From infiltration	1 WpI: 12/12 plants with systemic BMV infection
BMV plasmids C13/F1 +F2 and C13/F3-13m-GFP	*N. benthamiana*	From infiltration	5 WpI: 12/12 plants with systemic BMV infection
BMV - tobacco juice infected with plasmids C13/F1+F2 & C13/F3-13m	Maize Va35	Rub + carborundum	4 WpI: 1/2 plants with systemic BMV infection
BMV - tobacco juice infected with plasmids C13/F1+F2 & C13/F3-13m-GFP	Maize Va35	Rub + carborundum	4 WpI: 3/4 plants with systemic BMV infection
BMV virus particle DSMZ	*Spinacia oleracea*	Rub + carborundum	2 WpI: 5/5 plants with local BSMV infection; 3 of which also systemic
BMV virus particle DSMZ	Maize A188	Rub + carborundum	2 WpI: 4/6 plants with local BSMV infection

BMV - spinach juice infected with virus particles DSMZ	*Spinacia oleracea*	Rub + carborundum	2 WpI: 5/ plants with systemic BSMV infection
BSMV plasmids pCaBS-α & pCaBS-β & pCa-γLIC	*Spinacia oleracea*	Rub plasmid mix + carborundum	2 WpI: 11/11 plants with local BSMV infection
BSMV plasmids pCaBS-α & pCaBS-β & pCa-γLIC	*N. benthamiana*	From infiltration	2 WpI: 14/14 plants with systemic BSMV infection
BSMV plasmid pCaBS-α & pCaBS-β & pCa-γLIC	Maize A188	Vascular puncture innoculation	2 WpI: 1/15 plants with systemic BSMV infection
BMV virus particle DSMZ	Maize A188	Vascular puncture innoculation	2 WpI: 1/12 plants with systemic BSMV infection

The white background in table 2 indicates that for this experiment, a systemic infection could be achieved. A light grey background indicates a local infection, while a dark grey background indicates a low infection rate.

Proof of successful infection is obtained from either an ELISA or by means of an RT-PCR.

Example 6: 2-gRNA strategy

A so-called 2-gRNA strategy was established for the targeted control of genomic DNA and to specifically excise a target nucleic acid region of interest from the genome through the use of a CRISPR nuclease (see Figures 17 A and B). As shown in Figure 17 A, genomic DNA is first isolated, and then digested by a restriction enzyme (RE) of interest, the cleavage site of which lies within the PCR product of interest. Any RE can be used that can cleave between the two gRNA target regions. In this manner, an accumulation of potentially edited DNA takes place due to the absence of the region between the gRNA target regions, and the fact that the selected restriction enzyme cannot cleave this DNA. Subsequently, PCR amplification takes place with primers that bind upstream and downstream from the two gRNA target regions, i.e. they can accumulate under suitable reaction conditions under hybridization. If necessary, a re-PCR can be carried out with a nested primer set. After the successful editing process, the resulting PCR

product is smaller than the product from non-edited DNA (see Fig. 17 B). Figure 17 B shows the results of the analysis of an edit after the use of the 2-gRNA strategy with genomic DNA of a maize plant. The genomic DNA was isolated from maize plants and the target gene hmg13-gene (HMG-transcription factor 13; GRMZM2G066528) was amplified with PCR. The sequence of the HMG-transcription factor 13 gene without editing is shown in SEQ ID NO: 60.

The nucleotide positions 1-98 of the SEQ ID NO: 60 and the nucleotide positions 912-1023 of the SEQ ID NO: 60 correspond to the region of the *hmg* gene that remains after a successful edit. Nucleotide positions 82-101 of the SEQ ID NO: 60 and the nucleotide positions 909-928 of the SEQ ID NO: 60 are each gRNA target regions.

Fig. 17 B shows the results of a separation in a 1% gel with standard parameter 100 V and visualization via fluorescence obtained with ethidium bromide, with different contrast levels. Bands 1 and 2 show the results for non-edited maize plants, and band 4 shows the results after successful editing. The PCR product is smaller because the region between the two gRNA target regions has been excised. This approach therefore represents a quick and efficient strategy for experimentally confirming a successful genome edit.

SEQ ID NO: 61 shows the results of the sequencing of the small PCR product after *hmg*13 editing with the 2-gRNA strategy. The deletion has taken place through a targeted edit between the two bases, C and T, at positions 98 and 99 of the SEQ ID NO: 61.

Example 7: Genome Editing in Tobacco (not inventive)

NbTTG1 was selected as the target gene in *Nicotiana benthamiana* for the genome editing work, the ortholog gene of which results in a trichome phenotype in *Arabidopsis thaliana* when dysfunctional. Mutants are described for the corresponding Arabidopsis gene AtTTG1 (AT5G24520):

- ttg1(EMS mutants):	no trichomes on the leaf surfaces and stem. Yellow seeds resulting from the absence of brown pigments.
- ttg1-13 (fast neutron mutants):	no trichomes, transparent seed casings, increased number of root hairs.

The ortholog in *Nicotiana benthamiana* was identified by means of sequence comparison and the genomic locus was amplified by means of PCR. The section in question is shown in Figure 18. Appropriate gRNAs were selected on the basis of this sequence, as described above. The components for the genome editing were introduced into the plant via TRV (tobacco rattle virus) (see example 8, below). The 2-gRNA strategy outlined above in example 6 was also used here for the purpose of analysing a successful edit.

As shown in table 3 below, different combinations of two gRNAs achieved deletions in the NbTTG1 gene of varying sizes. A Cas9 nuclease was used for this test, although the approach can be used for any of the CRISPR nucleases.

Table 3:

gRNAs	Deletion
gRNA1 + gRNA4	232 bp
gRNA2 + gRNA4	216 bp
gRNA3 + gRNA4	206 bp
gRNA4 + gRNA5	446 bp
gRNA1 + gRNA3	25 bp

Example 8: Tobacco Rattle Virus (TRV) mediated expression of CRISPR-Cas in *Nicotiana benthamiana* (not inventive)

For the leaf inoculation of tobacco, first *Agrobacterium* (Ab) cultures were cultivated overnight at 28° C in 30 ml liquid Luria broth (LB) medium, which contains a selective antibiotic. The next day, the overnight cultures were centrifuged at 4,400 rpm for 15 minutes. The supernatant was discarded and the pellet was again centrifuged at 4,400 rpm for 2 minutes. The remaining supernatant was discarded, and the pellet was re-suspended in 5 ml re-suspension medium (10 mM MES, 10 mM $MgCl_2$, 20 µM ACE). The optical density at 600 nm (OD_{600}) of the suspension was adjusted to 0.8 using the re-suspension medium. The diluted Ab suspension was then incubated for 4 hours at room temperature. The Ab suspension was subsequently infiltrated with a syringe or cannula on the undersurface of a leaf of interest, e.g. a leaf from *Nicotiana benthamiana*, wherein 3 leafs of each plant were normally inoculated. In order to visualise the systemic spreading efficiency of TRV, the leaves were inoculated with an RFP/pTRV2 (red fluorescent marker+TRV as a viral vector) and with pZFN-tDT-nptII as a

control. As can be seen in Figure 19, a clear RFP fluorescence can be detected in the directly inoculated leaves, as well as in the non-inoculated distal leaves (original red fluorescence is indicated by the light and/or white regions in Fig, 19). The construct pZFN-tDT-nptII functions as a control, which only allows the expression of the RFP in the inoculated leaves, but not in the distal leaves.

It was also confirmed that even meristematic tissue can be activated through these TRV methods, allowing for a targeted modification of this type of tissue through the specific CRISPR methods. For this purpose, *Nicotiana benthamiana* plants were infected with TRV, wherein the construct comprises a gene that codes a red fluorescent protein, e.g. tdT or the like. It was possible to determine the location of TRV in the plant through the detection of the red fluorescence with appropriate means (fluorescence microscope, binocular). It may be advantageous here to use fluorescent markers with a high level of intensity, as these can also be readily detected in deeper tissue layers. Fig. 20 A to H show images of a flower meristem, a flower bud, a pistil, or a prepared pistil with exposed ovaries. All of the images demonstrate the successful expression of the fluorescent marker in the respective plant meristematic cells, or tissues as a target structure, and thus the efficiency of the selected introduction method.

Lastly, TRV titres were quantified in the inoculated and non-inoculated tobacco leaves by means of a standard double antibody sandwich (DAS) ELISA. 10-12 dpl leaf material was harvested from each plant that was to be analysed as the starting material for the ELISA, wherein for each plant, the following mixture samples were created: (i) mixture sample, each from two TRV inoculated leaves; (ii) mixture sample, each from two non-inoculated leaves. The harvested leaf material was pressed and the collected plant sap was used at a dilution of 1:50 in the DAS-ELISA. The DAS-ELISA was carried out using a polyclonal antiserum from rabbits. The antiserum was obtained from the company Loewe®, and is named "Tobacco Rattle Tobravirus BroadRange TRV" (catalogue no. 07152S). The ELISAs were assessed 60 minutes after application of the substrate 4-nitrophenylphosphate by means of the photometric measurement of the OD_{405}. In this manner, the TRV titres were quantified in *N. Benthamiana* inoculated with (i) pTRV1 (=negative control); (ii) pTRV1+pTRV2-tDTco (=positive control) and (iii) pTRV1+pTRV2-Cas9. The results are shown in FIG. 21.

Example 9: Quantification of CRISPR Tools

By way of example, Cas9 transcripts were detected by means of RT-PCR. For this purpose, 10-12 dpl leaf material was harvested from each plant that was to be analysed as the starting material for the ELISA, wherein the following mixture samples were created for each plant: (i) mixture sample, each from two TRV inoculated leaves; (ii) mixture sample, each from two non-inoculated leaves (see Example 8). First, RNA was extracted from the harvested leaf material, using an RNeasy Mini Kit (Qiagen). In each case, 500 ng RNA was subsequently transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA served as a template in a subsequent PCR for detecting Cas9. Cas9-specific primers were used.

Protein extracts were produced from leaf material of transgenic maize plants, and separated on a 4%-20% SDS-PAGE gradient gel. The 160 kDa Cas9 was dedected with a monoclonal antibody from ActiveMotif (catalogue no. 61577). The documentation of this detection system is shown in Fig 22.

Quantitative SYBR green-based RT-PCR systems were established in order to quantify RNAs and to determine whether or not an expression by the gRNAs, conveyed through a sub-genomic promoter, takes place. When amplified with the same PCR efficiency, a quantification can be performed by comparing the gRNA quantity with the transcription level of viral proteins. This system is shown in Figure 23, using the brome mosaic virus (BMV) by way of example.

Example 10: Viral expression systems (not inventive)

In addition to the aforementioned viral vectors, the CRISPR tools and methods of this disclosure can likewise be virally introduced into other plant systems. Different methods may be used, depending on the target plant of interest, the type of transformation, and the target tissue that is to be infected. The system from Ugaki et al. (1991, Nucleic Acids Res., Replication of a geminivirus derived shuttle vector in maize endosperm cells) is suitable for maize endosperm cells serving as the primary target structure. Using the wheat dwarf virus (WDV) as a vector, an infected culture can thus be obtained through protoplast transformation of maize endosperm cultures. For this purpose, a modified virus is used, which carries a neomycin phosphotransferase gene II (*npt*II) in place of the coat protein (CP). A transient replication system with the wheat dwarf virus as cargo, in accordance with Matzeit et al. (1991, Nucleic Acids Res., 19(2), 371-377) can be used for *Triticum* target plants. In this system,

protoplasts derived from *Triticum* suspension cultures are transfected. The CP gene of the virus is again replaced by a marker gene of interest.

Systems based on the *Maize Streak Virus* can be used, which are known to the person skilled in the art, and described in Palmer & Rybicki (2000, Archives of Virology, 146 (6), 1089-1104) and elsewhere. Three-day old seedlings are infected at coleoptile nodes, and a transient expression of a recombinant construct of interest can be obtained. By exchanging the viral CP and MP genes, a systematic spreading of the virus can be prevented, such that only the first two or three leaves are infected.

As explained above, the *barley stripe mosaic virus* (BSMV) is also suitable as a viral vector. The BSMV genome was intensively transformed in order to establish a known plant protoplast vector (see Joshi et al., 1991, EMBO J., 9(9), 2663-2669.). This vector, which carries a luciferase (luc) reporter gene according to Joshi et al., 1990, is suitable for protoplast transfection of maize and tobacco protoplasts.

BSMV (see also Manning et al., 2010, New Phytologist, 187 (4), 1034-1047), WDV, Wheat Strike Mosaic Virus (WSMV) (Choi et al., 2000, Plant J., 23(4), 547-55), Tomato Yellow Leaf Curl Virus (TYLCV) (Peretz et al., 2007, Plant Physiol., 145 (4), 12514-1263) and Brome Mosaic Virus (BMV) (French et al., 1986, Science 231(4743), 1294-7) are also suitable as systems for transfection of protoplasts, seedlings, petioles and other plant cells or tissues in wheat, as well as barley and tobacco, as described at length in the literature. BSMV vectors (see Manning et al., 2000, supra) in a modified form are particularly well-suited as vectors for virus-induced gene silencing. To this end, the BSMV genome is modified through site-oriented mutagenesis by suppressing the expression of the viral coat protein. TYLCV (see Peretz et al., supra, or EP 2 436 769 A1)) was attenuated and made available for use as a viral shuttle vector for plants, as well as *E. Coli*, by deleting a sequence comprising 60 base pairs in the viral coat protein in the proximity of the N-terminus of the gene.

Specific approaches for sugar beet transformation on the basis of viral vectors are likewise known. The beat curly top virus (BCTV) (Kim et al., Plant Mol. Biol., 2007, 64(1-2):103-12), the beet yellows virus (BYV) (Prokhnevsky et al., Molecular Biotechnology, 57 (2), 101-110, 2015), the beet soil-borne mosaic virus (BSBMV) (Dach et al., 2015 ASSBT proceedings conference transcript, 47th annual meeting of the work group, Viral Diseases of Plants, Section

C), or the beet necrotic yellow vein virus (BNYVV) (Hamza et al., 2015, ASSBT Proceedings)are particularly well-suited for this purpose. These vectors are not only suitable as vectors for sugar beets, but also for other dicotyledons, e.g. spinach.

Numerous methods are likewise available for transformation by means of viral vectors when using tobacco as the model plant. Many of these methods are based on *Agrobacterium*-mediated infiltration. Suitable viruses comprise the tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), bean yellow dwarf virus (BeYDV), plum pox virus (PPV) (see Gleba et al., 2014, Gleba et al., 2014 or Salzar-Gonzalez et al., 2015, Plant Mol. Biol., 87:203-217). Moreover, various other systems have been described, that use, e.g., cabbage leaf curl virus (CaLCuV) (Yin et al., 2015, Nature Scientific Reports, 5:14926, 2015), Tobacco Rattle Virus (TRV) (Ali et al., 2015, Genome Biology, 16:238) orTobacco Yellow Dwarf Geminivirus (TYDV) (Dugdale et al., 2014, Nat. Protoc., 9(5), 1010-27) as a virus.

All of the aforementioned above contain cloning sites for introducing target genes of interest. Specific cleavage sites can also be introduced easily into a viral genome of interest through available mutagenesis methods.

Example 11: Optimized methods for windowing plants (not inventive)

To further optimize the targeted introduction of CRISPR constructs, and thus the effect of the genome editing, further imporvements were made to the method outlined above in Example 3. The original method comprises the closing of the artificially inserted window with a closure, such as a special tissue paper. This may, however, involve the disadvantage, depending on the exposure, that the injured and exposed plant tissue can be more easily infected with fungi, or that a portion of the exposed tassel, the bombarded portion, comes into too much contact with the air, which may result in a drying out of the exposed tassel structures, or immature flowers, and thus the individual tassel branches. For this reason, the exposed tassel tissue, transformed as described above, was covered with a moistened cotton pad or tissue in a first step. As a result, the drying could be significantly reduced, although this method is still prone to fungal infections. In order to address this problem, waxes or Vaseline-like substances were applied to the injured site (after transformation). A variety of substances were tested, comprising Vaseline, mixtures of natural waxes with Vaseline and other commercially available products for healing injuries, specifically in trees. This approach is well known to the person skilled in the art, particularly in the field of grafting. In addition, the injured site was wrapped with a

special grafting tape or parafilm which significantly improves the closure of the wound, and thus the protection against fungal infection, such that the transformed meristematic tissue can develop to full maturity. With this optimised strategy, a majority of the tassels in their transformed form were able to develop to maturity. Success rates of 75% and more were obtained with this optimised strategy, i.e. events in which the exposed and transformed tassel tissue was able to develop to full maturity in planta.

Example 12: ***Agrobacterium*** **injection (not inventive)**

In order to further expand the possible field of application, the method outlined in Example 3 was carried out, modified in such a way that instead of the particle bombardment, *Agrobacterium* (Ab)-mediated transformation was used. In a preliminary test, the susceptibility of immature tassel tissue to Ab was tested. For this purpose, a red fluorescent protein was transformed *in vitro* into immature tassel tissue, which had been previously isolated from the plant. At the time of isolation, the plants were in the V6-V7 stage, and the tassels were approx. 2-3 cm long. Ab was set to an OD600 of 1.0, and the tassels were incubated for 10 minutes with the Ab suspension. The red fluorescence was observed two days after the infection. Numerous red fluorescent dots were observed in the tassels, confirming the suitability of Ab infiltration for the transformation of tassel tissue. In a next step, plants in the V6-V7 stage were used, and the plants were windowed, as described above, in the region of the immature tassel tissue. Ab containing a red fluorescent expression construct, was injected directly into the tassel tissue at an OD of 0.7. Approximately 100-200 µl of the Ab suspension was injected into each tassel. The windowing sequence as well as the Ab injection sequence are shown in Figure 24. At this point, the tassels were covered with Vaseline/paraffin, as described above, and the development of red fluorescence was monitored for two days after the injection. In order to suppress an excessive growth of Ab, an antibiotic solution (e.g. Timentin, Carbenicillin, Cefotaxim) was applied to the infected tissue 2 to 7 days after the initial injection. The treated tassels were able to ripen to maturity, and self-pollination was carried out. Molecular analyses in the T1 generation confirmed the successful transformation. This confirmed that an *in planta* method is suitable for transforming meristematic cells *in planta*, without impairing the further development of the tassels, such that the resulting pollen can be obtained directly from the plant, without lengthy (*in vitro*) cultivation processes, and can be used directly for pollination.

Example 13: Applicability to different maize genotypes

The tassel transformation experiments outlined above were tested for different maize genotypes, specifically A188, Va35 and A632. For each genotype, the vegetation stage in which the tassel tissue can be transformed varies naturally. However, it is easy to determine. In A188, the stage is V6-V7, by way of example, while A632 was targeted in stages V7 to V9. It was possible in all of these genotypes to expose the tassels in a suitable manner, i.e. it was possible to window the plants without damaging them or the tassel tissue, and to obtain mature, pollen-producing anthers.

Example 14: Embryo meristem bombardment

In order to further optimize the methods described herein, a so-called embryo meristem bombardment was established, which allows for plants to be efficiently obtained directly from immature embryos, without a time consuming and contamination-prone cell culture as an intermediate step. For this, the particle bombardment of meristem regions of embryos was carried out in the pipeline mode for the genotypes A188 and A632. Approximately 100 embryos (Fig. 25 A) were bombarded with CRISPR/Cas9 constructs, together with a red fluorescent protein-expressing plasmid. The development of fluorescence was observed one day after the bombardment (Fig 25 B). Numerous embryos demonstrated fluorescence, and thus the successful and functional introduction of the CRISPR construct. Work was continued with the successfully transformed embryos. After germination, 25% of the plants were analysed on the molecular level. All of the other fluorescent-positive plants were allowed to grow to maturity in a greenhouse. As soon as the plants reached the reproductive stage, a sample was removed from the tassel, as well as the ears, and examined for CRISPR/Cas9 activity. When a successful result was identified, by means of PCR, for example, the plants were used for self-pollination, and the resulting descendants were likewise analysed.

The plants produced in this manner produced seeds for both genotypes, and were fertile. The plants exhibited a slower growth rate and a slightly curved growth (Fig. 25 C). Nevertheless, fertile plants could be produced through this method without difficulty, the pollen of which could be used directly in further experiments. This type of transformation is thus also a highly efficient method for quickly and effectively introducing CRISPR constructs, or the genome edits obtained therefrom, into a meristematic tissue or a cell of interest, and to then be able to directly obtain and further use reproductive tissue from this tissue.

Example 15: Meristem access in different types of plants

As specified above, it is desirable to bring about an *in planta* transformation for numerous different plants and to combine this with the methods disclosed herein, such that a targeted modification of numerous meristematic target structures can be obtained through the CRISPR systems. Specifically, the transient introduction of CRISPR constructs of interest into a plant meristematic target structure is of great interest, as this would allow for a targeted modification of a target nucleic acid region of interest, and this modification, but not the CRISPR construct itself, would then be passed on to further generations.

The tissues that can develop *in planta* into reproductive organs are limited. The most important is the shoot meristem. This meristem is defined by the group of cells that can differentiate into all vegetative organs and cells, as well as reproductive organs and cells that are above ground. It is composed of a limited number of cells that can be (re)programmed, in order to differentiate themselves into all of the organs of a plant. This meristem normally has the shape of a dome. The outer lines of the cells, called the L1 layer, form the basis for all epidermal tissue. The inner layers (L2 and L3) of the meristem form the rest of the organs, and are thus interesting targets for the purposes of the present invention. The meristem is formed very early in the development of the embryo. After the vegetative growth, the meristem develops in the flower meristem in order to generate the reproductive organs of the plant. The tissues that can produce the modified reproductive organs are: (1) the shoot meristem of the embryo, (2) the shoot meristem of plantlets or plants in the vegetative stage, and (3) the flower meristems or the inflorescences.

When the genetic information of this tissue is modified by non-viral approaches (gene guns, microinjections, Agrobacterium, etc.) it may be the case that not all of the cells of these meristems are modified in a targeted manner. Consequently, some of the differentiating plant organs are modified, and some retain the wild genotype. Chimeras are obtained in this manner.

One alternative for the targeted manipulation of numerous different grain plants is to transform microspores (immature pollen) or pollen grains. These tissues can then be used to pollinate further plants and obtain modified descendants. There are only a small number of relevant examples in the literature, most of which are in the context of bombardment and transient expression analysis of the inserted genes (Twell et al., 1989, Obert et al., 2008). Nevertheless, the technology has been further developed in order to allow microspores or pollen to ripen in a targeted manner, and to obtain modified descendants through subsequent pollination. The

method for this technology us very similar for various crop plants. Microspores can be targeted directly in immature anthers, or by releasing microspores into a culture medium. This targeting can take place through bombardment or microinjections, by way of example. This technique has been used successfully for producing transgenic tobacco plants (Touraev et al., 1997) and cotton (Gounaris et al., 2005). As with the targeting of mature pollen, recently obtained pollen can be treated through bombardment, or sonically (Eapen (2011)), and used immediately for the pollination of, maize ears (Horikawa et al., 1997), for example. The descendants can then be analysed for the presence of transgenes or genomic events introduced in a targeted manner.

***Beta vulgaris*:**

Immature embryos may be obtained for the transformation of meristematic tissues in sugar beets, as described in Zhang et al. (2008). Flower spikes were obtained from plants grown in a greenhouse, 14 days after anthesis. They were sterilized in a 30% bleaching agent for 30 minutes. Immature embryos (IEs) were isolated, and subsequently cultivated for 4 weeks on a solid MS medium with various plant growth regulators. An image of an immature embryo of this kind is shown in Figure 26. The apical shoot meristems can be treated in a targeted manner, directly in these immature embryos, wherein the meristem regions can be targeted with the aid of a microscope. Alternatively, random targeting technologies, such as bombardment, can be implemented. The plants continue to mature after targeting. This embryo maturation takes place in an incubator, in the dark, at a temperature of approx. 20°-30° C. The maturation period lasts approximately 1 to 4 weeks. As soon as the embryo has reached maturity and begins to germinate, it is transferred to a solid MS medium and exposed to light, so that the plantlets can develop. When these plantlets are sufficiently robust, they are transferred into soil, after an acclimation phase of approx. 1 to 4 weeks. These plants are then cultivated and the descendants are analysed.

The targeting of mature embryos from sugar beets requires the removal of the hard pericarp (Hermann et al., 2007). The embryo is located in the middle and the apical shoot meristem is accessible. Prior to removal of the pericarp, the seed must be sterilized by bleaching with ethanol. The pericarp can then be removed with scalpels or other sharp tools to expose the embryo. This embryo is then placed in a suitable medium for the specific transformation methods of interest. The meristem of the mature embryo, or the entire embryo, can then be subjected directly to a transformation that randomly activates meristem regions with the use of a microscope. After a resting phase of approx. 1 to 10 days in an incubator, in the dark, at 20°-

30° C, the embryo germinates, and the plantlets can be planted. The sugar beet plants are then grown to maturity, and the descendants are analysed.

The shoot meristems in sugar beet shoots can be targeted by making targeted incisions in meristem regions (Artschwager, 1926). These types of shoot meristems were already targeted, e.g., through particle bombardment. Particle penetration tests were carried out prior to assessing gene expression. Transient GUS expression was detected in the first and second cell layers of the meristem. Dividing cells with GUS activity showed that the cells survived the bombardment (Mahn et al., 1995). It was also proposed that meristems with attenuated Agrobacterium strains could be used for *Beta vulgaris* transformation (Kerns et al., 1988). Different methods (microinjection, Agrobacterium) and different plant tissues at different stages of development could be used for this purpose. For the purposes of the present invention, a bombardment of meristematic tissues from seedlings that were grown *in vitro* was carried out. The leaf material was removed until the meristematic tissue was exposed. Vertical incisions were then made in the meristematic regions, or the regions were provided vertically, without an incision. Following bombardment with a gene cannon, the explants were left *in vitro*. One day after the bombardment of the cells, it was shown that the cells exhibited beta-glucuronidase activity, which had been introduced as a marker into the cells, confirming that meristematic regions of sugar beets are suitable for particle bombardment and thus for the introduction of genome editing tools, e.g. CRISPR tools.

In addition, the inflorescence of sugar beets can also be modified in a targeted manner, during maturation, either prior to flowering, or directly in the immature flowers. The buds can then continue to mature. After pollination, the seeds are harvested, and the descendants are analysed. In *Beta vulgaris*, the inflorescence is composed of an open main axis with numerous closed, dichasial and sympodial inflorescences. The terminal bud of each inflorescence unit and lateral buds merge at a later developmental stage. The five stamen primordia come from one another, and they occur in the course of bud development through the formation of an (intra)staminal ring from an annular intercalary meristem (Olvera et al., 2008).

***Triticum aestivum*:**

Another approach has been developed involving wheat as the target plant. In this approach, immature kernels from immature ears were collected 5 to 20 days after flowering. These kernels were sterilized through surface treatments with bleaching agents and ethanol. The

immature embryos were then extracted with a scalpel under a microscope. The meristems of these embryos were exposed to varying extents. These meristems were then subjected to various transformation methods, such as described in Sautter et al., (1995). Figures 27 A and B provide images related to this approach. After the embryos were treated in this manner, they were cultivated further, specifically in an embryo cultivation medium, as described in Matthys-Rochon et al., (1998). The germinating plantlets (Figure 27 C) were then transferred to the greenhouse after acclimation. The plants were cultivated in the standard manner, and the descendants were subsequently analysed.

The shoot meristem in wheat can likewise be targeted for modification, as described in Sautter et al. 1995. To this end, seeds were sterilized and washed through soaking in 70% ethanol for 2 minutes, followed by a sodium hypochlorite treatment and four rinses in water for the production of vegetative shoot apical meristems. The sterile seeds were then sown in test tubes on an MS medium, supplemented with 100 mg/l cefotaxime, 2% sucrose, and 0.8% Difco agar. Shoot apical meristems from 6-10 day old plants were subsequently exposed through the removal of the coleoptiles and the first three to five leaves. Roots were then trimmed to approximately 5 mm. The explants were then supplemented with different sucrose concentrations (optimum: 10%) and placed on an MS base medium (0.8% agarose). Following particle bombardment, the explants were then transferred to a further culture on MS agarose.

The flower organs can also be targeted in wheat. In wheat, the shoot meristem differentiates itself at a very early stage of development to form immature ears or shoots a few weeks after seeding. These immature ears can be found on the bottom of the shaft (see Fig. 28, left-hand image). A window is formed in these immature ears through an incision in the shoot. The meristematic tissues can be accessed through this window with various transformation techniques. Following transformation, the wound was closed, and the transformed system was cultivated further until the seeds matured (see Fig. 28 centre left, centre right and right-hand image) and the descendants were subsequently analysed. Alternatively, the immature ears can be removed from the inflorescence, sterilized *in vitro*, and detached in a targeted manner. The maturation and seed production can then be carried out *in vitro*, on the basis of Barnabas et al., 1992.

It has furthermore been shown that it is possible to target immature inflorescence in wheat using a gene gun (Leduc et al., 1994, Sautter et al., 1995). It was shown that, following

bombardment, the cells in the tissue treated in this manner express inserted reporter genes, and can continue to divide.

***Brassica napus*:**

It has also been shown for rapeseed that the shoot apical meristem develops in the so-called "heart stage." A transformation process for rapeseed in this stage that can also be used with the method disclosed herein is described in Huang et al., 2009.

Moreover, when rapeseed is used as the target plant, the shoot meristem can be transformed in a targeted manner after germination, or when the plants have reached the 2-8 leaf stage. To this end, the leaf primordials covering the meristem are carefully removed with a scalpel. The exposed meristems are then preferably treated with an antioxidant for their protection. Subsequently, the meristematic regions can be transformed by means of various transformation techniques. The plants can also be cultivated until the reproductive organs have reached maturity, and the descendants thereof can be analysed for the presence of the modification that has been introduced in a targeted manner.

Rapeseed buds can also be targeted. The buds in a rapeseed inflorescence are produced continuously. New buds are produced on the tips of the racemes. Two approaches can be used for the transformation of floral organs in rapeseed. In the first approach, immature buds can be opened *in situ*, and the reproductive tissues can be activated in a targeted manner. Following treatment, untreated inflorescence and pods are removed, and the inflorescence is then covered in order to promote self-pollination. The seeds are harvested, and the descendants are analysed. In the second approach, all of the differentiated buds are carefully removed from the raceme/panicle, and the floral meristem is left exposed. These meristems are then treated with various types of transformations. The meristems are then covered so that normal development can continue. All of the husks/pods are harvested, and the descendants are tested in terms of their molecular biology and their phenotypes.

***Glycine max*:**

Meristematic regions can likewise be activated in a targeted manner to bring about transformation in soya beans.

The shoot meristem from an embryo is exposed to light and transformed, as described in McCabe (McCabe et al., 1988). To this end, mature soya seeds (BR-16, Doko RC, BR-91 and Conquista) are subjected to surface sterilization in 70% ethanol for 1 minute, followed by immersion in 1% sodium hypochlorite for 20 minutes, and then three rinses in sterilized distilled water. The seeds were soaked in distilled water for 18-20 hours. The embryonic axes were cut out of the seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were placed in a bombardment medium (BM: MS (Murashige and Skoog, 1962) basal salt mixture, 3% sucrose, and 0.8% Phytagel sigma, pH 5.7), the apex oriented upwards in 5 cm culture dishes with 12 ml culture medium. As soon as the shoots derived from the embryonic axes reached a length of 2-3 cm, a 1 mm long section was removed from the base of each leaf for the analysis of GUS (beta-blucuronidase) (McCabe et al., 1988). The shoots, or sprouts that express the exogenous DNA were transferred individually into a plastic pot containing 0.2 l autoclaved, fertilized soil (vermiculite (1:1)), and then kept covered and sealed with a plastic bag and rubber band in the greenhouse. The rubber band was removed after 1 week. After another week, the plastic bag was removed. As soon as the acclimated plants reached a length of about 10 cm, they were transferred to pots with 5 l of fertilized soil until seeds start to develop (McCabe et al., 1988). As soon at the plants were grown, leaf samples were removed for analysis. The plants were then grown to maturity in order to analyse their descendants for the targeted modification.

Alternatively, the meristems of the immature embryos were targeted for transformation. To this end, the pods were harvested 5 to 20 days after flowering, and the embryos were extracted with a scalpel and gripping tool between the heart or cotyledon stages. These embryos were placed on an embryo growth medium, and the shoot apical meristem is transformed in a targeted manner with various delivery/transformation methods. The embryos were then grown in the dark for 1 to 10 weeks until they reached full maturity, as described in Buchheim et al., (1989), and exposed to light for embryo germination. The plantlets grown in this manner were cultivated to maturity in a greenhouse. The seeds were harvested, and the descendants were analysed. Recombinant constructs were introduced into the shoot meristems of the germinating soya bean plants as described above (Chee et al., 1989). Seeds from *Glycine max* L. Merr (Cv A0949) were sterilized by immersion for 15 minutes in a 15% Clorox solution, followed by numerous rinses with sterilized distilled water. Seeds were placed for 18 to 24 hours on sterilized, moistened paper towels in Petri dishes for germination, at 26° C in the dark. The seed cases were removed, one of the two cotyledons of each germinated seed was removed,

and the half-seeds, together with the shoot bud of the seedling (Plumula), the cotyledon nodes, and the neighbouring cotyledon tissue, were inoculated overnight with liquid cultures of an avirulent Agrobacterium strain, C58Z707, which contains the binary plasmid pGA482G. Other introduction or transformation methods can be used instead of Agrobacterium transformation. Another approach is described in Chowrira et al., 1995. In this approach, the terminal buds of plantlets (7-10 days old) are exposed through the removal of the surrounding leaf tissue. Foreign DNA is injected with a syringe, also containing lipofectin as a transfection agent, and the meristem is subsequently electroporated with the mixture. The plants are grown to maturity without selection, and chimeric plants can subsequently be obtained in this manner. The descendants of these plants are then analysed.

Access to the stigmas of the soya bean flowers to be pollinated is obtained as described in Shou et al., (2002). In summary, all of the experiments were carried out in the late afternoon, with flowers that had been naturally pollinated that same morning. Two petals and one keel were removed, in order to expose the stigmas of soya bean flowers to light. Stigmas were severed at the border between the ovary and the stigma, and plasmid DNA (concentrations of 25, 80, 100 or 150 µg/ml) was applied to the exposed stigma. The treated flowers were tagged, and untreated flowers and buds on the same node were removed. The shoots that developed from the treated flowers were harvested individually. Alternatively, the floral meristem of the soya bean can be transformed in a targeted manner before the development of terminal buds by removing the primordials or if the flowers begin to develop further. This exposure is obtained through the excision of the primordial with a scalpel. The floral meristem is covered as soon as it has been transformed. Once the inflorescence has developed and self-pollination has begun, the shoots of the treated plants can be harvested, the seeds processed, and the descendants can be tested for the targeted genomic modification.

***Gossypium sp.*:**

Experiments according to the present invention for introducing a targeted modification of interest can also be performed for cotton by treating the meristems of embryos as described in Aragão et al., (2005). In this approach, seeds (variants 7mH, CD-401, Antares and ITA94) were harvested by hand, and fibrous material was removed with an acid treatment. Concentrated sulphuric acid was added, and the seeds (3 ml/g seeds) were stirred thoroughly for 1 minute with a glass rod. The seeds were then transferred immediately into 5 l water, rinsed three times with distilled water, and dried on a paper towel. Mature seeds were surface-

sterilized with 70% ethanol for 10 minutes, followed by 1 minute treatment in 2.5% calcium hypochlorite, and rinsed three times in sterilized distilled water. The seeds were then soaked in distilled water for 24 hours, upon which the seeds were left to germinate for 16 hours at room temperature in darkness. Embryonic axes were cut out of seeds, and apical meristems were exposed by removal of the cotyledons. Explants were transferred to an MS medium, containing 3% glucose (5 mg/l benzylaminopurine (BAP), 0.8% Phytagel (Sigma)). The pH was set to 5.7 prior to autoclaving. Embryonic axes were produced as described above and positioned in a bombardment medium (MS basal salt mixture medium, 3% glucose, 5 mg/l BAP, and 0.8% Phytagel Sigma, pH 5.7), with the apex at the top, in 5 cm culture dishes with 12 ml culture medium. At this point, the meristems could be transformed or transfected. The treated apical meristems were cultivated in darkness. The meristems exhibiting growth were transferred to a growth chamber. The plantlets were then transferred to a greenhouse and cultivated to maturity. The descendants were analysed.

Alternatively, embryo meristems were transformed, as described in Rajasekaran (2013).

In another approach, the meristems of immature embryos were targeted *in vitro*, according to the protocol described by Mauney (1961). The culture medium that was used was composed of White's nutrient mixture, with all of its ingredients, plus five times the normal concentration and supplements (40 mg/l adenine sulphate, 250 mg/l casein hydrolysate, 150 ml/l coconut milk, and 7 g/l NaCl). The medium was stiffened with 8 g/l bacto-agar, and 20 g/l sucrose as a carbohydrate source. The feature of this medium which plays the most significant role in the success of the cultivation method is the adjustment of the osmotic pressure to a high level, which can be achieved through the addition of 7 gm/l NaCl. After the embryos grew on this medium for 3-4 weeks, they were transferred to a medium with a medium osmotic pressure (3 g/l NaCl instead of 7 g/l), and then into a medium without NaCl after a further growth period of 2 weeks. The successfully cultivated embryos in the latter medium germinated, and were planted in soil.

In order to transform shoot meristems into cotton plantlets, meristems were transformed in an initial approach as described in Zapata et al., (1999). Seeds were surface-sterilized with concentrated sulphuric acid (1 hour) at 50 rpm in a rotary shaker (50% Clorox (1 hour)), and rinsed at least three times with sterilized double-distilled water. The seeds were then placed on a medium stifferend with 0.15% (mass/volume) Gelrite, pH 5.7, containing Murashige and

Skoog (MS) inorganic salts (Murashige and Skoog, 1962), and 2% sucrose, for germination. The seeds were incubated for about 3-4 days at 28° C in the dark. The shoot tips were isolated, and then transferred into MS inorganic salts (Murashige and Skoog, 1962), with 100 mg/l myo-inositol, 0.5 mg/l thiamine/HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine/HCl, 3% sucrose, and 0.15% (mass/volume) Gelrite, at pH 5.7. After isolation, a recombinant construct of interest can be delivered, and the plant material can subsequently be transferred to a new medium. The vital transformed apices are transferred to a fresh medium. The surviving apices are then transferred to the same medium, but without kanamycin. As soon as roots develop, the plants with roots (T0) were transferred to soil, and allowed to grow to maturity in a greenhouse. The descendants could then be analysed.

Alternatively, the cotton meristems were transformed as described in Keshamma et al., (2008). To this end, seeds from a strain, viz. NC-71, were soaked overnight in distilled water and surface-sterilized, first with 1% Bavistin for 10 minutes, and then with 0.1% $HgCl_2$ for a few seconds, and subsequently washed thoroughly with distilled water. The seeds were left to germinate later at 30° C in darkness on Petri dishes. Two day old seedlings were used as explants. The seedlings with straight plumules/primary buds were infected by separating them from the cotyledons without damaging them, such that the meristem was visible. A transfection or transformation method of interest can then be used. The seedlings were subsequently transferred to autoclaved Soilrite (Vermiculite equivalent), watered, and covered for germination under aseptic conditions in a growth chamber, 5 seedlings per pot. After 5 to 6 days, the seedlings were transferred into pots with Soilrite, and allowed to grow for at least 10 days before they were transferred to the greenhouse. The mature plants could then be analysed.

In another approach, cotton meristems were transformed according to the protocol described in McCabe et al., (1993).

In another approach, cotton flowers could be transformed in a targeted manner according to the method described in Gounaris et al., (2005). In this method, cotton plants of the Christina type are used for the transformation. Flowers that are to be used as pollen receptors have to be separated from male plants two days prior to the expected dehiscence. On the morning of the pollination day, donor flowers with intact stamens were collected 1-2 hours after blooming. Each donor flower could then be treated with a transformation or transfection method of interest. These inflorescences were used for further pollination of receptor flowers after the

removal of the male germ cell. The pollinated flowers were allowed to develop and produce seeds. The descendants could then be analysed in terms of their molecular biology. In another approach, flower meristems or immature flowers can be genetically modified in a targeted manner. For this, the meristem forming the fruiting branch, the fruit bud, and the immature flower were exposed by removing the primordials and bracts. A delivery method (transformation/transfection, biological, chemical or mechanical) of interest can then be used according to the present disclosure. Subsequently, the treated zones were covered and allowed to continue to grow. The fruits were then harvested. The descendants could be analysed with regard to their molecular biology, as well as their phenotypes.

***Oryza sativa*:**

If the target plant of interest is rice, the following methods can be used for introducing a targeted genomic modification into a meristematic target structure.

According to the methods of Naseri et al., 2014, rice seeds (O. sativa, Hashimi) are sterilized through soaking in 90% ethanol (1 minute), and washed three times with water. Sterile seeds are placed on wet cotton for two days at 22° C. At this stage, the inoculation with *A. tumefaciens* was performed in embryonic apical meristems of the saturated seeds on the surface of the seed where a shoot would later develop. The surface is penetrated to a depth of approximately 1 to 1.5 mm with a needle (0.7 mm in diameter, previously immersed in *A. tumefaciens* inoculum) The inoculated seeds were then covered with aluminium foil in bottles, placed on filter paper on wet perlite, and incubated at 23° C in darkness for nine days. 70 to 75% of the inoculated seeds germinated. In order to kill off *A. tumefaciens*, the seedlings were immersed in an aqueous solution (1000 ppm) of cefotaxime for 1 hour at room temperature. For root formation, the seedlings were placed in a Yoshida solution. Lastly, the seedlings were planted in pots, and grown to maturity (T0) under non-sterile conditions. Self-pollination was permitted, producing a T1 generation.

In another approach, the meristems of immature embryos were treated to bring about a targeted modification with the CRISPR tools disclosed herein. Immature seeds were harvested 3-12 days after pollination. The immature embryos are placed in a maturation medium (Ko et al., 1983), after which the transformation/transfection methods of interest can be used. The embryos were brought to maturity, as described in Ko et al., (1983). The seeds were harvested, and the descendants analysed in terms of their molecular biology.

Meristems from rice plants were treated as described in Muniz de Pèadua et al., (2001). In this approach, rice seeds were surface-sterilized and brought to germination *in vitro*. After approx. 4 days, the shoot apices were excised from the distal part of the first internode of the epicotyl and the coleoptile. After exposure, a delivery system of interest can be used. This enables root formation. The plants obtained were then transferred to the greenhouse and cultivated further, and the descendants were subsequently analysed.

Immature ears of rice were treated for the targeting of flower organs as described in Rodin et al., (2014). The inflorescence is used in stage 51 (Beginning of panicle emergence: tip of inflorescence emerged from sheath) in accordance with the BBCH scale (Lancashire et al., 1991) and can then be treated, and the descendants can be subsequently analysed. The floral meristems can also be targeted. To this end, the meristems must be exposed by removing the surrounding tissue and subsequently transformed and cultivated further, after which the descendants are analysed. The immature ears can be treated by means of transformation or transfection, either before differentiation, or after the surrounding primordials are removed. The treated ears were then permitted to develop further. The resulting seeds of these flowers were harvested, and the descendants were examined for targeted editing events (Itoh et al., 2005).

Example 16: Transient transformation methods

Specifically in terms of the *in planta* transformation of meristematic tissue, there is significant interest in creating transient transformation methods, in particular for the introduction of CRISPR tools of interest, as only the targeted modification to be introduced is passed on to the descendants of a cell, and not the tools themselves. Methods that are controlled and can be controlled in this manner have become increasingly important in the field of plant breeding due to regulatory requirements and safety concerns. Both transient and stable transformation methods must, as a matter of course, be adapted to the tissue that is to be transformed. For this reason, the following experiments were carried out, some of which have broad and general applications, with others having applications for specific tissues (pollen, meristems, flowers, etc.).

Cas9:

Cas9 was obtained from New England Biolabs (NEB), PNA BIO, ToolGen, LDBIOPHARMA or ABM, or Cas9 was purified, as described in Liu et al., 2015.

In-vitro transcription of sgRNAs:

The in-vitro transcription was carried out as described by Zuris et al., 2015. Linear DNA fragments containing a T7 promoter binding site followed by the 20 bp sgRNA target sequence were transcribed *in vitro* using the T7 RNA High Yield Synthesis kit (NEB) in accordance with the directions of the manufacturer. RNA transcribed in vitro was precipitated with ethanol, and purified by gel electrophoresis on a 10% polyacrylamide Criterion TBE urea gel (Bio-Rad). Excised gel fragments were extracted in 420 µl 300 mM NaCl overnight on a shaker surface at 4° C. Gel-purified sgRNA was precipitated with ethanol and dissolved in water, and the sgRNA concentration was then quantified through UV absorption. The sgRNA can then be snap-frozen and stored at −80° C. Alternatively, gRNAs were obtained as described in Kim et al., 2014. For this, RNA was transcribed in vitro through T7-RNA-polymerase run-off reactions, using the MEGAshortscript T7 kit (Ambion). Templates for sgRNA or crRNA were created through the accumulation and extension of two complementary oligonucleotides. The transcribed RNA was purified through phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. The purified RNA was quantified through spectrometry.

Alternatively, another protocol (described in Ramakrishna et al., 2014) can be implemented, according to which the RNA was transcribed *in vitro*, through run-off reactions using T7 RNA polymerase. Templates for sgRNA transcription were created through annealing/hybridisation and the extension of two complementary oligonucleotides. The transcribed RNA was separated on a 8% denatured urea-PAGE gel. The RNA was placed in nuclease-free water, and subsequently purified and obtained through phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. The purified RNA was quantified through spectrometry.

Protein Cas9 and gRNA complexation:

As described in Zuris et al., (2015), 1 µl 200 µM Cas9 protein was mixed with 2 µl 50 µM sgRNA and incubated for 5 minutes at room temperature for the introduction of Cas9-sgRNA complexes. The complex was then mixed with 3 µl of either RNAiMAX or Lipofectamine 2000 and incubated for a further 30 minutes, prior to the injection. Alternatively, the complexation was carried out as described in Kim et al., 2014. In this approach, Cas9 protein (4.5-45 mg) was pre-mixed with in-vitro transcribed sgRNA (6-60 mg). Cas9 protein in a storage buffer (20

mM HEPES, pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerine) were dissolved with sgRNA in nuclease-free water, mixed, and incubated at room temperature for 10 minutes.

Agrobacterium (Ab)

Pollen transformation with Ab:

Pollen transformation was carried out as described in Li et al., (2004). In this approach, flowers with freshly developed and exposed anthers were collected. An aliquot of an Ab solution was transferred into sterile 1.5 ml test tubes, and centrifuged for 10 minutes at 3,000 rpm. The pellet was re-suspended (in pollen germination medium, with approx. 50 mg pollen/ml) and a vacuum (−80 Pa) was applied for 30 minutes, and then slowly released. The suspension was subsequently centrifuged at 3,000 rpm for 5 minutes, and the pellets, i.e. the pollen, were used directly for pollination.

Shoot apical meristem transformation with Ab:

Meristems from seedlings were transformed on the basis of the protocol described by Chee et al., 1989. Inoculations were carried out at three different sites by inserting a 30′/2 gauge needle into the plumule, cotyledonary nodes and adjacent regions, and injecting 30 ml of the Ab cells at each injection point. The germination process of the seeds infected with Ab was continued in that the seeds were transferred to sterilized moist paper and further incubated at 26° C in darkness for about 4 hours. The seedlings were then planted in the ground and allowed to develop fully. Alternatively, another protocol (as described in Ramakrishna et al., 2008) can be implemented. The seedlings are infected, as soon as the plumule emerges, by separating the cotyledons, without destroying them, such that the meristem is visible. The meristems are then pierced with a sterile sewing needle, and then dipped in an *Agrobacterium* culture for 60 minutes. After the infection, the seedlings were washed briefly with sterile water, and later placed on autoclaved Soilrite.

Particle bombardment:

Embryo meristems were bombarded as follows: embryos in the coleoptile stage, or the heart stage, were placed in in embryo maturation medium supplemented with osmotic agent 0-6 hours prior to the bombardment, as described in Vain et al., (1993). The particle processing and preparation process was implemented in accordance with a routine DNA precipitation process with calcium chloride and spermidine. With protein/RNA mixtures, the protocol described by Martin-Ortigosa et al., (2014), was carried out, wherein the mixture was dried or

freeze-dried together with the gold particles. 16-24 hours after the bombardment, the embryos were placed on a ripening medium without an osmotic agent.

Bombardment of anthers:

Bombardment of anthers can be carried out in accordance with Twell et al., 1989. For this, plant anthers were surface-sterilized 1d prior to releasing pollen in 10% Clorox for 10 minutes, and rinsed in sterile distilled water. The anthers were sliced transversely with a sterile razor blade, and 20 anther sections were placed on solid MSO medium with a surface area of 4 cm^2, with exposed thecae.

In another approach, anthers were bombarded as described in Obert (Obert et al., 2008). In this approach, spikes/ears were harvested as soon as the microspores were in the middle mononuclear development stage. Two different pre-treatments were used in our studies, together with the use of material which was not pre-treated. Cold pre-treatment involved placing the plant material on a moist filter paper in a cold room at 5° C (Dedicova et al, 1999). After the pre-treatment of the material (14 days in cold storage), the specific portions of the ears containing microspores were selected at the suitable stage and used as further experimental material. The material was surface-sterilized (in 70% (vol./vol.) alcohol), and washed three times with sterilized distilled water. For the mannitol pre-treatment, suitable portions of the fresh ears containing microspores in the correct stage were surface-sterilized (in 70% alcohol) and washed three times with sterile distilled water. Anthers were isolated in sterile conditions and placed in a liquid medium for pre-treatment (0.3 M mannitol or water) and stored in a cold room at 5°C. Anthers were either isolated prior to bombardment or placed on the surface of a cultivation medium (FHG media, Kasha et al., 2001) after pre-treatment. The bombardment conditions were: distance (macrocarrier—anthers in Petri dishes): 9 cm; pressure settings of 650, 900 and 1,100 psi. Anther cultures were then cultivated at 26° C in the dark in a tissue culture growth chamber.

In another approach (Touraev et al., 1997), single-cell microspores and pollen grains in a middle bi-cellular stage were bombarded immediately after isolation in the respective culture media. The suspension (0.7 ml), containing approx. 5×10^5 cells, was evenly spread on a sterile filter paper (Whatman No. 1), and transferred into a 10 cm Petri dish (Sterilin, UK). The helium-driven PDS-1000/He particle discharge system (Bio-Rad, USA) was used for the biolistic transformation. The bombardment was substantially carried out as described in

Sanford et al., (1993). Plasmid DNA was precipitated onto gold particles (Bio-Rad, USA) with a mean diameter of 1.1 μm. Each transformation comprised three bombardments. The bombarded microspores, or mid-bi-cellular pollen grains, were washed off of the filter paper, and incubated in a relevant maturation medium.

Bombardment of flowers:

On the basis of a protocol described by Twell et al., 1989, groups of 10 flowers with intact curved petals were bombarded, wherein the cut stems were suspended in distilled water.

Bombardment of pollen:

On the basis of a protocol described by Twell et al., 1989, pollen from mature flowers was collected in sterile micro-centrifuge test tubes. Prior to bombardment, dry pollen samples were suspended in a liquid MSO medium with a density of approx. 106 grains/ml. The pollen suspension (1 ml) was immediately pipetted onto the surface of a 9 cm Petri dish, containing an MSO medium thickened with agar, on which a sterile Whatman no. 1 filter paper with a nylon membrane (Genescreen, NEN) was previously placed. The bombardment was carried out within 60 minutes after the plant material was transferred to the MSO medium. The precipitation of plasmid DNA onto tungsten micro-projectiles and the bombardment took place as described in Klein et al. After bombardment, the Petri dishes or intact flowers were incubated in distilled water at 26° C in the light.

In another approach, pollen bombardment was carried out as described in Horikawa et al., (1997). In this approach, mature pollen grains were collected from extruded tassels. The subsequent bombardment preparation steps were carried out very quickly, because the life expectancy of the pollen decreases quickly. The pollen was immersed in a liquid MS medium containing 30 g/l sucrose (pH 5.8). The 4.0×10^5 pollen grains (in 1 ml medium) were adsorbed onto the surface of a piece of microfilter (pore size 0.45 um, Fuji Film Co., Tokyo) through vacuum filtration. The microfilter was placed on 1% agar plates in a Petri dish in preparation for bombardment with a particle cannon.

Pollination with the treated pollen:

The protocol described in Touraev et al., 1997, was implemented for pollination with the bombarded pollen. This protocol involved emasculating mature flowers shortly before flowering, while the anthers were still closed, one day prior to pollination. The pollen that

matured *in vitro* was washed repeatedly in a GK medium without quercetin, and then transferred to stigma in droplets of 3 µl. Stigmas with good stigma exudate production were selected for the pipette pollination process. To prevent cross-pollination, all of the other flower buds in the climatic chamber were removed one day before opening. Mature seed capsules or pods were collected 3-4 weeks later.

In another approach, pollen bombardment was carried out as described in Horikawa et al., (1997). For this, pollen was placed in 1 ml liquid MS medium. The pollen was used immediately for pollination by pipetting it onto the awns of an ear (previously covered with sacs), three days after awn emergence. The pollination treatments were carried out on 20 ears. As control, pollen was pollinated by a sample without DNA.

Bombardment of flowers with HELIOS:

The flowers or inflorescence were bombarded with the "Helios" hand pistol from Bio-Rad, in accordance with the manufacturer's instructions. As soon as the inflorescence or the flowers were exposed, they were bombarded with 1 to 5 shots at 50-300 psi. The exposed meristems were then covered and the inflorescence or flowers were able to continue maturing.

In another approach, the protocol described by Gounaris et al., 2005, was carried out. The flowers used as pollen receptors were emasculated two days prior to the expected pollination date. On the morning of the pollination day, intact stamens were collected from the donor flowers 1-2 hours after opening. Each of the donor flowers was treated with 4-5 shots from the particle cannon while on a flat surface in a Petri dish covered with a nylon net. The particle cannon was operated with a helium pressure of 400 psi, and was equipped with a particle diffusion screen. The helium gas purity was class 4.5 (99.994%). Each bombarded inflorescence was used to pollinate approx. 15-20 emasculated receptor flowers. The pollinated flowers were able to continue developing, and produce seeds.

Microinjection: DNA/RNA/protein and combinations

Embryo microinjection:

Embryo microinjection was performed as described in Neuhaus et al., 1987. Embryos positioned on a cover glass were individually selected visually using a manual micro-capillary, connected to a silicone tube, and transferred into a medium on a specimen holder in approx. 2 µl droplets for the microinjection (Spangenberg et al., 1986). The microinjection was carried

out by securing the embryoid bodies in place with a retention capillary, and performing a microinjection into the respective cells. Exogenous DNA was injected as a 1:1 mixture of linearized (through cleavage of the plasmids outside the inserted genes) and super-coiled molecules, in a quantity of approx. 0.5 µg/µl in 50 mM NaCl, 50 mM tris-HCl, pH 7.8.

Microinjection of shoot meristems with *Agrobacterium* (Ab):

Shoot meristem microinjection with Ab was carried out as described by Sivakumar et al., (2014). 100 µl of the culture was microinjected with an insulin syringe into the embryonic shoot apical meristems of germinated cotton seeds. The culture was microinjected 1-5 times (0.5-1.0 mm depth), in order to assess the effect of the number of microinjections in and around the embryonic shoot apical meristem. Excess bacteria culture was removed after dabbing the infected seeds on sterile filter paper (Whatman no. 1). The seeds were co-cultivated in darkness for two days on a ½ strength MS medium. After co-cultivation, the seedlings were washed with cefotaxime (200 mg/l), and transferred into an antibiotic selection medium containing cefotaxime and hygromycin B.

Microinjection of DNA in shoot meristems:

The microinjection was carried out as described in Lusardi et al., (1994). Mature, dried seeds were washed for 20 seconds with absolute ethanol, followed by sterilization with commercial bleach (2.5% NaClO), supplemented with 0.01% Tween 80 (20 minutes while shaking). The seeds were then rinsed four to five times with sterile distilled water. The germination was induced by incubating the seeds in a 9 cm Petri dish between filter papers with sterile distilled water in the dark for 3-4 days at 27° C. During this time, the shoot passed through the integument and reached a length of approx. 0.8 cm to 1.0 cm. At this point, the shoot was removed from the seed at the scutellar node level. The coleoptile and the five or six embryonic cotyledons were removed under a stereomicroscope. After the embryonic cotyledons were removed, the uncovered apices, surrounded by two leaf layers, were exposed at various stages of development. The isolated apices were cultivated in 9 cm Petri dishes in an MS medium (Murashige and Skoog, 1962), supplemented with 2% sucrose and thickened with 0.8% Difco Bacto-Agar (Difco Lab., Detroit), and cultivated further with a 27° C/22° C temperature regimen and a 16/8 hour light/dark lighting schedule. Normal plantlets developed within 10 days. Over the next 15-20 days, they reached a sufficient size for transferring into pots, and were placed in the greenhouse. For the microinjection, the plasmids for the injection were dissolved in injection buffer (10 mM Tris-HCl and 0.1 M EDTA, pH 7.5). The injection buffer

was filtered through a 0.2 µm disposable filter unit (Schleicher and Schuell, Germany), in order to sterilize the solution and prevent particle contamination. All of the injections were carried out under sterile conditions. The isolated shoot meristems from maize were transferred into 9 cm Petri dishes. The MS medium was supplemented with 2% sucrose, and thickened with 0.8% Difco Bacto Agar. The apices were oriented on the medium such that the apical domes were clearly visible. The cells of the L2 layers of the meristems were injected using a (high power) stereomicroscope (up to 200× enlargement; SV 8, Zeiss, Germany) equipped with an embryo splitter system from Research Instruments (UK). In some experiments, a co-injection of FITC dextran was used in order to better identify the injected cells (Neuhaus et al., 1993; Schnorf et al., 1991). An injection capillary (tip diameter of less than 1 µm) was mounted on the mechanical micromanipulator of the system and was connected to a microinjector (Eppendorf 5242 microinjector), which delivered approximately 3 µl into the cells at a constant volume (Neuhaus et al., 1986, 1987; Schnorf et al., 1991). The second manipulator of the embryo splitter system was used to stabilize and move the apices during the injection. This manipulator was also equipped with a micro-needle for this purpose so that the apical meristems could be moved and fixed in place, so that they could be treated in the correct position.

WHISKERS

Whisker-mediated delivery into the various meristems was performed as described by Frame et al., (1994). The exposed tissue was treated with 40 µl 5% Whisker suspension and 25 µl of plasmid DNA. The contents of the reaction vessel were first lightly stirred, and then placed either upright in a multi-sample head on a Vortex Genie II vortex mixer (Scientific Industries Inc., Bohemia, N.Y.), or horizontally in the holder of a Mixomat amalgam mixer (Degussa Canada Ltd. Burlington, Ontario). The transformation was carried out for 60 seconds by mixing at full speed (Vortex Genie II), or at a fixed speed for 1 second (Mixomat).

Alternatively, whiskers were loaded, together with DNA/RNA or protein mix whiskers, into the pipette of a micromanipulator, and then macroinjected into meristematic tissue. Cell penetrating peptides: DNA/RNA/protein and combinations thereof

Mixing of cell penetrating peptides and Cas9 protein and gRNA:

A protocol on the basis of Ramakrishna et al., 2014, was implemented for the use of cell penetrating peptides. One day after plating, the cells were washed with Opti-MEM and with Cas9-M9R and sgRNA:9R, either successively or simultaneously. The sgRNA:9R complex

was formed during a 30 minute incubation of 10 mg sgRNA and 30-50 mg 9R peptide in 250 ml (for the sequential treatment) or 100 ml (for the simultaneous treatment) of Opti-MEM medium at room temperature.

Embryo:

TAT peptides (Tat, Tat2, M-Tat) were used for introducing GUS enzymes into wheat embryos. The TAT peptide and GUS enzyme were first prepared in separate micro-centrifuge test tubes. An unmarked TAT peptide (4 µg) was added to sterile water (final volume: 100 µl). Likewise, 1 µg of the GUS enzyme (Sigma Aldrich) was added to sterile water, to obtain a final volume of 100 µl. The contents of the two test tubes were mixed together, resulting in a 4:1 ratio of peptide to protein in the mixture. The mixture was incubated for 1 hour at room temperature, and then added to the isolated, immature embryos (in a 2 ml micro-centrifuge test tube) in the presence or absence of the permeabilization agent (toluol/ethanol 1:40, vol./vol. with respect to the total volume of the peptide/protein mixture). After 1 hour incubation at room temperature, the embryos were washed twice with the buffer, and subjected to a permeability and trypsin treatment (1:1 (vol./vol.) permeabilization buffer) for 5 minutes at room temperature. The embryos were washed twice with permeabilization buffer, followed by a histochemical GUS analysis of the embryos. 1 µg of the GUS enzyme was transfected for the delivery by the Chariot Protein Transduction kit (Active Motif, Carlsbad, Calif., USA), in accordance with the manufacturer's instructions. Permeabilized and non-permeabilized embryos were incubated for 1 hour with the Chariot-GUS complex. All of the post-incubation steps were the same as those described for the TAT peptides.

Transformation of microspores:

The transformation of microspores using cell-penetrating peptides was carried out in accordance with Shim et al., (2012). The microspore extraction was performed in accordance with Eudes and Amundsen (2005), and all of the steps for isolating microspores were carried out using the NPB-99 liquid medium (Zheng et al., 2001; Eudes and Amundsen, 2005). After washing the microspores with NPB-99, 2-3 ml microspore solution were layered onto 2 to 3 ml 30% Percoll solution, containing 400 mM mannitol and 10 mM MES, pH 7.0. The microspores were centrifuged for 5 minutes at 100 × g, at 4° C. The cells that formed a band at the Percoll/NPB-99 cleavage site were diluted to 15 ml with NPB-99 in a fresh 15 ml centrifuge test tube, and then centrifuged again. The supernatant was decanted off, and the microspores were re-suspended in approximately 1 ml NPB-99 medium. The microspore concentration was

determined using a hemocytometer, and adjusted to 2.5×10^5 cells/ml. Five treatments, including the control, were applied to microspore suspensions of the same extraction as follows: T1, control treatment, comprising 200 µl sterilized water; T2, 1 µg dsDNA in 100 µl sterilized water was added to 4 µg TAT2, diluted in 100 µl sterilized water, and mixed lightly, resulting in a 1:4 ratio of dsDNA to TAT2 (dsDNA:TAT2); T3, 1 µg dsDNA, diluted in 100 µl sterile water, and 6 µl Chariot (Active Motif, Carlsbad, Calif.), diluted in 100 µl sterilized water, were mixed together (dsDNA-Pep1); T4, 4 µg RecA (MJS Biolynx, Brockville, Canada; #UB70028Z) in 50 µl sterile water and 1 µg dsDNA in 50 µl sterile water were mixed together for 15 minutes, and 6 µl Chariot in 100 µl sterile water was added to the dsDNA-RecA solution, to obtain a final volume of 200 µl in a 2 ml micro-centrifuge test tube (dsDNA-RecA-Pep1); T5, µg RecA in 50 µl sterile water and 1 µg dsDNA in 50 µl sterile water were mixed together for 15 minutes. 4 µg Tat2 in 100 µl sterile water was added to the dsDNA-RecA solution to achieve a final volume of 200 µl in a 2 ml micro-centrifuge test tube (dsDNA-RecA-Tat2). After incubation for 15 minutes at room temperature (RT), 5 µl Lipofectamine (Invitrogen, Carlsbad, Ca; #11,668 to 019) was added to all of the preparations, and they were then incubated for a further five minutes at RT. The mixtures were then added immediately to 50,000 pelleted microspores in 2 ml micro-centrifuge test tubes, and incubated for 15 minutes. 100 µl NPB-99 was then added to each test tube, and they were incubated for 45 minutes at RT. The transfected microspores were pelletised, the supernatant was removed, and the cells were washed twice with NPB-99. 1 ml NPB-99 was then added to the microspores in each of the test tubes. They were carefully mixed, and aliquots of 500 µl were pipetted into 35 mm Petri dishes, containing 3 ml NPB-99+10% Ficoll (Sigma, St. Louis, Mo.; F4375; NPB-99-10F) and 100 mg/l of the antibiotic cefotaxime (Sigma; # C7039).

Electroporation

Pollen transformation:

Pollen transformation by means of electroporation was carried out according to the protocol established by Shi et al., (1996). This involved electroporating mature pollen, germinating pollen, or pollen without an exine layer with a field strength of 750-1250 V/cm and a constant impulse of 13 ms.

Ultrasonic treatment

Pollen ultrasonic treatment:

Pollen transformation through ultrasonic treatment was carried out as described by Wang et al., (2000). 0.3 g of fresh pollen was collected in the morning, and mixed in 20 ml of a solution containing 5% sucrose with approximately 10 µg of the plasmid DNA of interest. The solution was treated with ultrasound, both before and after the plasmid DNA was added. A JY92-II ultrasound device from Ningbo Xinzi Scientific Instrument Institute was used for the ultrasonic treatment with the following parameters: intensity of 300 W, eight treatments for 5 and 10 second intervals. Subsequently, clipped maize silk was pollinated with the treated pollen.

References cited in the Examples section:

Aragäo, F. J., et al. (2005). "Germ line genetic transformation in cotton (Gossypium hirsutum L.) by selection of transgenic meristematic cells with a herbicide molecule." Plant Science 168(5): 1227-1233 / Record #: 2408

Artschwager, E. (1926). "Anatomy of the vegetative organs of the sugar beet." J. agric. Res 33: 143-176 / Record #: 2352

Barnabas, B., et al. (1992). "In vitro pollen maturation and successful seed production in detached spikelet cultures in wheat (Triticum aestivum L.)." Sexual Plant Reproduction 5(4): 286-291 / Record #: 2374

Buchheim, J. A., et al. (1989). "Maturation of Soybean Somatic Embryos and the Transition to Plantlet Growth." Plant Physiology 89(3): 768-775 / Record #: 2402

Chee, P. P., et al. (1989). "Transformation of soybean (Glycine max) by infecting germinating seeds with Agrobacterium tumefaciens." Plant Physiology 91(3): 1212-1218 / Record #: 2392

Chowrira, G., et al. (1995). "Electroporation-mediated gene transfer into intact nodal meristemsin planta." Molecular Biotechnology 3(1): 17-23 / Record #: 2393

Eapen, S. (2011). "Pollen grains as a target for introduction of foreign genes into plants: an assessment." Physiology and Molecular Biology of Plants 17(1): 1-8 / Record #: 2384

Frame, B. R., et al. (1994). "Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation." The Plant Journal 6(6): 941-948 / Record #: 1755

Gounaris, Y., et al. (2005). "Pollen-mediated genetic transformation of cotton with the Arabidopsis thaliana hmgr cDNA using the particle gun." Journal of Food Agriculture and Environment 3(2): 157-160 / Record #: 2330

Grandjean, O., et al. (2004). "In Vivo Analysis of Cell Division, Cell Growth, and Differentiation at the Shoot Apical Meristem in Arabidopsis." The Plant Cell 16(1): 74-87 / Record #: 2387

Hermann, K., et al. (2007). "1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (Beta vulgaris L.): a comparative study of fruits and seeds." Journal of Experimental Botany 58(11): 3047-3060 / Record #: 2370

Horikawa, Y., et al. (1997). "Transformants through pollination of mature maize (Zea mays L.) pollen delivered bar gene by particle gun." Journal of Japanese Society of Grassland Science (Japan) / Record #: 2383

Huang, Y., et al. (2009). "Probing the endosperm gene expression landscape in Brassica napus." BMC Genomics 10(1): 256 / Record #: 2377

Itoh, J.-I., et al. (2005). "Rice Plant Development: from Zygote to Spikelet." Plant and Cell Physiology 46(1): 23-47 / Record #: 2416

Keshamma, E., et al. (2008). "Tissue culture-independent in planta transformation strategy: an Agrobacterium tumefaciens-mediated gene transfer method to overcome recalcitrance in cotton (Gossypium hirsutum L.)." Journal of Cotton Science 12(3): 264-272 / Record #: 2405

Kim, S., et al. (2014). "Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins." Genome Research 24(6): 1012-1019 / Record #: 2288

Ko, S.-W., et al. (1983). "A simplified method of embryo culture in rice of Oryza sativa L." Bot. Bull. Acad. Sin 24: 97-101 / Record #: 2415

Krens, F. A., et al. (1988). "Transformation and regeneration in sugar beet (Beta vulgaris L.) induced by 'shooter' mutants of Agrobacterium tumefaciens." Euphytica 39(3): 185-194 / Record #: 1426

Leduc, N., et al. (1994). "Gene transfer to inflorescence and flower meristems using ballistic micro-targeting." Sexual Plant Reproduction 7(2): 135-143 / Record #: 2345

Li, X., et al. (2004). "Improvement of cotton fiber quality by transforming the acsA and acsB genes into Gossypium hirsutum L. by means of vacuum infiltration." Plant Cell Reports 22(9): 691-697 / Record #: 2419

Liu, J., et al. (2015). "Efficient delivery of nuclease proteins for genome editing in human stem cells and primary cells." Nat. Protocols 10(11): 1842-1859 / Record #: 2421

Lusardi, M. C., et al. (1994). "An approach towards genetically engineered cell fate mapping in maize using the Lc gene as a visible marker: transactivation capacity of Lc vectors in differentiated maize cells and microinjection of Lc vectors into somatic embryos and shoot apical meristems." The Plant Journal 5(4): 571-582 / Record #: 82

Mahn, A., et al. (1995). "Transient gene expression in shoot apical meristems of sugarbeet seedlings after particle bombardment." Journal of Experimental Botany 46(10): 1625-1628 / Record #: 2367

Martin-Ortigosa, S., et al. (2014). "Proteolistics: a biolistic method for intracellular delivery of proteins." Transgenic Research: 1-14 / Record #: 2260

Matthys-Rochon, E., et al. (1998). "In vitro development of maize immature embryos: a tool for embryogenesis analysis." Journal of Experimental Botany 49(322): 839-845 / Record #: 2285

Mauney, J. R. (1961). "The Culture In vitro of Immature Cotton Embryos." Botanical Gazette 122(3): 205-209 / Record #: 2409

McCabe, D. E., et al. (1993). "Transformation of elite cotton cultivars via particle bombardment of meristems." Nature Biotechnology 11(5): 596-598 / Record #: 2404

McCabe, D. E., et al. (1988). "Stable transformation of soybean (Glycine max) by particle acceleration." Nature Biotechnology 6(8): 923-926 / Record #: 2391

Muniz de Péadua, V., et al. (2001). "Transformation of Brazilian elite Indica-type rice (Oryza sativa L.) by electroporation of shoot apex explants." Plant Molecular Biology Reporter 19(1): 55-64 / Record #: 2412

Naseri, G., et al. (2014). "In planta transformation of rice (Oryza sativa) using thaumatin-like protein gene for enhancing resistance to sheath blight." African Journal of Biotechnology 11(31) / Record #: 2411

Neuhaus, G., et al. (1987). "Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids." Theoretical and Applied Genetics 75(1): 30-36 / Record #: 2123

Obert, B., et al. (2008). "Genetic transformation of barley microspores using anther bombardment." Biotechnology Letters 30(5): 945-949 / Record #: 2068

Olvera, H. F., et al. (2008). "Floral and Inflorescence Morphology and Ontogeny in Beta vulgaris, with Special Emphasis on the Ovary Position." Annals of Botany 102(4): 643-651 / Record #: 2372

Rajasekaran, K. (2013). Biolistic Transformation of Cotton Zygotic Embryo Meristem. T Transgenic Cotton. 958: 47-57 / Record #: 2403

Ramakrishna, S., et al. (2014). "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA." Genome Research 24(6): 1020-1027 / Record #: 2287

Ritchie, G. L., et al. (2007). "Cotton growth and development." from http://cotton.tamu.edu/mwg-internal/de5fs23hu73ds/progress?id=-gCnQaUKI-guBsRa08L1m_QGIEFw5-bHQ3iPfT8R-CI, / Record #: 2410

Rod-in, W., et al. (2014). "The floral-dip method for rice (Oryza sativa) transformation." Journal of Agricultural Technology 10(2): 467-474 / Record #: 2414

Sautter, C., et al. (1995). Ballistic microtargeting of visible marker genes to the shoot meristem of wheat. Gene Transfer to Plants, Springer: 152-156 / Record #: 2339

Sautter, C., et al. (1995). "Shoot apical meristems as a target for gene transfer by microballistics." Euphytica 85(1-3): 45-51 / Record #: 2373

Shi, H., et al. (1996). "Exine-detached pollen of Nicotiana tabacum as an electroporation target for gene transfer." Acta Botanica Sinica 38(8): 626-630 / Record #: 2417

Shim, Y.-S., et al. (2012). "dsDNA and protein co-delivery in triticale microspores." In Vitro Cellular & Developmental Biology - Plant: 1-10 / Record #: 1253

Shou, H., et al. (2002). "Irreproducibility of the soybean pollen-tube pathway transformation procedure." Plant Molecular Biology Reporter 20(4): 325-334 / Record #: 2399

Sivakumar, S., et al. (2014). "Optimization of factors influencing microinjection method for Agrobacterium - Mediated transformation of Embryonic Shoot Apical Meristem in Cotton (Gossypium hirsutum L. cv.SVPR-2)." International Journal of Current Biotechnology / Record #: 2420

Touraev, A., et al. (1997). "Plant male germ line transformation." The Plant Journal 12(4): 949-956 / Record #: 2380

Twell, D., et al. (1989). "Transient expression of chimeric genes delivered into pollen by microprojectile bombardment." Plant Physiology 91(4): 1270-1274 / Record #: 2316

Vain, P., et al. (1993). "Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize." Plant Cell Reports 12(2): 84-88 / Record #: 20

Wang, J., et al. (2000). "Transgenic maize plants obtained by pollen-mediated transformation." Acta Botanica Sinica 43(3): 275-279 / Record #: 2418

Wiebold, W. J. (2012). "Arrested Development in the Soybean Field." from http://ipm.missouri.edu/IPCM/2012/10/Arrested-Development-in-the-Soybean-Field/ / Record #: 2400

Zapata, C., et al. (1999). "Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex." Theoretical and Applied Genetics 98(2): 252-256 / Record #: 2407

Zhang, C.-L., et al. (2008). "Efficient somatic embryogenesis in sugar beet (Beta vulgaris L.) breeding lines." Plant Cell, Tissue and Organ Culture 93(2): 209-221 / Record #: 1461

Zuris, J. A., et al. (2015). "Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo." Nat Biotech 33(1): 73-80 / Record #: 2303

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<210> 9

<211> 377

<212> DNA

<213> Oryza spp. U3

<400> 9

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<211> 763

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<213> Zea spp. U3

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<210> 11

<211> 1880

<212> DNA

<213> Brachypodium spp. EF1

<400> 11

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<210> 12

<211> 2690

<212> DNA

<213> Maize Streak Virus (MSV) South African Strain

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<210> 13

<211> 6337

<212> DNA

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<211> 3249

<212> DNA

<213> Neisseria cinerea

<400> 20

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<211> 3294

<212> DNA

<213> Staphylococcus aureus

<400> 21

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<211> 3012

<212> DNA

<213> Campylobacter lari

<400> 22

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<210> 29

<211> 2145

<212> DNA

<213> Brome mosaic virus strain RNA3 R-BMV cDNA

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<210> 30

<211> 2111

<212> DNA

<213> Brome mosaic virus strain RNA3 gi|331498|gb|J02042.1|MBRCG3Z Brome mosaic virus 3a and coat protein genes, complete cds

<400> 30

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<211> 2150

<212> DNA

<213> Brome mosaic virus C-BMVA/G

<400> 31

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<211> 3787

<212> DNA

<213> Barley stripe mosaic virus BSMV ND18 RNA1 U35767

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<211> 2790

<212> DNA

<213> Barley stripe mosaic virus BSMV ND18 RNA3 U13917

<400> 34

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<211> 3768

<212> DNA

<213> Barley stripe mosaic virus BSMV_RNA1_gi|19744917|ref|NC_003469.1|

<400> 35

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<211> 3289

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<213> Barley stripe mosaic virus BSMV_RNA2_gi|19744922|ref|NC_003481.1|

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<211> 3164

<212> DNA

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cggttatact gttgctacgg aggacattaa catggatgtt caggattgta gacttaagtt 1080

cgggaagact tttcgacctt atgaatttaa ggaatcactg aaaccagtac tgaggacagc 1140

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gaagaaggcg ttaaaggtat tcttctttga agatttaatt gatcgaacgg atcattgcac 1320

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atttaggaat tggtatgtaa gctacaactt ccggtagctg cgtcacactt taagagtgtg     3120
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<210> 38

<211> 2360

<212> DNA

<213> Maize stripe virus segment 3, complete sequence GenBank: AJ969410.1

<400> 38

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gtcttgaatg atctcaccaa cattcatatc acttgtgagg atgtccactg ctctgcaaag 180

ctcctctgct acatatatga tatctacaag tctaggtacc catcaatgga tgagcattct 240

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aattgttgga catgcagaat gtccaaaaac aatctacctt tccaagtccc ctcaataaag 480

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agtagtatct gtagaatccc ctttctagga gtgtacggat aatgtaacta agttacattt 720

taagaataaa aaagtgtgct aagcacacta tccgaatagc aatcggatga aggccttaac 780

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ctatatgtac atatcaataa tagtgaacta aaaagcacaa gcactaaaat ataatagaag 960

caaaagcaaa gcataataaa catcataaac tataataaga taatattgtt aaaccataat 1020

aataatgaca cacaaacact caatactaat attatcatac tctcattcat acttgttttt 1080

gattttccat tttcccattt ttttgttttt ttgtataatc taatttttat ggttttcttg 1140

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cgattaccca gactttgtgt                                               2360
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<210> 39

<211> 1368

<212> PRT

<213> Streptococcus pyogenes

<400> 39

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Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
            20                  25                  30

Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
        35                  40                  45

Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
```

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    50                  55                  60

Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65                  70                  75                  80

Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
                85                  90                  95

Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
            100                 105                 110

His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
        115                 120                 125

His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140

Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160

Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175

Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190

Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205

Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220

Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240

Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255

Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270

Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285

Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300
```

```
Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320

Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335

Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350

Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365

Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380

Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400

Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415

Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430

Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445

Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460

Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480

Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495

Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510

Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525

Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
    530                 535                 540

Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560
```

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Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575

Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590

Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605

Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620

Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640

His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655

Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
            660                 665                 670

Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
        675                 680                 685

Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
    690                 695                 700

Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
705                 710                 715                 720

His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
                725                 730                 735

Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
            740                 745                 750

Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
        755                 760                 765

Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
    770                 775                 780

Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785                 790                 795                 800

Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
                805                 810                 815
```

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Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
            820                 825                 830

Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
        835                 840                 845

Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
    850                 855                 860

Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
865                 870                 875                 880

Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
                885                 890                 895

Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
            900                 905                 910

Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
        915                 920                 925

Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
    930                 935                 940

Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945                 950                 955                 960

Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
                965                 970                 975

Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
            980                 985                 990

Val Gly Thr Ala Leu Ile Lys Lys  Tyr Pro Lys Leu Glu  Ser Glu Phe
        995                 1000                1005

Val Tyr  Gly Asp Tyr Lys Val  Tyr Asp Val Arg Lys  Met Ile Ala
    1010                1015                1020

Lys Ser  Glu Gln Glu Ile Gly  Lys Ala Thr Ala Lys  Tyr Phe Phe
    1025                1030                1035

Tyr Ser  Asn Ile Met Asn Phe  Phe Lys Thr Glu Ile  Thr Leu Ala
    1040                1045                1050

Asn Gly  Glu Ile Arg Lys Arg  Pro Leu Ile Glu Thr  Asn Gly Glu
```

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    1055                1060                1065

Thr Gly  Glu Ile Val Trp Asp  Lys Gly Arg Asp Phe  Ala Thr Val
    1070                1075                1080

Arg Lys  Val Leu Ser Met Pro  Gln Val Asn Ile Val  Lys Lys Thr
    1085                1090                1095

Glu Val  Gln Thr Gly Gly Phe  Ser Lys Glu Ser Ile  Leu Pro Lys
    1100                1105                1110

Arg Asn  Ser Asp Lys Leu Ile  Ala Arg Lys Lys Asp  Trp Asp Pro
    1115                1120                1125

Lys Lys  Tyr Gly Gly Phe Asp  Ser Pro Thr Val Ala  Tyr Ser Val
    1130                1135                1140

Leu Val  Val Ala Lys Val Glu  Lys Gly Lys Ser Lys  Lys Leu Lys
    1145                1150                1155

Ser Val  Lys Glu Leu Leu Gly  Ile Thr Ile Met Glu  Arg Ser Ser
    1160                1165                1170

Phe Glu  Lys Asn Pro Ile Asp  Phe Leu Glu Ala Lys  Gly Tyr Lys
    1175                1180                1185

Glu Val  Lys Lys Asp Leu Ile  Ile Lys Leu Pro Lys  Tyr Ser Leu
    1190                1195                1200

Phe Glu  Leu Glu Asn Gly Arg  Lys Arg Met Leu Ala  Ser Ala Gly
    1205                1210                1215

Glu Leu  Gln Lys Gly Asn Glu  Leu Ala Leu Pro Ser  Lys Tyr Val
    1220                1225                1230

Asn Phe  Leu Tyr Leu Ala Ser  His Tyr Glu Lys Leu  Lys Gly Ser
    1235                1240                1245

Pro Glu  Asp Asn Glu Gln Lys  Gln Leu Phe Val Glu  Gln His Lys
    1250                1255                1260

His Tyr  Leu Asp Glu Ile Ile  Glu Gln Ile Ser Glu  Phe Ser Lys
    1265                1270                1275

Arg Val  Ile Leu Ala Asp Ala  Asn Leu Asp Lys Val  Leu Ser Ala
    1280                1285                1290
```

```
Tyr Asn  Lys His Arg Asp Lys  Pro Ile Arg Glu Gln  Ala Glu Asn
    1295                 1300                 1305

Ile Ile  His Leu Phe Thr Leu  Thr Asn Leu Gly Ala  Pro Ala Ala
    1310                 1315                 1320

Phe Lys  Tyr Phe Asp Thr Thr  Ile Asp Arg Lys Arg  Tyr Thr Ser
    1325                 1330                 1335

Thr Lys  Glu Val Leu Asp Ala  Thr Leu Ile His Gln  Ser Ile Thr
    1340                 1345                 1350

Gly Leu  Tyr Glu Thr Arg Ile  Asp Leu Ser Gln Leu  Gly Gly Asp
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<210> 40

<211> 1880

<212> DNA

<213> Brachypodium distachyon

<400> 40

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<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> Protospacer Region guide RNA 14

<400> 41

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<210> 42

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Protospacer Region guide RNA 16

<400> 42
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<210> 43
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<213> Artificial Sequence

<220>
<223> Protospacer Region guide RNA 37

<400> 43
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<210> 44
<211> 20
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<220>
<223> Protospacer Region guide RNA 38

<400> 44
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<210> 45
<211> 20
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<223> Protospacer Region guide RNA 39

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<210> 46

<211> 20

<212> DNA

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<223> Protospacer Region guide RNA 43

<400> 46

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<210> 47

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Protospacer Region guide RNA 18

<400> 47

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<212> DNA

<213> Artificial Sequence

<220>

<223> Protospacer Region guide RNA 52

<400> 48

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<210> 49

<211> 9

<212> PRT

<213> Simian virus 40

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1 5

<210> 50

<211> 18

<212> PRT

<213> Agrobacterium tumefaciens

<400> 50

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Ala Arg

<210> 51

<211> 18

<212> PRT

<213> Agrobacterium tumefaciens

<400> 51

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1 5 10 15

Glu Arg

<210> 52

<211> 17

<212> PRT

<213> Agrobacterium tumefaciens

<400> 52

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Arg
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<210> 53

<211> 17

<212> PRT

<213> Agrobacterium tumefaciens

<400> 53

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Lys Leu Arg Pro Glu Asp Arg Tyr Ile Gln Thr Glu Lys Tyr Gly Arg
1               5                   10                  15

Arg
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<210> 54

<211> 15

<212> PRT

<213> Agrobacterium tumefaciens

<400> 54

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1               5                   10                  15
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<210> 55

<211> 15

<212> PRT

<213> Agrobacterium tumefaciens

<400> 55

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Lys Thr Lys Tyr Gly Ser Asp Thr Glu Ile Lys Leu Lys Ser Lys
1               5                  10                  15
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<210> 56

<211> 20

<212> PRT

<213> Agrobacterium rhizogenes

<400> 56

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Lys Arg Lys Arg Ala Ala Ala Lys Glu Glu Ile Asp Ser Arg Lys Lys
1               5                  10                  15

Met Ala Arg His
            20
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<210> 57

<211> 20

<212> PRT

<213> Agrobacterium rhizogenes

<400> 57

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1               5                  10                  15

Met Ala Arg Arg
            20
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<210> 58

<211> 18

<212> PRT

<213> Agrobacterium rhizogenes

<400> 58

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Lys Arg Pro Arg Val Glu Asp Asp Gly Glu Pro Ser Glu Arg Lys Arg
1               5                   10                  15

Ala Arg
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<210> 59

<211> 3738

<212> DNA

<213> Artificial Sequence

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<210> 61

<211> 210

<212> DNA

<213> Artificial Sequence

<220>

<223> genome edited hmg13 gene

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ctcgttggcg ccactggcgg gtggttctgc                                     210
```

PATENTKRAV

1. Fremgangsmåde til fremstilling af en majsplante, omfattende følgende trin:

(i) tilvejebringelse af en plantemålstruktur,

der omfatter mindst én meristematisk celle,

hvor plantemålstrukturen er et modent eller umodent majskim, og den mindst ene meristematiske celle omfatter mindst et nukleinsyreområde;

(ii) tilvejebringelse af mindst ét gRNA eller tilvejebringelse af et eller flere rekombinante konstrukter, hvor det eller de rekombinante konstrukter omfatter

(a) mindst ét gRNA eller en sekvens, der koder for et gRNA, og

(b) eventuelt mindst én regulatorisk sekvens og/eller én lokaliseringssekvens og

(c) eventuelt mindst én DNA-reparationstemplate, og tilvejebringelse af mindst én CRISPR-nuklease, fortrinsvis en Cas eller en Cpf1-nuklease, eller et katalytisk aktivt fragment deraf og/eller et effektor-domæne eller tilvejebringelse af et eller flere rekombinante konstrukter,

hvor det eller de rekombinante konstrukter omfatter

(A) mindst én CRISPR-nuklease, eller et katalytisk aktivt fragment deraf eller en sekvens, der koder for en CRISPR-nuklease, eller en sekvens, der koder for et katalytisk aktivt fragment deraf og/eller mindst ét effektordomæne, eller en sekvens, der koder for et effektordomæne, og

(B) eventuelt mindst én regulatorisk sekvens og/eller én lokaliseringssekvens,

hvor gRNA'et er i stand til at hybridisere såvel til et segment af nukleinsyreområdet som med CRISPR-nukleasen eller det katalytisk aktive fragment deraf og/eller til at interagere med effektordomænet;

hvor, når gRNA'et eller sekvensen, der koder for gRNA'et og CRISPR-nukleasen eller det katalytisk aktive fragment deraf eller CRISPR-nukleasen eller sekvensen, der koder for det katalytisk aktive fragment deraf og/eller effektordomænet eller sekvensen, der koder for effektordomænet, tilvejebringes via ét eller flere rekombinante konstrukter, gRNA'et eller sekvensen, der koder for gRNA'et og CRISPR-nukleasen eller det katalytisk aktive fragment deraf eller CRISPR-nukleasen eller sekvensen, der koder for det katalytisk aktive fragment deraf og/eller effektordomænet, eller sekvensen, der koder for effektordomænet, er lokaliseret på eller i samme eller på eller i forskellige rekombinante konstrukter;

(iii) indføring af gRNA'et, CRISPR-nukleasen eller det katalytisk aktive fragment deraf og/eller effektordomænet eller det eller de rekombinante konstrukter i plantemålstrukturens mindst ene meristematiske celle;

(iv) dyrkning af plantemålstrukturen under betingelser, der fremmer aktivering af det indførte gRNA, CRISPR-nukleasen eller det katalytisk aktive fragment deraf og/eller af effektordomænet eller det eller de indførte rekombinante konstrukter og derved muliggør en målrettet ændring af nukleinsyreområdet i plantemålstrukturens mindst ene meristematiske celle, for at opnå en plantemålstruktur, der omfatter mindst én meristematisk celle, som omfatter den målrettede ændring af nukleinsyreområdet;

(v) opnåelse af en majsplante ud fra den målrettede, ændrede mindst ene meristematiske celle, hvor majsplanten opnås direkte via celledeling og differentiering, uden som mellemtrin at gå via en cellekultur i form af en kallusproduktion og -regeneration, fra den målrettede ændrede mindst ene meristematiske celle,

hvor den opnåede majsplante omfatter den målrettede ændring af nukleinsyreområdet, og hvor i majsplanten fra trin (v) det eller de rekombinante konstrukter, som omfatter (s)

(a) mindst ét gRNA eller en sekvens, der koder for et gRNA, eller

(b) mindst én CRISPR-nuklease, fortrinsvis en Cas eller en Cpf1-nuklease, eller et katalytisk aktivt fragment deraf eller en sekvens, der koder for en CRISPR-nuklease, eller en sekvens, der koder for et katalytisk aktivt fragment deraf og/eller mindst ét effektordomæne, eller en sekvens, der koder for et effektordomæne, ikke integreres kromosomalt eller extrakromosomalt.

2. Fremgangsmåde ifølge krav 1, hvor i trin (ii) gRNA'et eller sekvensen, der koder for gRNA'et og/eller CRISPR-nukleasen, fortrinsvis en Cas eller en Cpf1-nuklease, eller det katalytisk aktive fragment deraf eller sekvensen, der koder for CRISPR-nukleasen eller det katalytisk aktive fragment deraf og/eller effektordomænet eller en sekvens, der koder for effektordomænet, er tilpasset til anvendelse i en plantecelle.

3. Fremgangsmåde ifølge et af kravene 1 eller 2, hvor der mellem trin (ii) og (iii) tilvejebringes mindst én vektor til indføring af det eller de rekombinante konstrukter.

4. Fremgangsmåde ifølge et af de foregående krav, hvor der mellem trin (ii) og (iii) tilvejebringes mindst ét yderligere rekombinant konstrukt omfattende et rekombinant nukleinsyresegment til målrettet homologistyret reparation af nukleinsyreområdet i plantemålstrukturen eller indsætning i nukleinsyreområdet i plantemålstrukturen og

eventuelt mindst én yderligere vektor til indføring af det mindst ene yderligere rekombinante konstrukt.

5. Fremgangsmåde ifølge et af de foregående krav, hvor den mindst ene vektor vælges fra gruppen bestående af ét virus, valgt fra gruppen bestående af SEQ ID NO:12-15 og 25-38, og fra sekvenser med mindst 66 %, 67 %, 68 %, 69 %, 70 %, 71 %, 72 %, 73 %, 74 %, 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %,90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 % eller 99 % sekvenshomologi med disse sekvenser, eller et agens, der er egnet til transfektion af en peptid- eller polypeptidsekvens eller af nukleinsyresekvenser, eller en kombination deraf.

6. Fremgangsmåde ifølge et af de foregående krav, hvor gRNA'et direkte som en naturlig eller syntetisk nukleinsyre og/eller CRISPR-nukleasen, fortrinsvis en Cas eller en Cpf1-nuklease, eller det katalytisk aktive fragment deraf direkte som et polypeptid og/eller effektordomænet direkte som en nukleinsyre eller et polypeptid indføres i plantemålstrukturen.

7. Fremgangsmåde ifølge et af de foregående krav, hvor gRNA'et eller sekvensen, der koder for gRNA'et eller CRISPR-nukleasen, fortrinsvis en Cas eller en Cpf1-nuklease, eller det katalytisk aktive fragment deraf eller sekvensen, der koder for CRISPR-nukleasen, eller sekvensen, der koder for det katalytisk aktive fragment deraf eller effektordomænet eller sekvensen, der koder for effektordomænet, endvidere omfatter en lokaliseringssekvens, valgt blandt en kernelokaliseringsekvens, en plastidlokaliseringssekvens, fortrinsvis en mitochondrielokaliseringssekvens og en chloroplastlokaliseringsekvens.

8. Fremgangsmåde ifølge et af de foregående krav, hvor der endvidere indføres en hæmmer af den endogene ikke-homologe endesamlings- (non-homologous end joining - NHEJ) reparationsmekanisme i plantemålstrukturen.

9. Fremgangsmåde ifølge et af de foregående krav, hvor det rekombinante konstrukt vælges blandt SEQ ID NO: 23 og 24, og blandt sekvenser med mindst 66 %, 67 %, 68 %, 69 %, 70 %, 71 %, 72 %, 73 %, 74 %, 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %,90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 % eller 99 % sekvenshomologi med disse sekvenser.

Figure 1

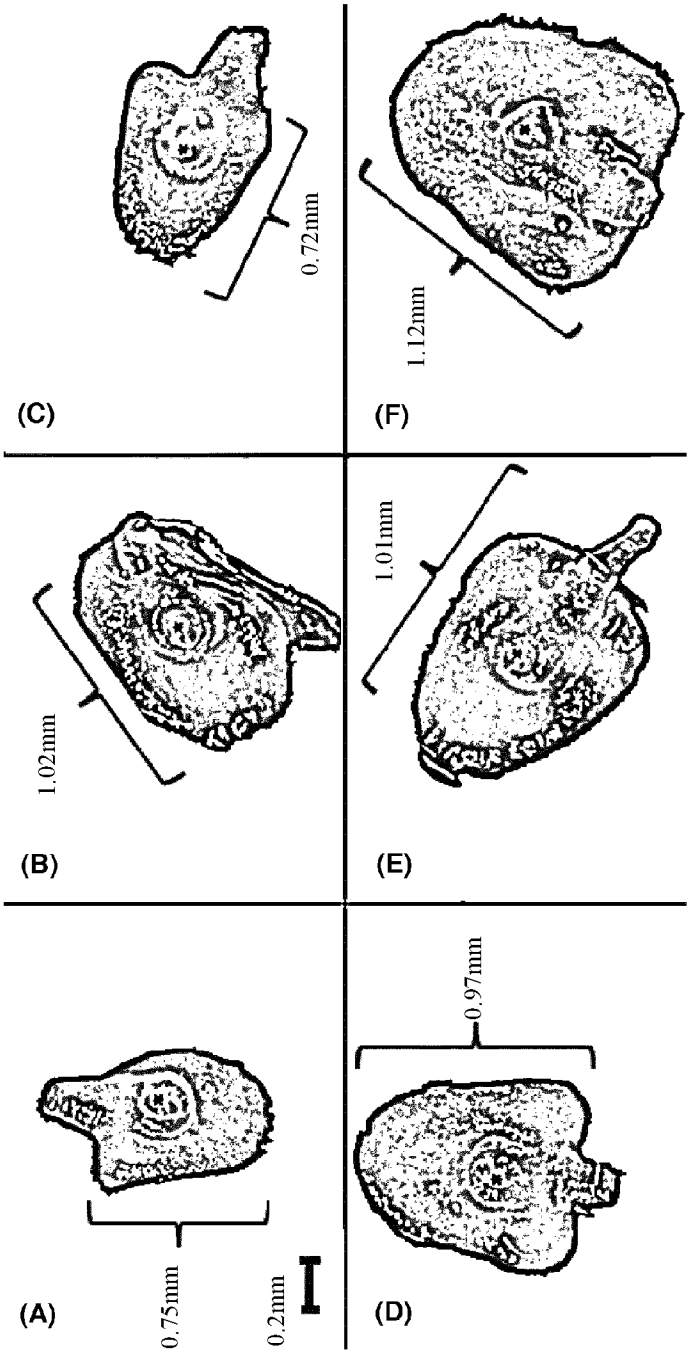


Figure 2

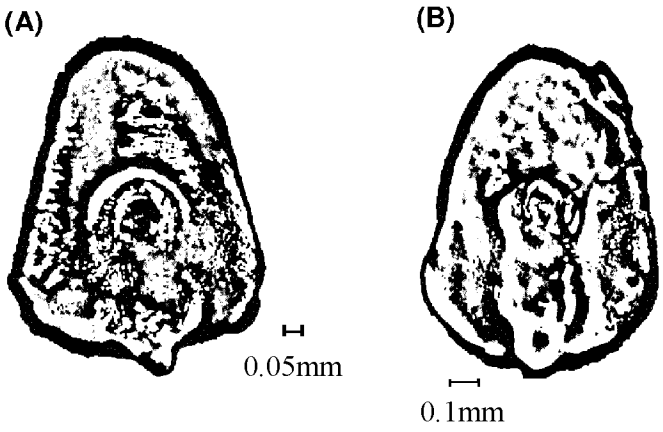
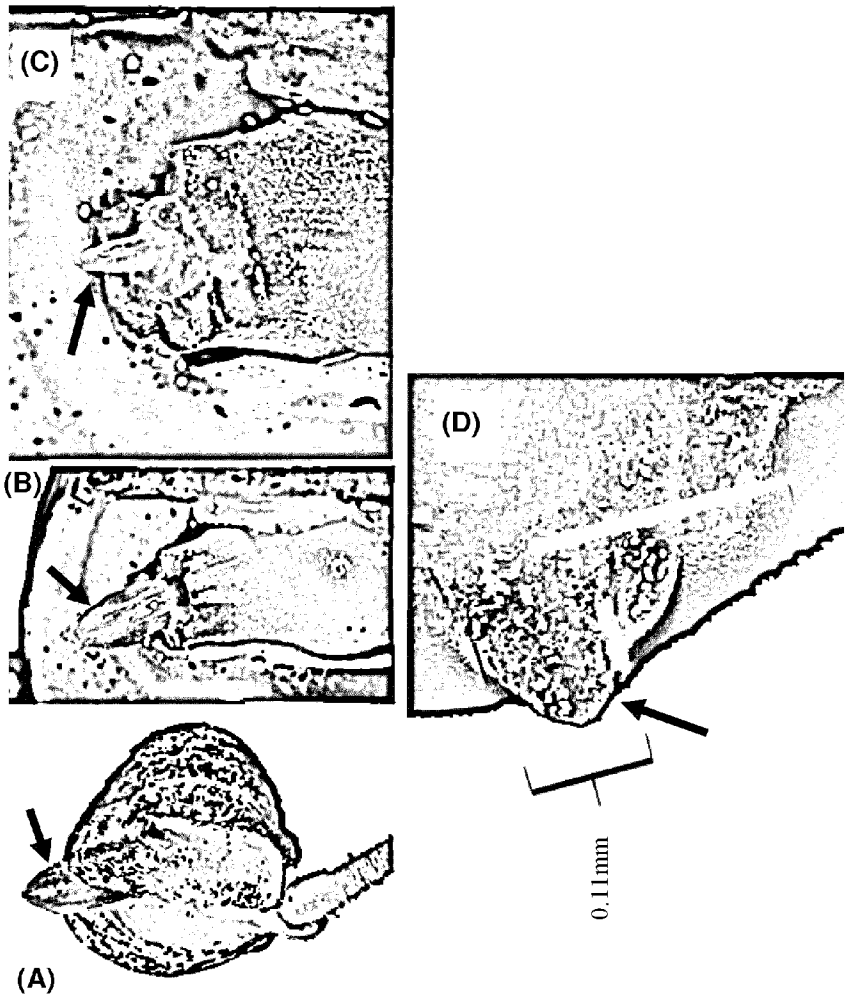


Figure 3



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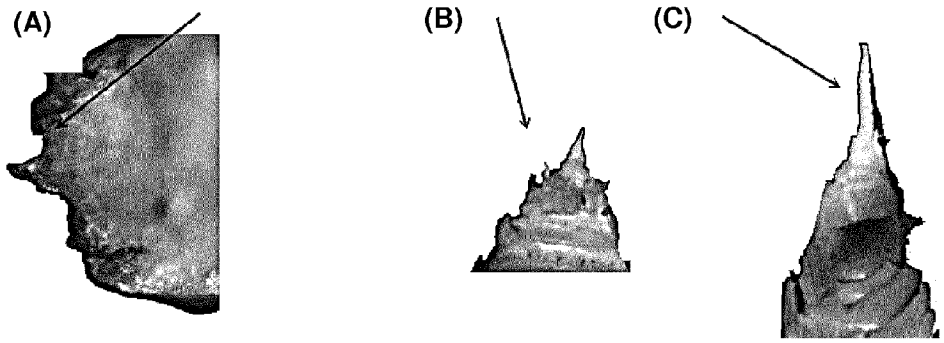

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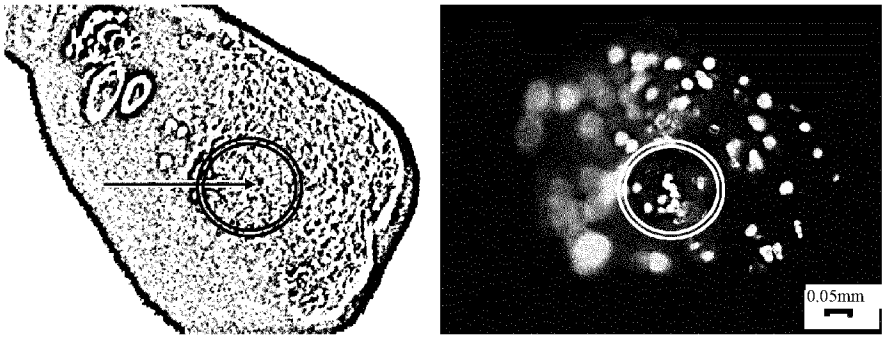
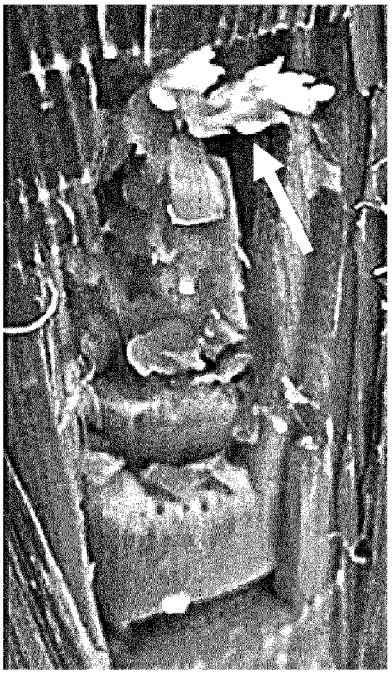


(A) (B)

Figure 6



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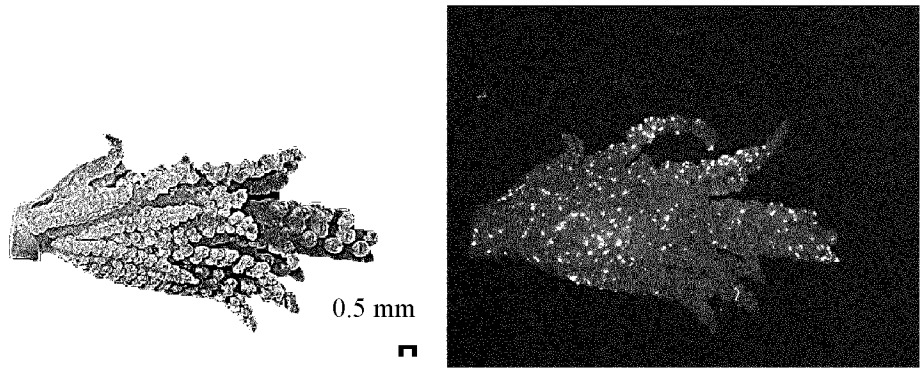
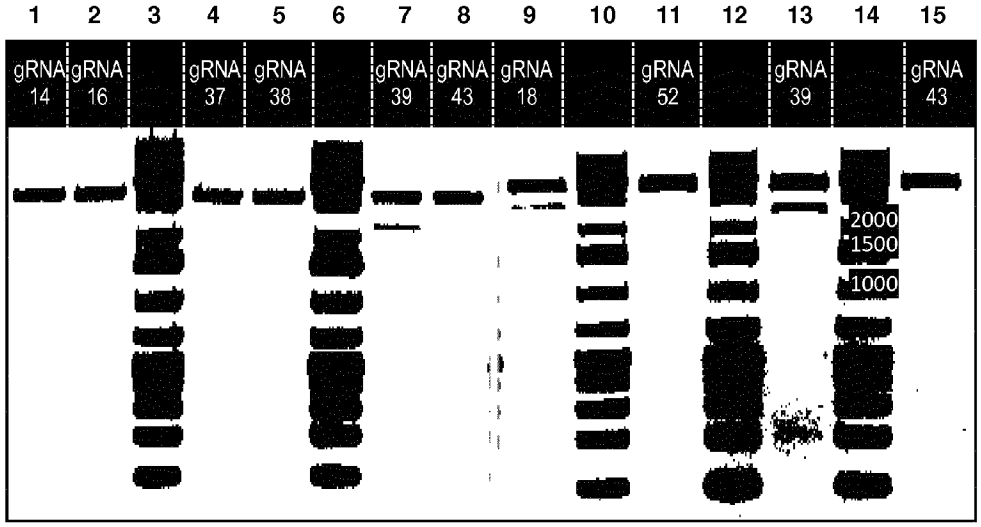
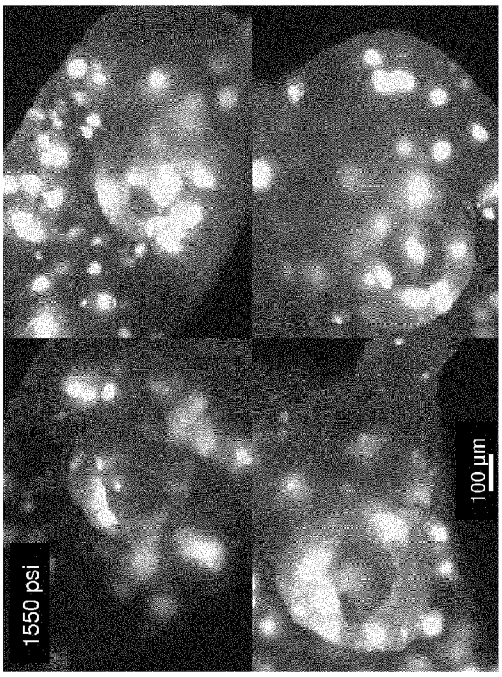


Figure 8

Band:





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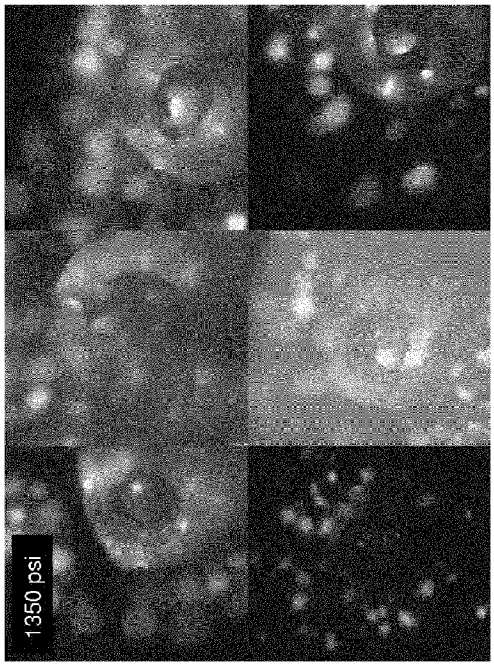
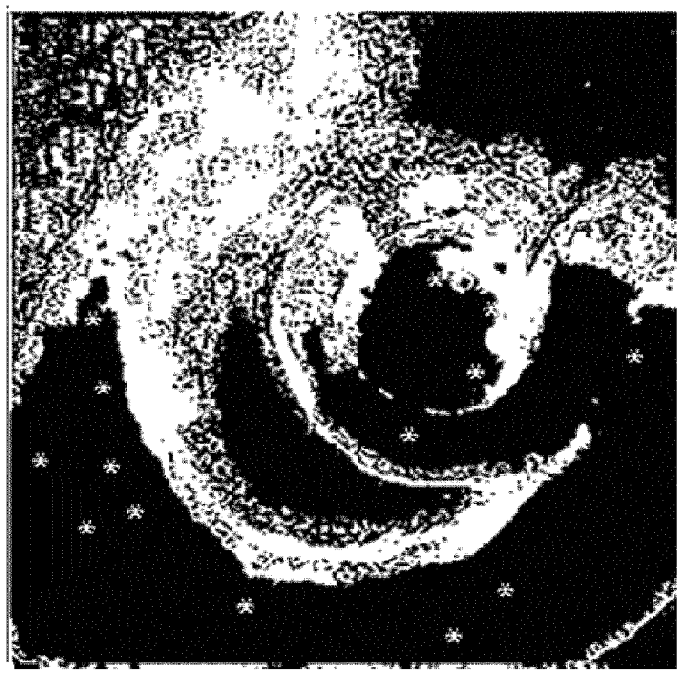


(A)

Figure 9



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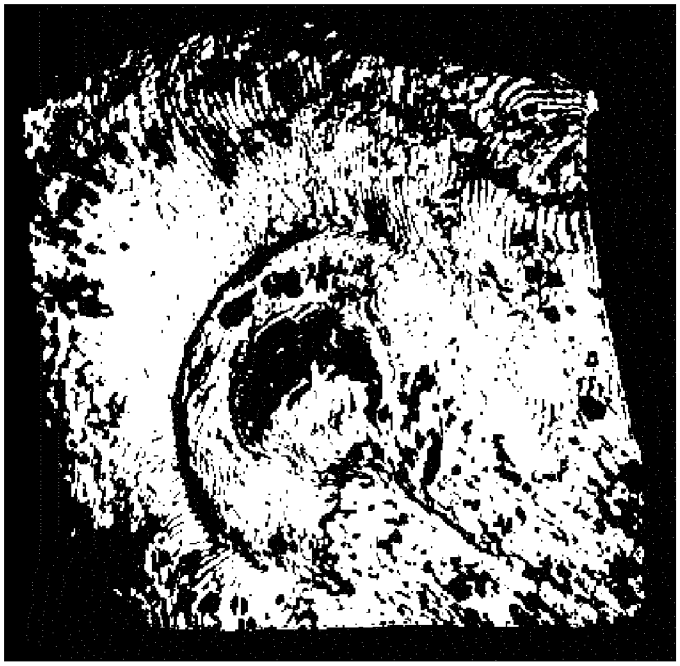
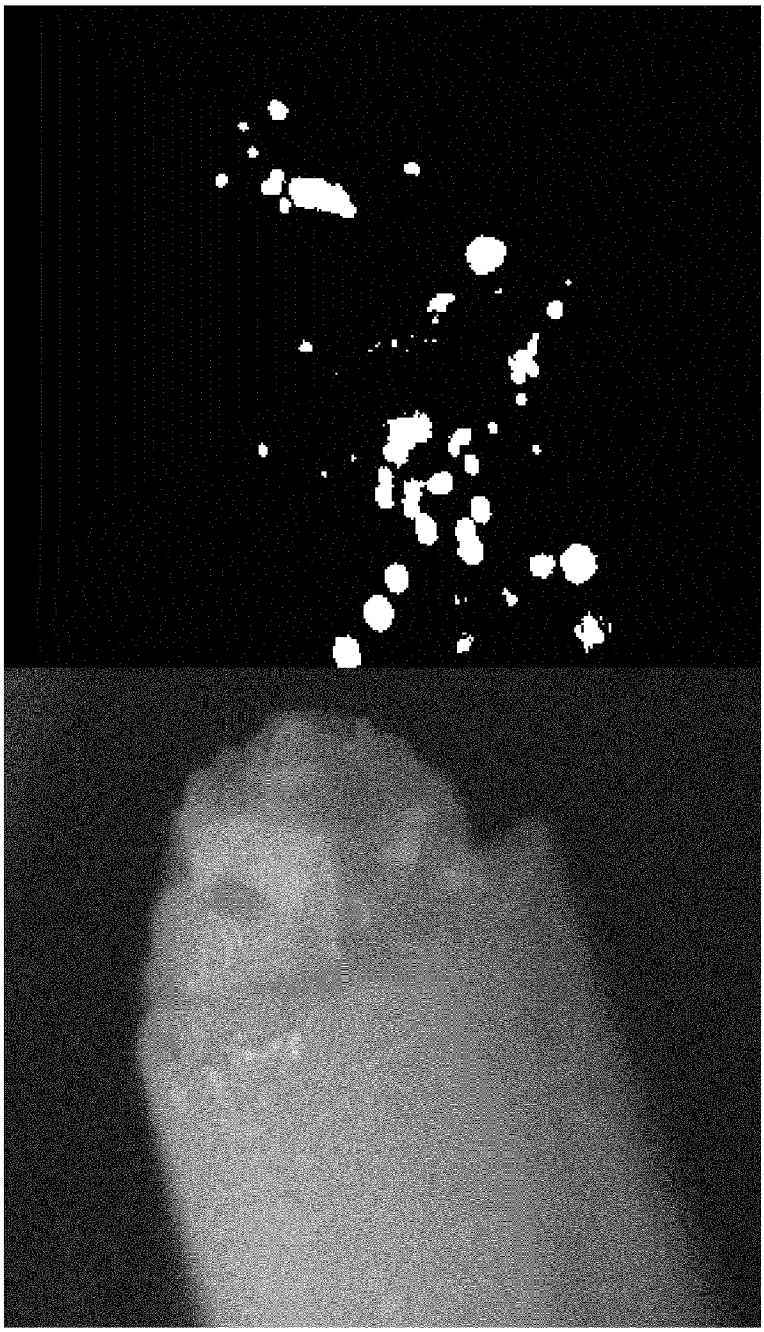


(A)

Figure 10



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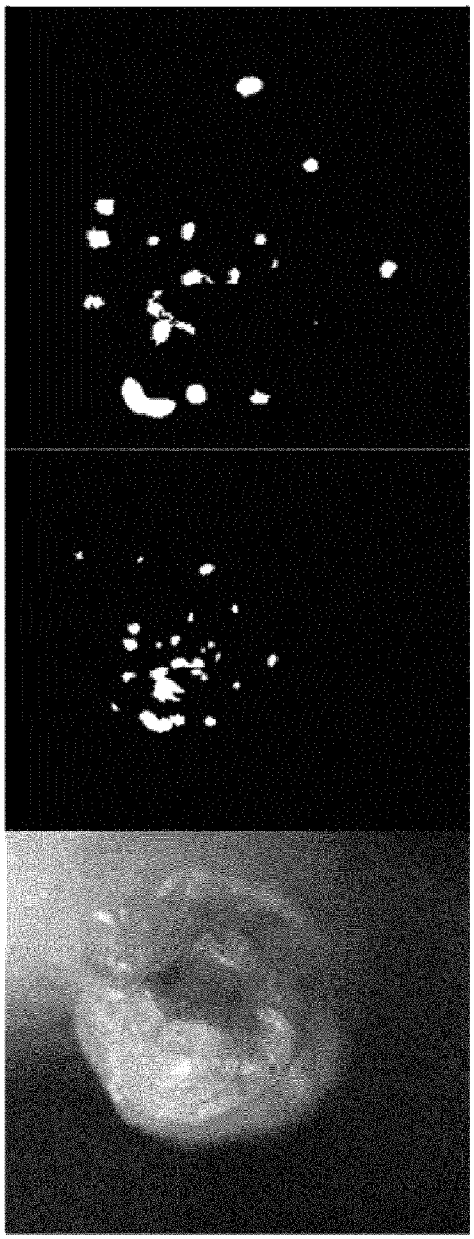


Figure 12

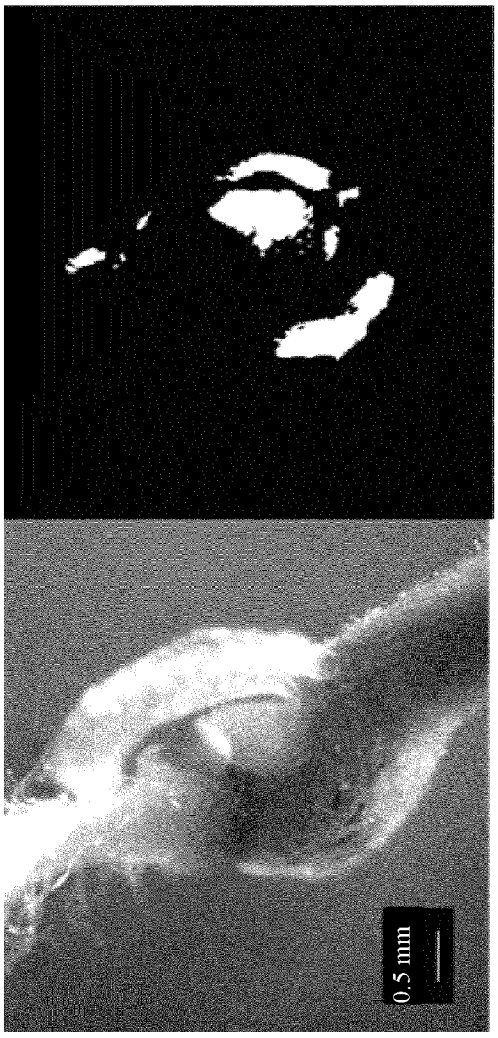
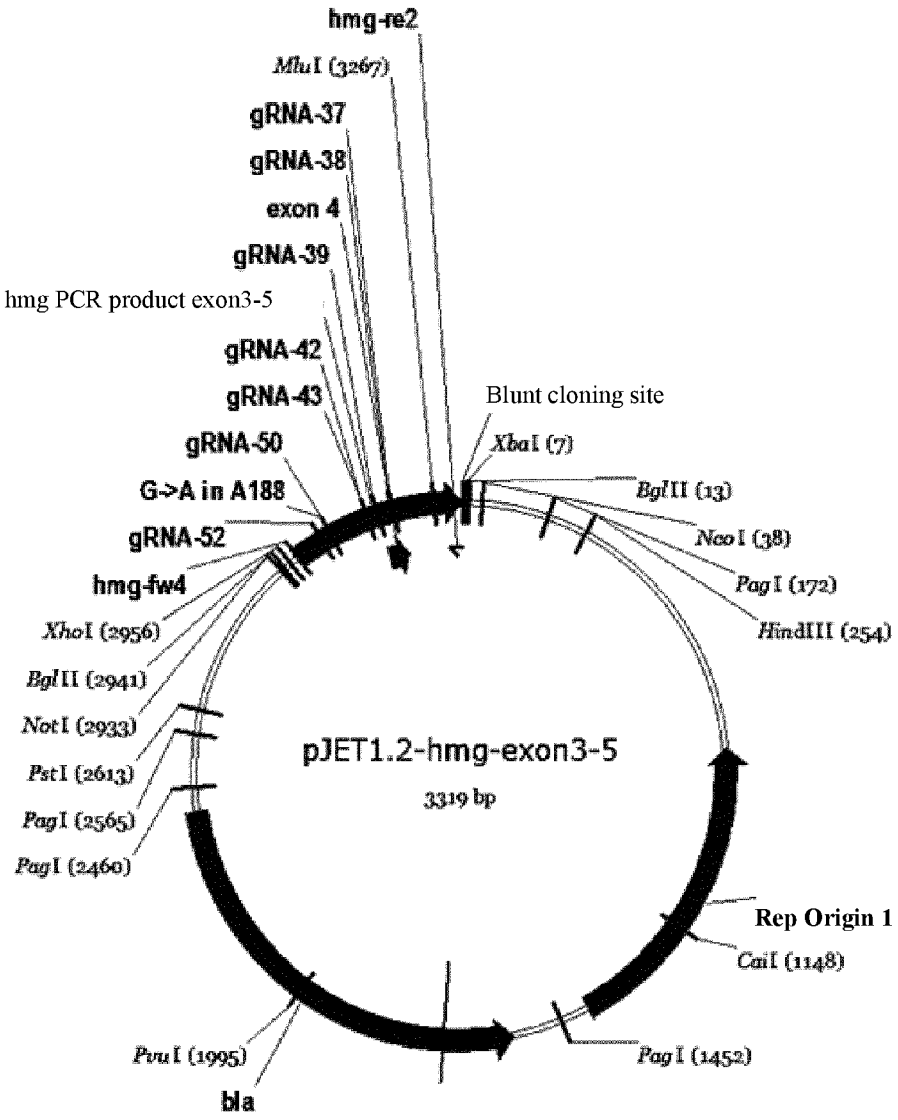


Figure 13

Figure 14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gRNA-52
hmg-fw4
XhoI (2956)
BglII (2941)
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PstI (2613)
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bla

Figure 15

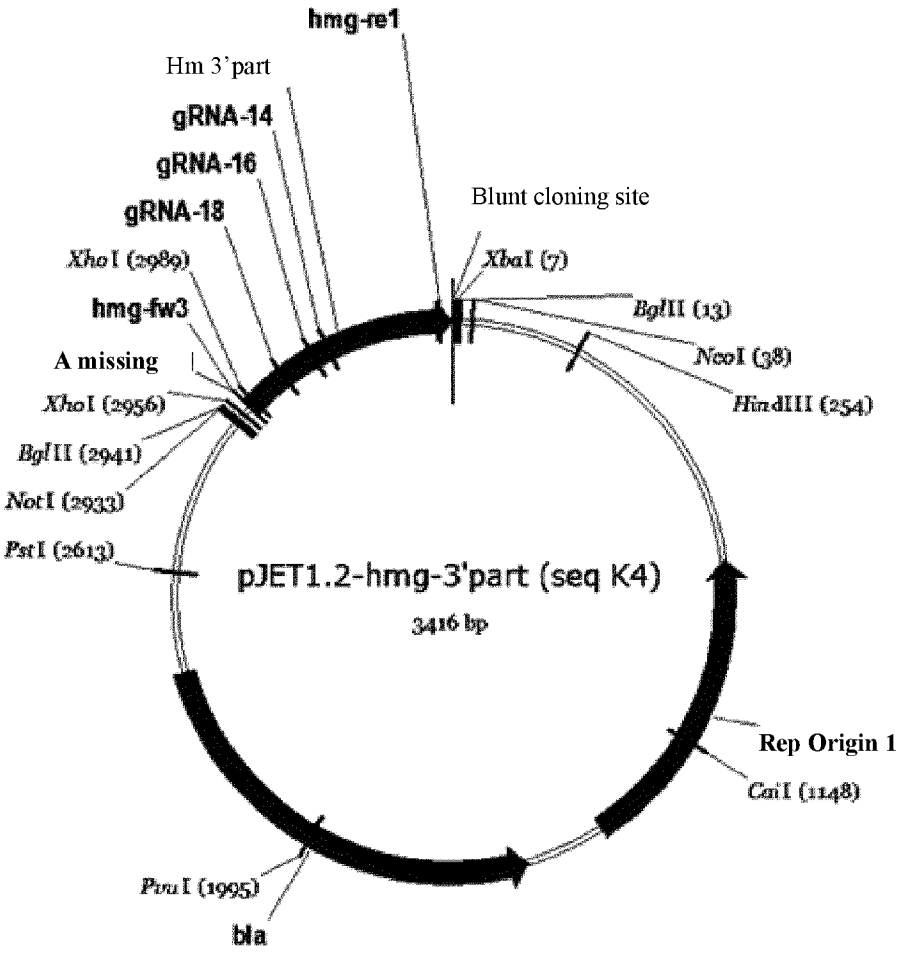

hmg-re1
Hm 3'part
gRNA-14
gRNA-16
gRNA-18
Blunt cloning site
XhoI (2989)
XbaI (7)
hmg-fw3
BglII (13)
NcoI (38)
A missing
HindIII (254)
XhoI (2956)
BglII (2941)
NotI (2933)
PstI (2613)
pJET1.2-hmg-3'part (seq K4)
3416 bp
Rep Origin 1
CaiI (1148)
PmaI (1995)
bla

Figure 16

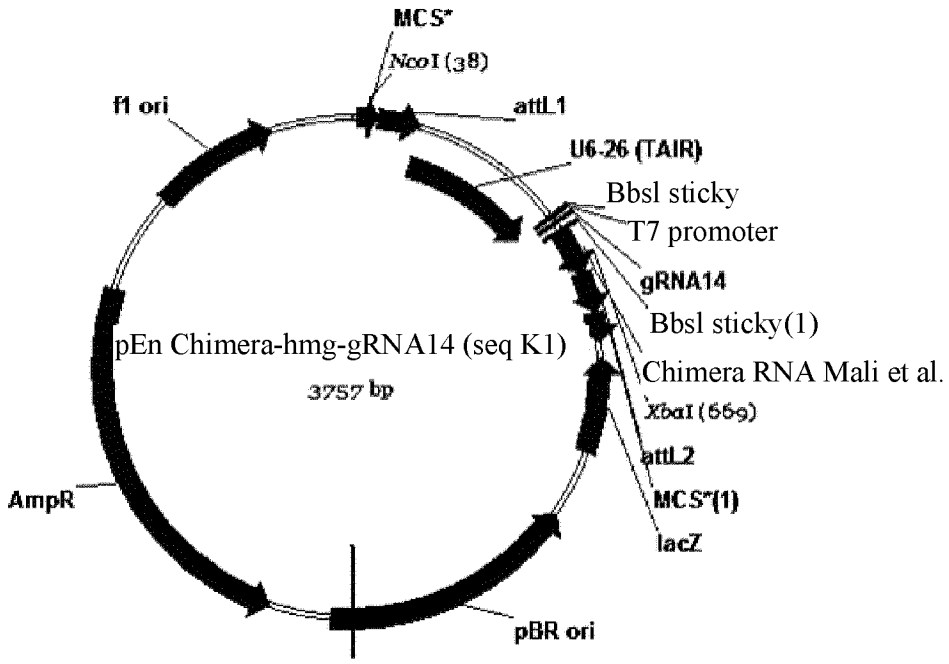
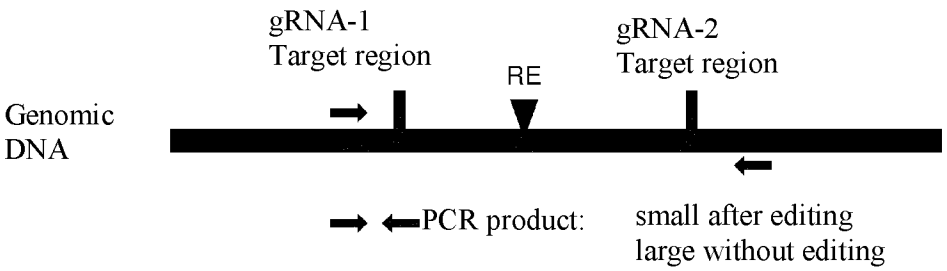
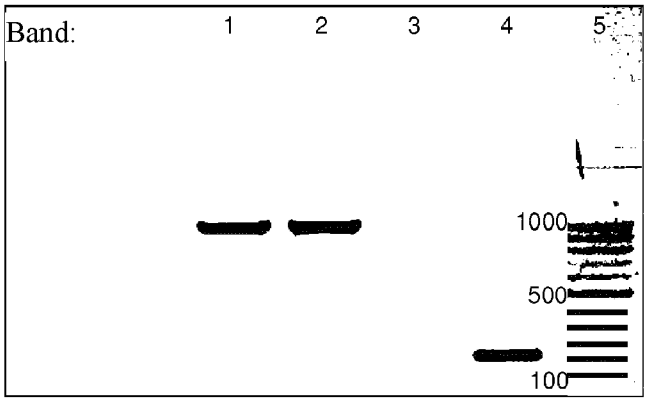

MCS*
NcoI (38)
attL1
U6-26 (TAIR)
Bbsl sticky
T7 promoter
gRNA14
Bbsl sticky(1)
Chimera RNA Mali et al.
XbaI (669)
attL2
MCS*(1)
lacZ
pBR ori
AmpR
f1 ori
pEn Chimera-hmg-gRNA14 (seq K1)
3757 bp

Figure 17


gRNA-1
Target region
RE
gRNA-2
Target region
Genomic
DNA
PCR product:
small after editing
large without editing

(A)


Band:
1
2
3
4
5
1000
500
100

(B)

Figure 18

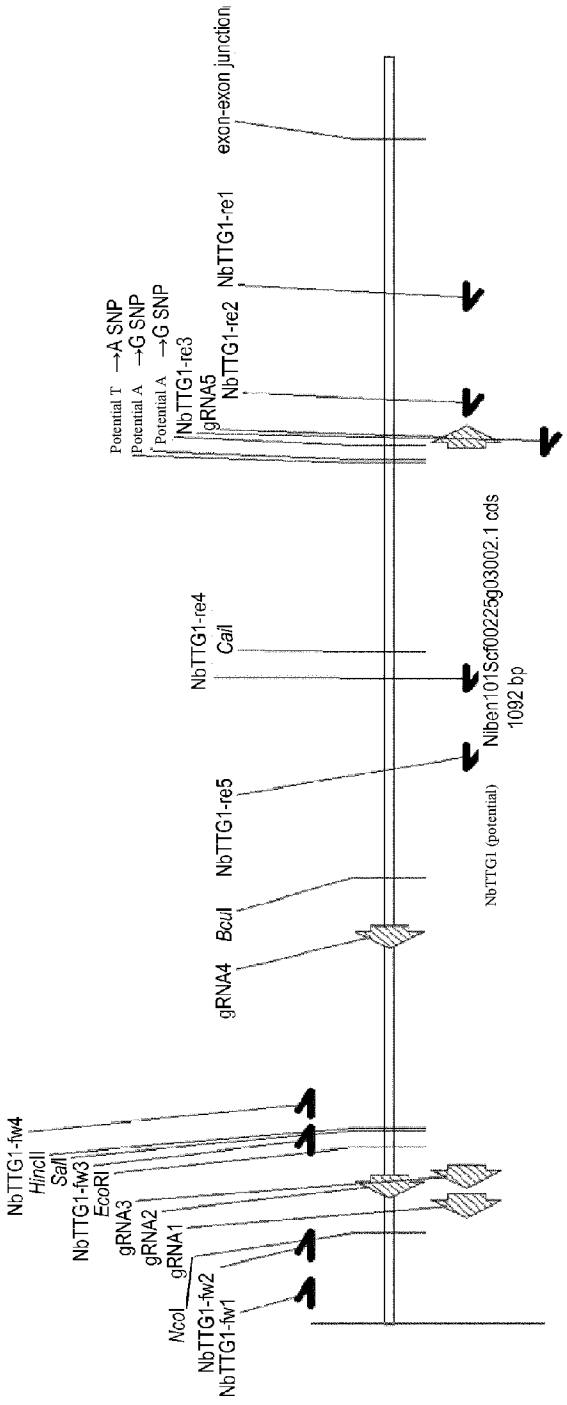
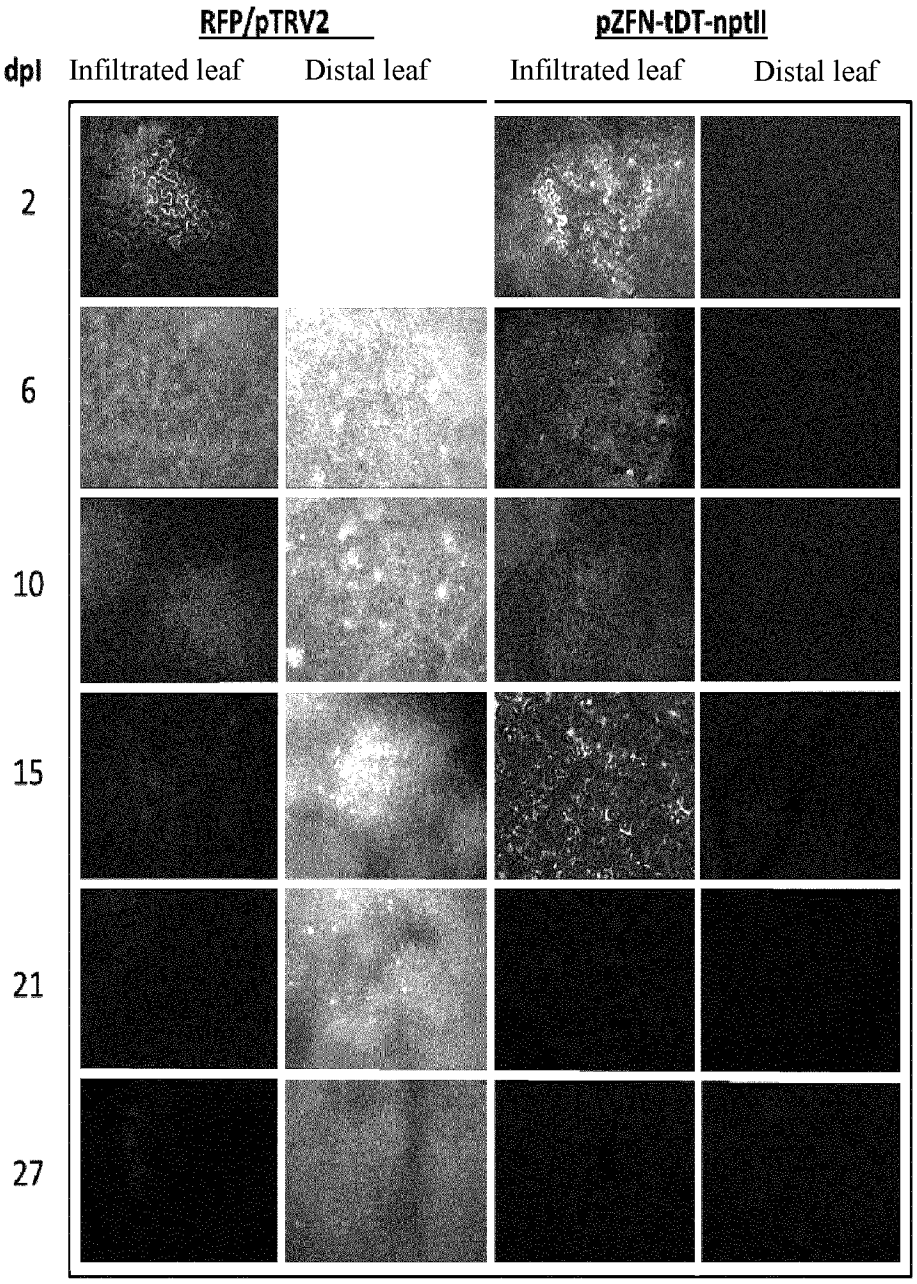
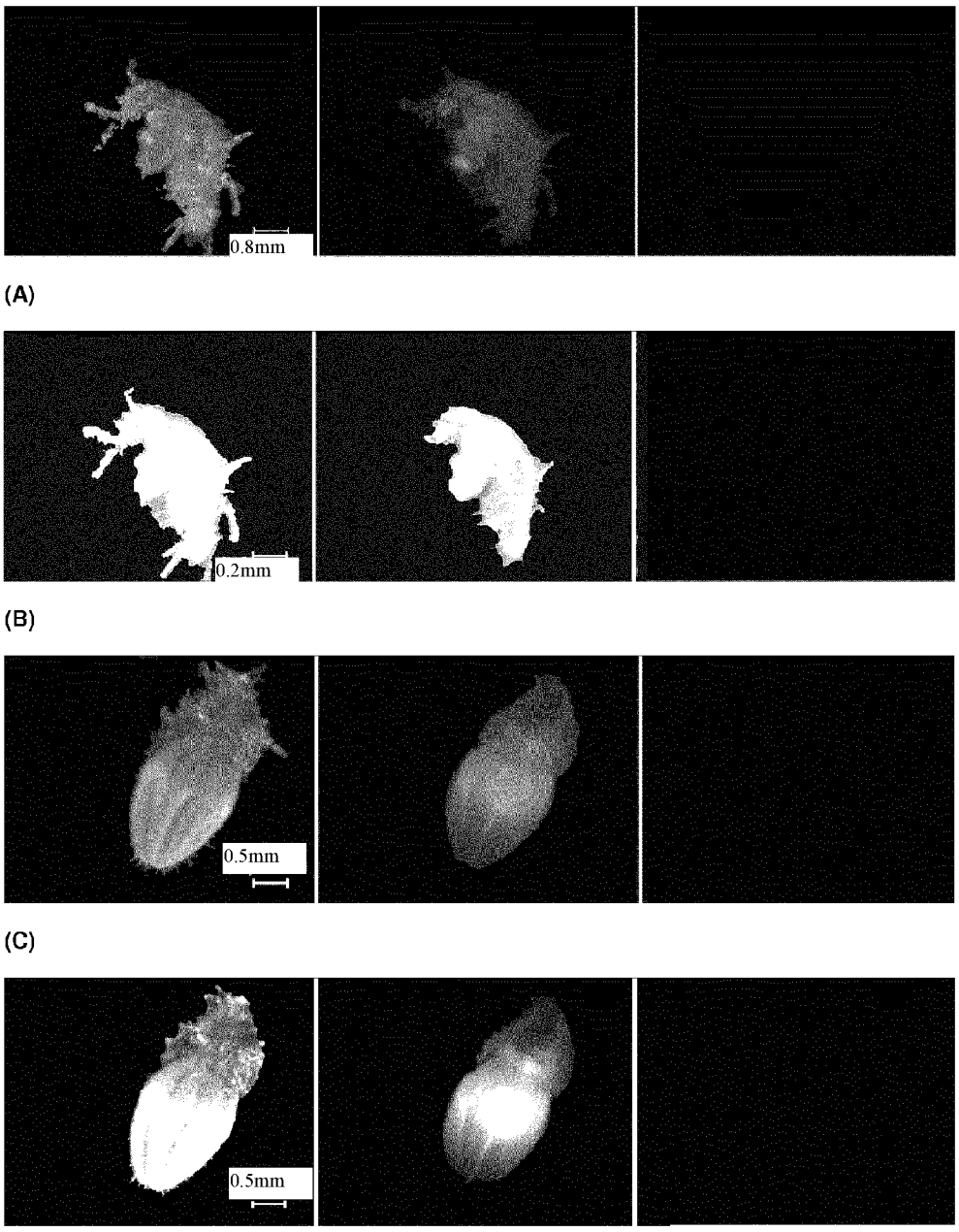

exon-exon junction
NbTTG1-re1
Potential T →A SNP
Potential A →G SNP
Potential A →G SNP
NbTTG1-re2
NbTTG1-re3
gRNA5
NbTTG1-re4
CaiI
Niben101Scf00225g03002.1 cds
1092 bp
NbTTG1 (potential)
NbTTG1-re5
BcuI
gRNA4
NbTTG1-fw4
HincII
SalI
NbTTG1-fw3
EcoRI
gRNA3
gRNA2
gRNA1
NcoI
NbTTG1-fw2
NbTTG1-fw1

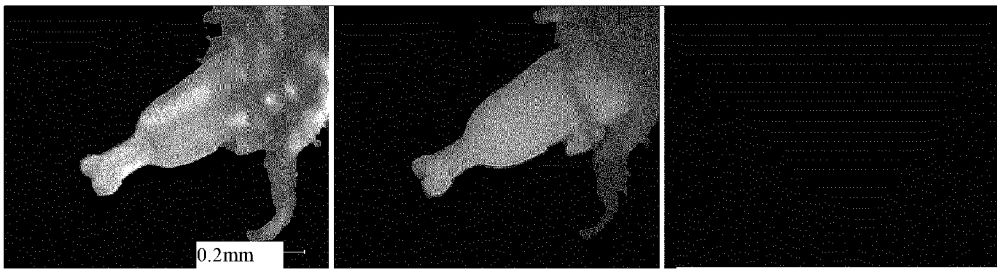
Figure 19



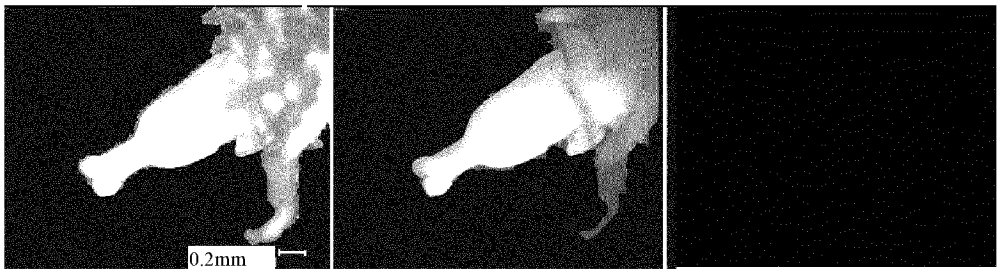
Dpi = Days post infiltration

Figure 20

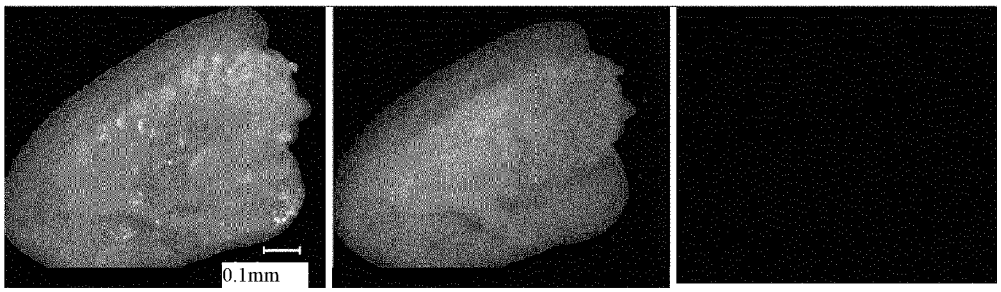




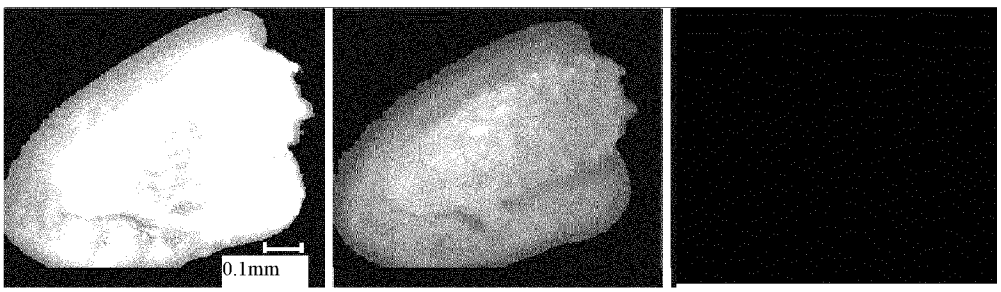
(E)



(F)



(G)



(H)

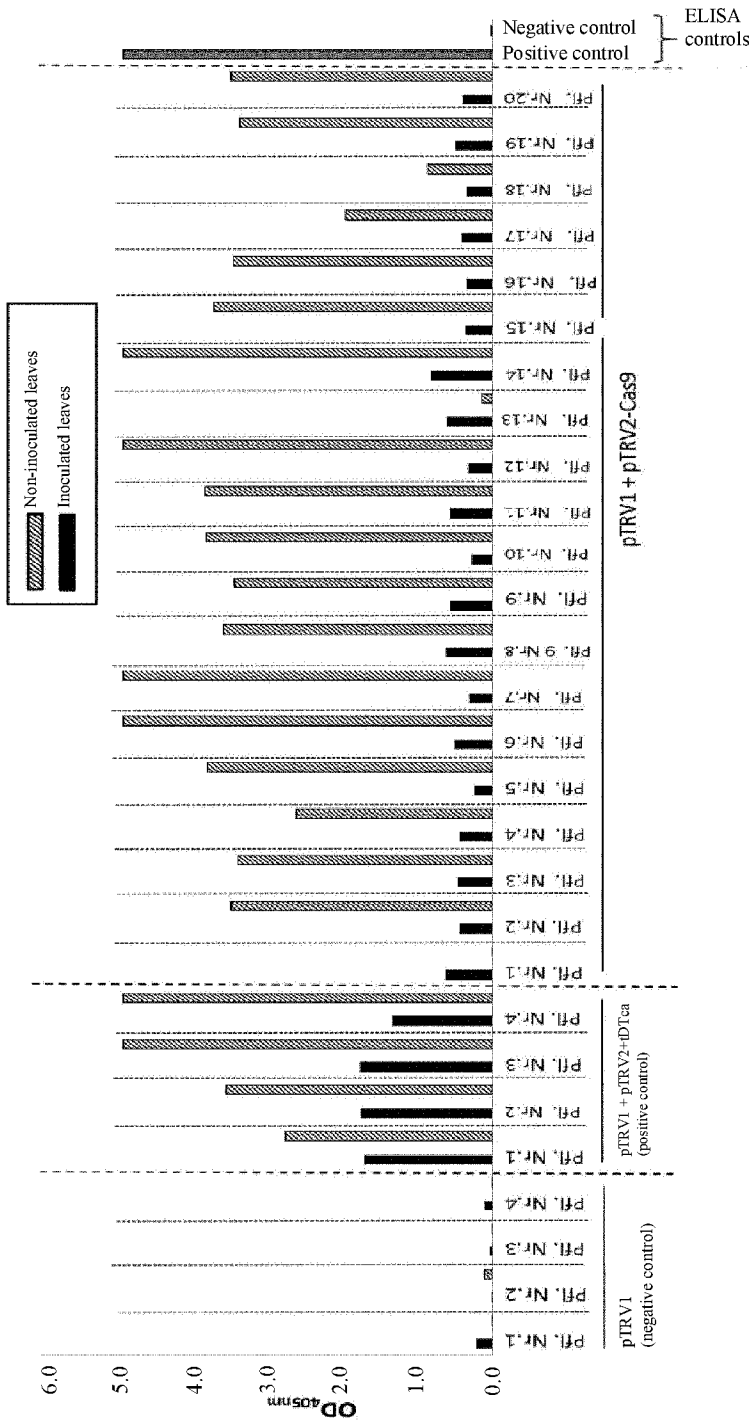

Negative control
Positive control
ELISA controls
Non-inoculated leaves
Inoculated leaves
pTRV1 + pTRV2-Cas9
pTRV1 + pTRV2+tDTca (positive control)
pTRV1 (negative control)
OD405nm
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 21

Figure 22

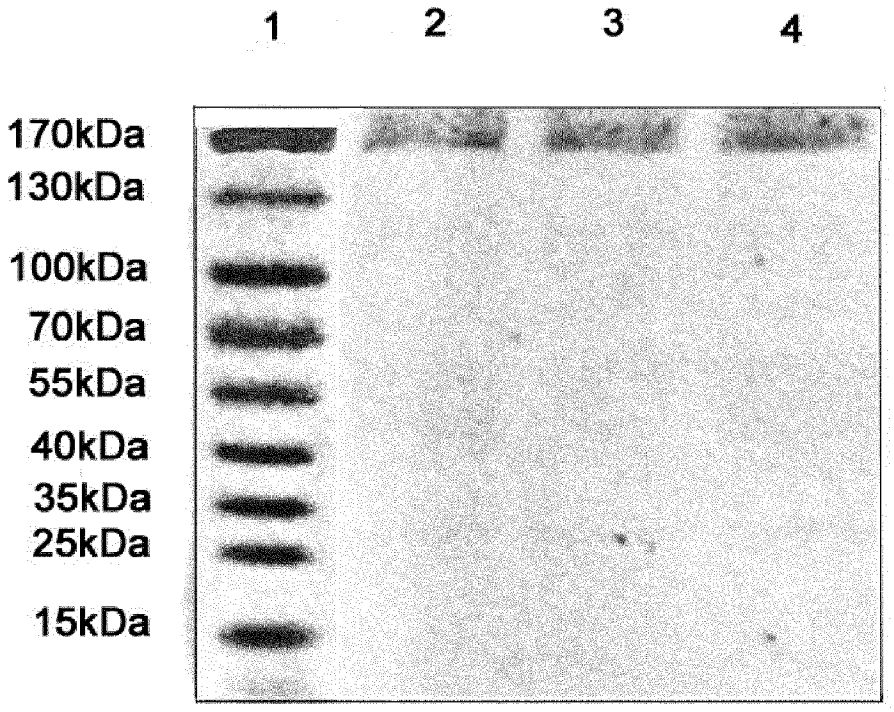

1
2
3
4
170kDa
130kDa
100kDa
70kDa
55kDa
40kDa
35kDa
25kDa
15kDa

Figure 23

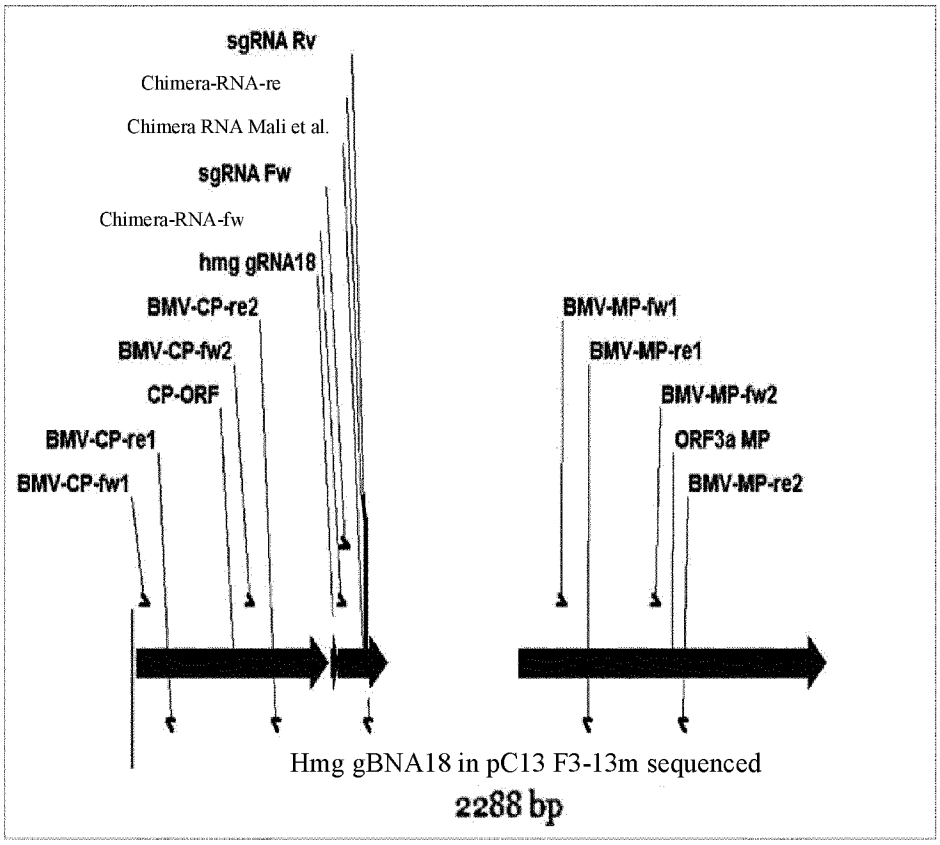
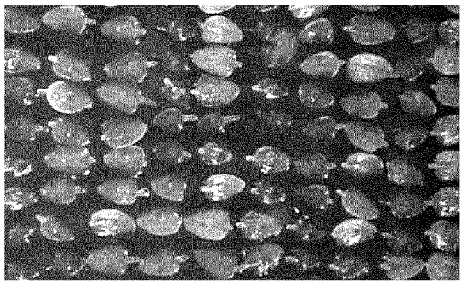
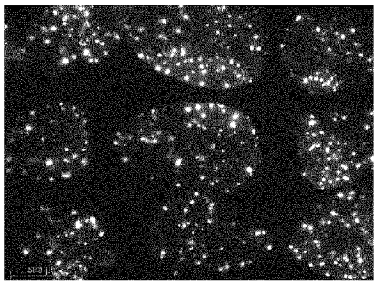

sgRNA Rv
Chimera-RNA-re
Chimera RNA Mali et al.
sgRNA Fw
Chimera-RNA-fw
hmg gRNA18
BMV-CP-re2
BMV-CP-fw2
CP-ORF
BMV-CP-re1
BMV-CP-fw1
BMV-MP-fw1
BMV-MP-re1
BMV-MP-fw2
ORF3a MP
BMV-MP-re2
Hmg gBNA18 in pC13 F3-13m sequenced
2288 bp

Figure 24

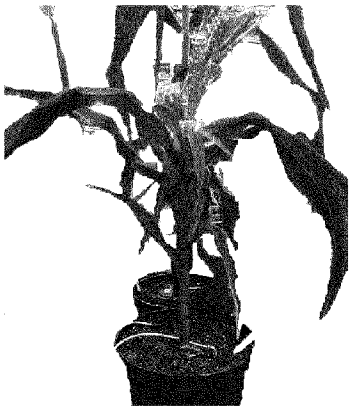
Figure 25



(A)



(B)



(C)

Figure 26

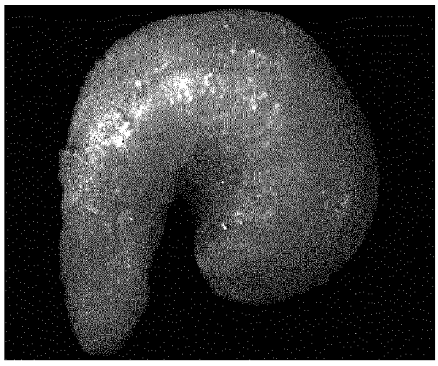
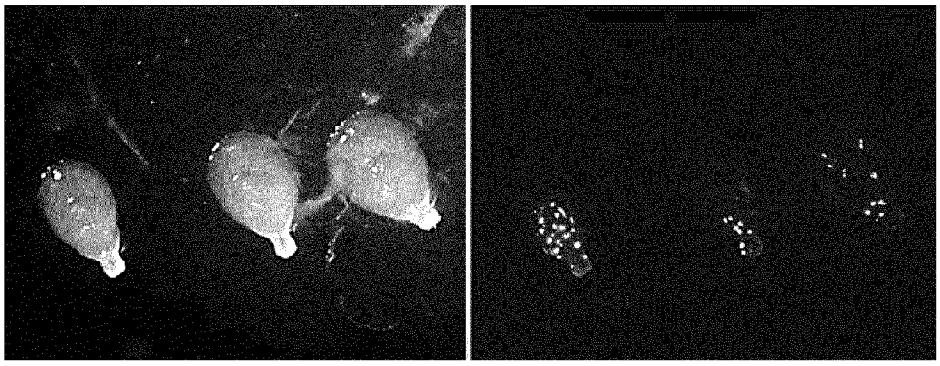


Figure 27



(A) (B)

Figure 28