GENOME MODIFICATION USING GUIDE POLYNUCLEOTIDE/CAS ENDONUCLEASE SYSTEMS AND METHODS OF USE

Abstract: Compositions and methods are provided for genome modification of a target sequence in the genome of a cell. The methods and compositions employ a guide polynucleotide/Cas endonuclease system to provide an effective system for modifying or altering target sites within the genome of a cell or organism. Once a genomic target site is identified, a variety of methods can be employed to further modify the target sites such that they contain a variety of polynucleotides of interest. Compositions and methods are also provided for editing a nucleotide sequence in the genome of a cell. Breeding methods and methods for selecting plants utilizing a two component RNA polynucleotide and Cas endonuclease system are also disclosed.

FIGURE 1A

Duplex Guide Polynucleotide

Cos Endonuclease Recognition (CER) Domain Variable Targeting (VT) Domain

FIGURE 1B

Single Guide Polynucleotide

Cas Endonuclease Recognition (CER) Domain Variable Targeting (VT) Domain
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GENOME MODIFICATION USING GUIDE POLYNUCLEOTIDE/CAS
ENDONUCLEASE SYSTEMS AND METHODS OF USE

This application claims the benefit of U.S. Provisional Application No. 61/868706, filed August, 22, 2013; U.S. Provisional Application No. 61/882532, filed September 25, 2013; U.S. Provisional Application No. 61/937045, filed February 07, 2014; U.S. Provisional Application No. 61/953090, filed March 14, 2014; and U.S. Provisional Application No. 62/023239, filed July 11, 2014; all of which are hereby incorporated herein in their entirety by reference.

FIELD

The disclosure relates to the field of molecular biology, in particular, to methods for altering the genome of a cell.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 20140815_BB2344PCT_ST25_SequenceListing created on August 15, 2014 and having a size of 82 kilobytes and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

BACKGROUND

Recombinant DNA technology has made it possible to insert foreign DNA sequences into the genome of an organism, thus, altering the organism's phenotype.

One method for inserting or modifying a DNA sequence involves homologous DNA recombination by introducing a transgenic DNA sequence flanked by sequences homologous to the genomic target. U.S. Patent No. 5,527,695 describes transforming eukaryotic cells with DNA sequences that are targeted to a predetermined sequence of the eukaryote's DNA. Specifically, the use of site-specific recombination is discussed. Transformed cells are identified through use of a selectable marker included as a part of the introduced DNA sequences.


Although several approaches have been developed to target a specific site for modification in the genome of a cell, there still remains a need for more efficient and effective methods for producing an organism, such as but not limited to yeast and fertile plants, having an altered genome comprising specific modifications in a defined region of the genome of the cell.

BRIEF SUMMARY

Compositions and methods are provided employing a guide polynucleotide/Cas endonuclease system for genome modification of a target sequence in the genome of a cell or organism, for gene editing, and for inserting a polynucleotide of interest into the genome of a cell or organism. The methods and compositions employ a guide polynucleotide/Cas endonuclease system to provide for an effective system for modifying or altering target sites and editing nucleotide sequences of interest within the genome of cell, wherein the guide polynucleotide is comprised of a DNA, RNA or a DNA-RNA combination sequence. Cells include, but are not limited to non-human, animal, bacterial, fungal, insect, yeast, and plant cells. Once a genomic target site is identified, a variety of methods can be employed to further modify the target sites such that they contain a variety of polynucleotides of
interest. Breeding methods and methods for selecting plants utilizing a guide polynucleotide and Cas endonuclease system are also disclosed. Also provided are nucleic acid constructs, cells, yeast, plants, plant cells, explants, seeds and grain having the guide polynucleotide/Cas endonuclease system. Compositions and methods are also provided for editing a nucleotide sequence in the genome of a cell. The nucleotide sequence to be edited (the nucleotide sequence of interest) can be located within or outside a target site that is recognized by a Cas endonuclease.

Thus in a first embodiment of the disclosure, the composition comprises a guide polynucleotide comprising: (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and, (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein the first nucleotide sequence domain and the second nucleotide sequence domain are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids. The % complementation between the first nucleotide sequence domain (Variable Targeting domain) and the target sequence can be at least 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%. The first nucleotide sequence domain (VT domain) comprises a contiguous stretch of 12 to 30 nucleotides.

In one embodiment, the first nucleotide sequence domain (VT domain) and the second nucleotide sequence domain of the guide polynucleotide are located on a single molecule. In another embodiment, the second nucleotide sequence domain (Cas Endonuclease Recognition domain) comprises two separate molecules that are capable of hybridizing along a region of complementarity.

In another embodiment, the composition comprises a guide polynucleotide, wherein the first nucleotide sequence domain is a DNA sequence and the second nucleotide sequence domain is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

In another embodiment, the composition comprises a guide polynucleotide, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain comprises at least one modification that optionally provides for an
additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer molecule, a 5' to 3' covalent linkage, or any combination thereof. The additional beneficial can be a modified or regulated stability, a subcellular targeting, tracking, a fluorescent label, a binding site for a protein or protein complex, modified binding affinity to complementary target sequence, modified resistance to cellular degradation, or an increased cellular permeability.

In another embodiment, the composition comprises a guide polynucleotide/Cas endonuclease complex wherein the guide polynucleotide comprises (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

In another embodiment, the composition comprises a guide polynucleotide/Cas endonuclease complex, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain of said guide polynucleotide comprises at least one modification that optionally provides for an additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC
nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5' to 3' covalent linkage, or any combination thereof.

In another embodiment, the composition comprises plant or seed comprising the guide polynucleotide or the guide polynucleotide/Cas endonuclease complex of the disclosure.

In another embodiment, the method comprises a method for modifying a target site in the genome of a cell, the method comprising introducing a guide polynucleotide into a cell having a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

In another embodiment, the method comprises a method for modifying a target site in the genome of a cell, the method comprising introducing a guide polynucleotide and a Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

In another embodiment, the method comprises a method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising: a) providing a guide polynucleotide, a donor DNA and a Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and, c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.
In another embodiment, the method comprises a method for modifying a target site in the genome of a cell, the method comprising: a) providing to a cell a crNucleotide, a first recombinant DNA construct capable of expressing a tracrRNA, and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said crNucleotide is a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said crNucleotide, said tracrRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and, b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

In another embodiment, the method comprises a method for modifying a target site in the genome of a cell, the method comprising: a) providing to a cell a tracrNucleotide, a first recombinant DNA construct capable of expressing a crRNA and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said tracrNucleotide is selected a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said tracrNucleotide, said crRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and, b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

In another embodiment, the method comprises a method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising: a) introducing into a cell a first recombinant DNA construct capable of expressing a guide polynucleotide, and a second recombinant DNA construct capable of expressing a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; b) contacting the cell of (a)
with a donor DNA comprising a polynucleotide of interest; and, c) identifying at least
one cell from (b) comprising in its genome the polynucleotide of interest integrated
at said target site.

In another embodiment, the method comprises a method for editing a
nucleotide sequence in the genome of a cell, the method comprising introducing a
guide polynucleotide, a polynucleotide modification template and at least one Cas
endonuclease into a cell, wherein said guide polynucleotide does not solely
comprise ribonucleic acids, wherein the Cas endonuclease introduces a double-
strand break at a target site in the genome of said cell, wherein said polynucleotide
modification template comprises at least one nucleotide modification of said
nucleotide sequence.

In another embodiment, the composition comprises a plant or seed
comprising a guide polynucleotide and a Cas endonuclease, wherein said guide
polynucleotide does not solely comprise ribonucleic acids, wherein said Cas
endonuclease and guide polynucleotide are capable of forming a complex and
creating a double strand break in a genomic target site of said plant.

In another embodiment, the composition comprises a plant or seed
comprising a recombinant DNA construct and a guide polynucleotide, wherein said
guide polynucleotide does not solely comprise ribonucleic acids, wherein said
recombinant DNA construct comprises a promoter operably linked to a nucleotide
sequence encoding a plant optimized Cas endonuclease, wherein said plant
optimized Cas endonuclease and guide polynucleotide are capable of forming a
complex and creating a double strand break in a genomic target site of said plant.

In another embodiment, the method comprises a method for selecting a plant
comprising an altered target site in its plant genome, the method comprising: a)
obtaining a first plant comprising at least one Cas endonuclease capable of
introducing a double strand break at a target site in the plant genome; b) obtaining a
second plant comprising a guide polynucleotide that is capable of forming a complex
with the Cas endonuclease of (a), wherein the guide polynucleotide does not solely
comprise ribonucleic acids, c) crossing the first plant of (a) with the second plant of
(b); d) evaluating the progeny of (c) for an alteration in the target site and e)
selecting a progeny plant that possesses the desired alteration of said target site.
Additional embodiments of the methods and compositions of the present disclosure are disclosed below.

**BRIEF DESCRIPTION OF THE DRAWINGS AND THE SEQUENCE LISTING**

The disclosure can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing, which form a part of this application. The sequence descriptions and sequence listing attached hereto comply with the rules governing nucleotide and amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §§1.821-1.825. The sequence descriptions contain the three letter codes for amino acids as defined in 37 C.F.R. §§ 1.821-1.825, which are incorporated herein by reference.

**Figures**

Figure 1A shows a duplex guide polynucleotide containing a double molecule comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA and a second nucleotide sequence domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide. The CER domain of the duplex guide polynucleotide comprises two separate molecules that are hybridized along a region of complementarity. The two separate molecules can be RNA, DNA, and/or RNA-DNA-combination sequences. The first molecule of the duplex guide polynucleotide comprising a VT domain linked to a CER domain (shown as crNucleotide) is referred to as "crDNA" (when composed of a contiguous stretch of DNA nucleotides) or "crRNA" (when composed of a contiguous stretch of RNA nucleotides), or "crDNA-RNA" (when composed of a combination of DNA and RNA nucleotides). The second molecule of the duplex guide polynucleotide comprising a CER domain (shown as tracrNucleotide) is referred to as "tracrRNA" (when composed of a contiguous stretch of RNA nucleotides) or "tracrDNA" (when composed of a contiguous stretch of DNA nucleotides) or "tracrDNA-RNA" (when composed of a combination of DNA and RNA nucleotides).
Figure 1B shows a single guide polynucleotide comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA and a second nucleotide domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide. By "domain" it is meant a contiguous stretch of nucleotides that can be RNA, DNA, and/or RNA-DNA-combination sequences. The single guide polynucleotide comprises a crNucleotide (comprising a VT domain linked to a CER domain) linked to a tracrNucleotide (comprising a CER domain) with a linker nucleotide sequence (shown as a loop).

The single guide polynucleotide being comprised of sequences from the crNucleotide and tracrNucleotide may be referred to as "single guide RNA" (when composed of a contiguous stretch of RNA nucleotides) or "single guide DNA" (when composed of a contiguous stretch of DNA nucleotides) or "single guide RNA-DNA" (when composed of a combination of RNA and DNA nucleotides).

Figure 2A-2C show expression cassettes for Cas9, crRNA and tracrRNA expression. Figure 2A shows a maize codon optimized Cas9 gene (encoding a Cas9 endonuclease) containing a potato ST-LS1 intron, a SV40 amino terminal nuclear localization sequence (SV40 NLS), and a VirD2 carboxyl terminal NLS (VirD2 NLS), operably linked to a plant ubiquitin promoter (UBI Pro) (SEQ ID NO: 5).

The maize optimized Cas9 gene (just Cas9 coding sequence, no NLSs) corresponds to nucleotide positions 2037-2411 and 2601-6329 of SEQ ID NO: 5 with the potato intron residing at positions 2412-2600 of SEQ ID NO: 5.SV40 NLS is at positions 201 0-2036 of SEQ ID NO: 5. VirD2 NLS is at positions 6330-6386 of SEQ ID NO: 5. Figure 2B shows a maize U6 polymerase III promoter operably linked to a nucleotide sequence encoding a crRNA molecule operably linked to a maize U6 terminator. The resulting maize optimized crRNA expression cassette is listed in SEQ ID NO: 8. Figure 2C shows a maize U6 polymerase III promoter operably linked to a nucleotide sequence encoding a tracrRNA molecule operably linked to a maize U6 PolIII terminator. The resulting maize optimized tracrRNA expression cassette is listed in SEQ ID NO: 9.

Figure 3A shows a duplex guide RNA/Cas9 endonuclease system and target DNA complex relative to the appropriately oriented PAM sequence (AGG) at the
maize LIGCas-3 target sequence (SEQ ID NO: 14, Table 1). The duplex guide RNA
(lighter gray backgrounds) comprises a crRNA molecule (SEQ ID NO: 10)
containing a variable targeting domain (VT domain) base-pairing to the
complementary strand of the LIGCas-3 target sequence, and a tracrRNA molecule
(SEQ ID NO: 11) comprising part of the CER domain. The Cas9 endonuclease is
depicted in dark gray. Triangles point towards the expected site of DNA cleavage
on both sense and anti-sense DNA strands.

Figure 3B shows a single guide RNA/Cas9 endonuclease complex interacting
with the genomic LIGCas-3 target site relative to the appropriately oriented PAM
sequence (AGG) at the maize genomic LIGCas-3 target site (SEQ ID NO: 14, Table
1). The single guide RNA (light gray background, SEQ ID NO: 96) is a fusion
between a crRNA and tracrRNA and comprises a variable targeting domain that is
complementary to one DNA strand of the double strand DNA genomic target site.
The Cas9 endonuclease is shown in dark gray. Triangles point towards the
expected site of DNA cleavage on both sense and anti-sense DNA strands.

Figures 4A-4C show an alignment and count of the top 10 most frequent
NHEJ mutations induced by the maize optimized guide RNA/Cas endonuclease
system described herein at the maize genomic Liguleless 1 locus. The mutations
were identified by deep sequencing. The PAM sequence and expected site of
cleavage are also indicated. Deletions or insertions as a result of imperfect NHEJ
are shown by a "-" or an italicized underlined nucleotide, respectively. In Figure 4A,
the reference sequence (SEQ ID NO: 23) represents the unmodified LIGCas-1 locus
with the target site underlined. The sequences comprising the mutations 1-10 of the
LIGCas-1 target site correspond to SEQ ID NOs: 24-33, respectively. In Figure 4B,
the reference sequence (SEQ ID NO: 23) represents the unmodified LIGCas-2 locus
with the target site underlined. The sequences comprising the mutations 1-10 of the
LIGCas-2 target site correspond to SEQ ID NOs: 34-43, respectively. In Figure 4C,
the reference sequence (SEQ ID NO: 44) represents the unmodified LIGCas-3 locus
with the target site underlined. The sequences comprising the mutations 1-10 of the
LIGCas-3 target site correspond to SEQ ID NOs: 45-54, respectively.

Figure 5 shows a duplex guide polynucleotide/Cas9 endonuclease system
and target DNA complex relative to the appropriately oriented PAM sequence at the
maize LIGCas-3 target sequence (SEQ ID NO: 14, Table 1). The duplex guide RNA (lighter gray backgrounds) comprises a crDNA molecule (SEQ ID NO: 55) containing a variable targeting domain (VT domain) base-pairing to the complementary strand of the LIGCas-3 target sequence and a tracrRNA molecule (SEQ ID NO: 11) comprising part of the CER domain. The Cas9 endonuclease is shown in dark gray. Triangles point towards the expected site of DNA cleavage on both sense and anti-sense DNA strands.

Figures 6A-6B show alignments and counts of the top 3 most frequent NHEJ mutations induced by either a maize optimized duplex guide RNA/Cas endonuclease system (Figure 6A) or a maize optimized duplex guide polynucleotide/Cas endonuclease system (Figure 6B) described herein at the maize genomic Liguleless 1 locus. The mutations were identified by deep sequencing. The PAM sequence and expected site of cleavage are also indicated. Deletions or insertions as a result of imperfect NHEJ are shown by a "-" or an italicized underlined nucleotide, respectively. In Figure 6A, the NHEJ mutations originated from synthetic crRNA plus tracrRNA and Cas9 expression cassettes. The reference sequence (SEQ ID NO: 44) represents the unmodified LIGCas-3 locus with the target site underlined. The sequences comprising the mutations 1-3 of the LIGCas-3 target site correspond to SEQ ID NOs: 56-58, respectively. In Figure 6B, the NHEJ mutations originated from synthetic crDNA plus tracrRNA and Cas9 expression cassettes. The reference sequence (SEQ ID NO: 44) represents the unmodified LIGCas-3 locus with the target site underlined. The sequences comprising the mutations 1-3 of the LIGCas-3 target site correspond to SEQ ID NOs: 59-61, respectively.

Figure 7 illustrates the disruption of the yeast ADE2 gene on chromosome 15 with URA3 coding sequence and 305 bp of duplicated ADE2 gene sequence resulting in the ADE:URA3:DE2 yeast screening strain.

Figure 8 illustrates the scheme by which cleavage activity may be monitored in the yeast ADE:URA3:DE2 screening strain. If the URA3 target site is cleaved, the ADE2 sequence duplications flanking the URA3 coding sequence may be used as template for homologous recombination repair of the DNA double strand break. As depicted by dashed lines leading from the regions of ADE2 sequence duplication in
the ADE:URA3:DE2 configuration to the ADE2 configuration, homologous recombination mediated repair of the double strand break results in the loss of the URA3 gene coding sequence and the gain of a functional ADE2 gene.

Figure 9 shows the numerical scale and corresponding red/white sectoring of yeast colonies used to quantify cleavage activity. Since the sectoring phenotype is a qualitative measure of cleavage activity, a 0-4 numerical scoring system was implemented. A score of 0 indicates that no white sectors (no cutting) were observed; a score of 4 indicates completely white colonies (complete cutting of the recognition site); scores of 1-3 indicate intermediate white sectoring phenotypes (and intermediate degrees of recognition site cutting).

**Sequences**

SEQ ID NO: 1 is the nucleotide sequence of the Cas9 gene from *Streptococcus pyogenes* M1 GAS (SF370).

SEQ ID NO: 2 is the nucleotide sequence of the potato ST-LS1 intron.

SEQ ID NO: 3 is the amino acid sequence of SV40 amino N-terminal.

SEQ ID NO: 4 is the amino acid sequence of *Agrobacterium tumefaciens* bipartite VirD2 T-DNA border endonuclease carboxyl terminal.

SEQ ID NO: 5 is the nucleotide sequence of an expression cassette expressing the maize optimized Cas9.

SEQ ID NO: 6 is the nucleotide sequence of the maize U6 polymerase III promoter.

SEQ ID NO: 7 is the amino acid sequence a SV40 nuclear localization signal.

SEQ ID NO: 8 is the nucleotide sequence of a maize optimized crRNA expression cassette containing the variable targeting domain targeting the LIGCas-3 target sequence.

SEQ ID NO: 9 is the nucleotide sequence of a maize optimized tracrRNA expression cassette.

SEQ ID NO: 10 is the nucleotide sequence of a crRNA containing a variable targeting domain targeting the LIGCas-3 target sequence.

SEQ ID NO: 11 is the nucleotide sequence of the tracrRNA from *Streptococcus pyogenes* M1 GAS (SF370)>
SEQ ID NO: 12 is the nucleotide sequence of the maize genomic target site LIGCas-1 plus PAM sequence.
SEQ ID NO: 13 is the nucleotide sequence of the maize genomic target site LIGCas-2 plus PAM sequence.
SEQ ID NO: 14 is the nucleotide sequence of the maize genomic target site LIGCas-3 plus PAM sequence.
SEQ ID NOs: 15-22 are nucleotide sequences of PCR primers.
SEQ ID NO: 23 is the nucleotide sequence of the unmodified reference sequence for LIGCas-1 and LIGCas-2 locus (Figure 4A-4B)
SEQ ID NOs: 24-33 are the nucleotide sequences of mutations 1-10 for the LIGCas-1 locus (Figure 4A).
SEQ ID NOs: 34-43 are the nucleotide sequences of mutations 1-10 for the LIGCas-2 locus (Figure 4B).
SEQ ID NO: 44 is the nucleotide sequence of the unmodified reference sequence for LIGCas-3 (Figure 4C)
SEQ ID NOs: 45-54 are the nucleotide sequences of mutations 1-10 for the LIGCas-3 locus (Figure 4C).
SEQ ID NO: 55 is the nucleotide sequence of a crDNA (comprised of deoxyribonucleic acids) containing a variable targeting domain targeting the LIGCas-3 target sequence
SEQ ID NOs: 56-58 are the nucleotide sequences of mutations 1-3 for the LIGCas-3 locus (originating from synthetic crRNA plus tracrRNA and Cas9 expression cassettes) (Figure 6A).
SEQ ID NOs: 59-61 are the nucleotide sequences of mutations 1-3 for the LIGCas-3 locus (originating from synthetic crDNA plus tracrRNA and Cas9 expression cassettes) (Figure 6B).
SEQ ID NO: 62 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crRNA) that does not include any modification to its ribonucleotide sequence.
SEQ ID NO: 63 is the nucleotide sequence of a CER domain of a crNucleotide (crRNA) that does not include any modification to its ribonucleotide sequence.
SEQ ID NO: 64 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crRNA), that includes phosphorothioate bonds at the 5' end of its nucleotide sequence (G°C'G'). In the sequence listing, the first N at the 5' end represents a G ribonucleotide with a phosphorothioate bond, the second N represents a C ribonucleotide with a phosphorothioate bond and third N represents a G ribonucleotide with a Phosphorothioate bond.

SEQ ID NO: 65 is the nucleotide sequence of a CER domain of a crNucleotide (crRNA) that includes phosphorothioate bonds near the 3' end of its nucleotide sequence (U'U'U'). In the sequence listing, the Ns at the nineteenth, twentieth and twenty-first positions represent U ribonucleotides with phosphorothioate bonds.

SEQ ID NO: 66 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crRNA) that includes 2'-O-methyl RNA nucleotides at its 5' end (mGmCmG). In the sequence listing, the first N at the 5' end represents a G 2'-O-methyl ribonucleotide, the second N represents a C 2'-O-methyl ribonucleotide and the third N represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 67 is the nucleotide sequence of a CER domain of a crNucleotide (crRNA) that includes 2'-O-methyl RNA nucleotides near the 3' end of its nucleotide sequence (mUmUmG). In the sequence listing, the N at the twentieth position represents a U 2'-O-Methyl ribonucleotide, the N at the twenty-first position represents a U 2'-O-Methyl ribonucleotide and the N at the twenty-second position represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 68 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crRNA) that includes 2'-O-Methyl RNA nucleotides for each nucleotide. In the sequence listing, the first N at the 5' end represents a G 2'-O-Methyl ribonucleotide, a N at the second position represents a C 2'-O-Methyl ribonucleotide, a N at the third position represents a G 2'-O-Methyl ribonucleotide, a N at the fourth position represents an U 2'-O-Methyl ribonucleotide, a N at the fifth position represents an A 2'-O-Methyl ribonucleotide, a N at the sixth position represents a C 2'-O-Methyl ribonucleotide, a N at the seventh position represents a G 2'-O-Methyl ribonucleotide, a N at the eighth position represents a C 2'-O-Methyl ribonucleotide, a
N at the tenth position represents an U 2'-O-Methyl ribonucleotide, a N at the eleventh position represents an A 2'-O-Methyl ribonucleotide, a N at the twelfth position represents C 2'-O-Methyl ribonucleotide, a N at the thirteenth position represents a G 2'-O-Methyl ribonucleotide, a N at the fourteenth position represents an U 2'-O-Methyl ribonucleotide, a N at the fifteenth position represents a G 2'-O-Methyl ribonucleotide, a N at the sixteenth position represents an U 2'-O-Methyl ribonucleotide and a N seventeenth position represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 69 is the nucleotide sequence of a CER domain of a crNucleotide (crRNA) that include 2'-O-Methyl RNA nucleotides for each nucleotide. In the sequence listing, the first N at the 5' end represents a G 2'-O-Methyl ribonucleotide, a N at the second position represents an U 2'-O-Methyl ribonucleotide, a N at the third position represents an U 2'-O-Methyl ribonucleotide, a N at the fourth position represents an U 2'-O-Methyl ribonucleotide, a N at the fifth position represents an U 2'-O-Methyl ribonucleotide, a N at the sixth position represents an A 2'-O-Methyl ribonucleotide, a N at the seventh position represents a G 2'-O-Methyl ribonucleotide, a N at the eighth position represents an A 2'-O-Methyl ribonucleotide, a N at the ninth position represents a G 2'-O-Methyl ribonucleotide, a N at the tenth position represents a C 2'-O-Methyl ribonucleotide, a N at the eleventh position represents an U 2'-O-Methyl ribonucleotide, a N at the twelfth position represents an A 2'-O-Methyl ribonucleotide, a N at the thirteenth position represents an U 2'-O-Methyl ribonucleotide, a N at the fourteenth position represents a G 2'-O-Methyl ribonucleotide, a N at the fifteenth position represents a C 2'-O-Methyl ribonucleotide, a N at the sixteenth position represents an U 2'-O-Methyl ribonucleotide, a N at the seventeenth position represents a G 2'-O-Methyl ribonucleotide, a N at the eighteen position represents an U 2'-O-Methyl ribonucleotide, a N at the nineteenth position represents an U 2'-O-Methyl ribonucleotide, a N at the twentieth position represents an U 2'-O-Methyl ribonucleotide, a N at the twenty-first position represents an U 2'-O-Methyl ribonucleotide and a N at the twenty-second position represents a G 2'-O-Methyl ribonucleotide.
SEQ ID NO: 70 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crDNA) that does not include any modification to its deoxyribonucleotide sequence.

SEQ ID NO: 71 is the nucleotide sequence of a CER domain of a crNucleotide (crDNA) that does not include any modification to its deoxyribonucleotide sequence.

SEQ ID NO: 72 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crDNA), which includes one Locked Nucleic Acid nucleotide (+T) in its nucleotide sequence. In the sequence listing, an N at the sixteenth position represents a T Locked Nucleic Acid base.

SEQ ID NO: 73 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crDNA), which includes three Locked Nucleic Acid nucleotide (+C,+T,+T) in its nucleotide sequence. In the sequence listing, an N at the twelfth position represents a C Locked Nucleic Acid base, a N at the fourteenth position represents a T Locked Nucleic Acid base and a N at the sixteenth position represents a T Locked Nucleic Acid base.

SEQ ID NO: 74 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crDNA), that includes six Locked Nucleic Acid nucleotide (+C,+C,+T,+C,+T,+T) in its nucleotide sequence. In the sequence listing, a N at the sixth position represents a C Locked Nucleic Acid base, a N at the eighth position represents a C Locked Nucleic Acid base, a N at the tenth position represents a T Locked Nucleic Acid base, a N at the twelfth position represents a C Locked Nucleic Acid base, a N at the fourteenth position represents a T Locked Nucleic Acid base and a N at the sixteenth position represents a T Locked Nucleic Acid base.

SEQ ID NO: 75 is the nucleotide sequence of a variable targeting domain of a crNucleotide (crDNA), that includes three Locked Nucleic Acid nucleotide (+C,+T,+T) in its nucleotide sequence and phosphorothioate bonds near the 5' end of its nucleotide sequence (G*C*G*). In the sequence listing, a first N at the 5' end represents a G deoxyribonucleotide with a phosphorothioate bond, a N at the second position represents a C deoxyribonucleotide with a phosphorothioate bond, a N at the third position represents a G deoxyribonucleotide with a phosphorothioate bond, a N at the twelfth position represents a C Locked Nucleic Acid base, a N at
the fourteenth position represents a T Locked Nucleic Acid base and a N at the
sixteenth position represents a T Locked Nucleic Acid base.

SEQ ID NO: 76 is the nucleotide sequence of a CER domain of a
crNucleotide (crDNA) that includes three Locked Nucleic Acid nucleotide (T\'T\'T)
5 near the 3' end of the nucleotide sequence. In the sequence listing, the Ns at the
nineteenth, twentieth and twenty-first positions represent T deoxyribonucleotides
with Phosphorothioate bonds.

SEQ ID NO: 77 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes one 5-Methyl dC nucleotide in its nucleotide
10 sequence. In the sequence listing, a N at the twelfth position represents a 5-Methyl
dC deoxyribonucleotide.

SEQ ID NO: 78 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes three 5-Methyl dC nucleotide in its nucleotide
sequence. In the sequence listing, Ns at the sixth, eighth and twelfth positions
15 represent 5-Methyl dC deoxyribonucleotides.

SEQ ID NO: 79 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes one 2,6-diaminopurine nucleotide in its
nucleotide sequence. In the sequence listing, a N at the eleventh position
20 represents a 2,6-Diaminopurine deoxyribonucleotide.

SEQ ID NO: 80 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes two 2,6-diaminopurine nucleotides in its
nucleotide sequence. In the sequence listing, a Ns at the fifth and eleventh positions
25 represent 2,6-diaminopurine deoxyribonucleotides.

SEQ ID NO: 81 is the nucleotide sequence of a variable targeting domain of
28 a crNucleotide (crDNA) that includes Locked Nucleic Acid nucleotides near the 5'
end of its nucleotide sequence. In the sequence listing, the first N at the 5' end
represent a G Locked Nucleic Acid base, second N represents a C Locked Nucleic
32 Acid base and third N represents a G Locked Nucleic Acid base.

SEQ ID NO: 82 is the nucleotide sequence of a CER domain of a
crNucleotide (crDNA) that includes Locked Nucleic Acid nucleotides near the 3' end
of the nucleotide sequence. In the sequence listing, a N at the twentieth position
37 represents a T Locked Nucleic Acid base, a N at the twenty-first position represents

17
a T Locked Nucleic Acid base and a N at the twenty-second position represents a G
Locked Nucleic Acid base.

SEQ ID NO: 83 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes phosphorothioate bonds near the 5' end of its
nucleotide sequence. In the sequence listing, a first N at the 5' end represents a G
deoxyribonucleotide with a phosphorothioate bond, second N represents a C
deoxyribonucleotide with a phosphorothioate bond and third N represents a G
deoxyribonucleotide with a phosphorothioate bond.

SEQ ID NO: 84 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes 2'-O-Methyl RNA nucleotides near the 5' end
of its nucleotide sequence. In the sequence listing, a first N at the 5' end represents
a G 2'-O-Methyl ribonucleotide, second N represents a C 2'-O-Methyl ribonucleotide
and third N represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 85 is the nucleotide sequence of a CER domain of a
crNucleotide (crDNA) that includes 2'-O-Methyl RNA nucleotides near the 3' end
of the nucleotide sequence. In the sequence listing, a N at the twentieth position
represents a U 2'-O-Methyl ribonucleotide, a N at the twenty-first position represents
a U 2'-O-Methyl ribonucleotide and a N at the twenty-second position represent G
2'-O-Methyl ribonucleotide.

SEQ ID NO: 86 is the nucleotide sequence of a variable targeting domain of
a crNucleotide (crDNA) that includes 2'-O-Methyl RNA nucleotides at each
nucleotide except T of its nucleotide sequence. In the sequence listing, a first N at
the 5' end represents a G 2'-O-Methyl ribonucleotide, a N at the second position
represents a C 2'-O-Methyl ribonucleotide, a N at the third position represents a G
2'-O-Methyl ribonucleotide, a N at the fifth position represents an A 2'-O-Methyl
ribonucleotide, a N at the sixth position represents a C 2'-O-Methyl ribonucleotide, a
N at the seventh position represents a G 2'-O-Methyl ribonucleotide, a N at the
eighth position represents a C 2'-O-Methyl ribonucleotide, a N at the ninth position
represents a G 2'-O-Methyl ribonucleotide, a N at the eleventh position represents
an A 2'-O-Methyl ribonucleotide, a N at the twelfth position represents C 2'-O-Methyl
ribonucleotide, a N at the thirteenth position represents a G 2'-O-Methyl
ribonucleotide, a N at the fifteenth position represents a G 2'-O-Methyl
ribonucleotide and a N at the seventeenth position represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 87 is the nucleotide sequence of a CER domain of a crNucleotide (crDNA) that includes 2'-O-Methyl RNA nucleotides at each nucleotide except T the nucleotide sequence. In the sequence listing, a first N at the 5' end represents a G 2'-O-Methyl ribonucleotide, a N at the sixth position represents an A 2'-O-Methyl ribonucleotide, a N at the seventh position represents a G 2'-O-Methyl ribonucleotide, a N at the eighth position represents an A 2'-O-Methyl ribonucleotide, a N at the ninth position represents a G 2'-O-Methyl ribonucleotide, a N at the tenth position represents a C 2'-O-Methyl ribonucleotide, a N at the twelfth position represents an A 2'-O-Methyl ribonucleotide, a N at the fourteenth position represents a G 2'-O-Methyl ribonucleotide, a N at the fifteenth position represents a C 2'-O-Methyl ribonucleotide, a N at the seventeenth position represents a G 2'-O-Methyl ribonucleotide and a N at the twenty-second position represents a G 2'-O-Methyl ribonucleotide.

SEQ ID NO: 88 is the nucleotide sequence of the Saccharomyces cerevisiae codon optimized Cas9.

SEQ ID NO: 89 is the nucleotide sequence of the T7 promoter from bacteriophage T7.

SEQ ID NO: 90 is the nucleotide sequence of the ADE:URA3:DE2 target sequence (PAM sequence not included)

SEQ ID NO: 91-95 are the nucleotide sequences of Cas9 endonucleases.

SEQ ID NO: 96 is the nucleotide sequence of a single guide RNA targeting the LIGCas-3 target sequence (Figure 3B).

DETAILED DESCRIPTION

The present disclosure includes compositions and methods for genome modification of a target sequence in the genome of a cell. The methods and compositions employ a guide polynucleotide/Cas endonuclease system to provide an effective system for modifying target sites within the genome of a cell. Cells include, but are not limited to, animal, bacterial, fungal, insect, yeast, and plant cells as well as plants and seeds produced by the methods described herein. Once a
genomic target site is identified, a variety of methods can be employed to further modify the target sites such that they contain a variety of polynucleotides of interest. Breeding methods utilizing a guide polynucleotide/Cas endonuclease system are also disclosed. Compositions and methods are also provided for editing a nucleotide sequence in the genome of a cell. The nucleotide sequence to be edited (the nucleotide sequence of interest) can be located within or outside a target site that is recognized by a Cas endonuclease.

CRISPR loci (Clustered Regularly Interspaced Short Palindromic Repeats) (also known as SPIDRs-SPacer Interspersed Direct Repeats) constitute a family of recently described DNA loci. CRISPR loci consist of short and highly conserved DNA repeats (typically 24 to 40 bp, repeated from 1 to 140 times—also referred to as CRISPR-repeats) which are partially palindromic. The repeated sequences (usually specific to a species) are interspaced by variable sequences of constant length (typically 20 to 58 by depending on the CRISPR locus (WO2007/025097 published March 1, 2007).


Cas gene includes a gene that is generally coupled, associated or close to or in the vicinity of flanking CRISPR loci. The terms "Cas gene", "CRISPR-associated (Cas) gene" are used interchangeably herein. A comprehensive review of the Cas protein family is presented in Haft et al. (2005) Computational Biology, PLoS Comput Biol 1(6): e60. doi:1 0.1371/journal.pcbi.001 0060. As described therein, 41
CRISPR-associated (Cas) gene families are described, in addition to the four previously known gene families. It shows that CRISPR systems belong to different classes, with different repeat patterns, sets of genes, and species ranges. The number of Cas genes at a given CRISPR locus can vary between species.

Cas endonuclease relates to a Cas protein encoded by a Cas gene, wherein said Cas protein is capable of introducing a double strand break into a DNA target sequence. The Cas endonuclease unwinds the DNA duplex in close proximity of the genomic target site and cleaves both DNA strands upon recognition of a target sequence by a guide polynucleotide, but only if the correct protospacer-adjacent motif (PAM) is approximately oriented at the 3' end of the target sequence (Figure 3A, Figure 3B).

In one embodiment, the Cas endonuclease is a Cas9 endonuclease that is capable of introducing a double strand break at a DNA target site, wherein the DNA cleavage at a specific location is enabled by a) base-pairing complementary between the DNA target site and the variable targeting domain of the guide polynucleotide, and b) the presence of a short protospacer adjacent motif (PAM) immediately adjacent to the DNA target site.

In one embodiment, the Cas endonuclease gene is a Cas9 endonuclease, such as but not limited to, Cas9 genes listed in SEQ ID NOs: 462, 474, 489, 494, 499, 505, and 518 of WO2007/025097 published March 1, 2007, and incorporated herein by reference. In another embodiment, the Cas endonuclease gene is plant, maize or soybean optimized Cas9 endonuclease (Figure 1A). In another embodiment, the Cas endonuclease gene is operably linked to a SV40 nuclear targeting signal upstream of the Cas codon region and a bipartite VirD2 nuclear localization signal (Tinland et al. (1992) Proc. Natl. Acad. Sci. USA 89:7442-6) downstream of the Cas codon region.

In one embodiment, the Cas endonuclease gene is a Cas9 endonuclease gene of SEQ ID NO: 1, 91, 92, 93, 94, 95 or nucleotides 2037-6329 of SEQ ID NO:5, or any functional fragment or variant thereof.

The terms "functional fragment", "fragment that is functionally equivalent" and "functionally equivalent fragment" are used interchangeably herein. These
terms refer to a portion or subsequence of a Cas endonuclease sequence in which the ability to create a double-strand break is retained.

The terms "functional variant" and "variant that is functionally equivalent" and "functionally equivalent variant" are used interchangeably herein. These terms refer to a variant of the Cas endonuclease in which the ability to create a double-strand break is retained. Fragments and variants can be obtained via methods such as site-directed mutagenesis and synthetic construction.

In one embodiment, the Cas endonuclease gene is a plant codon optimized *streptococcus pyogenes* Cas9 gene that can recognize any genomic sequence of the form N(1 2-30)NGG can in principle be targeted.

Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain, and include restriction endonucleases that cleave DNA at specific sites without damaging the bases. Restriction endonucleases include Type I, Type II, Type III, and Type IV endonucleases, which further include subtypes. In the Type I and Type III systems, both the methylase and restriction activities are contained in a single complex. Endonucleases also include meganucleases, also known as homing endonucleases (HEases), which like restriction endonucleases, bind and cut at a specific recognition site, however the recognition sites for meganucleases are typically longer, about 18 bp or more. (patent application WO-PCT PCT/US 12/30061 filed on March 22, 2012) Meganucleases have been classified into four families based on conserved sequence motifs (Belfort M, and Perlman P S J. Biol. Chem. 1995;270:30237-30240). These motifs participate in the coordination of metal ions and hydrolysis of phosphodiester bonds. HEases are notable for their long recognition sites, and for tolerating some sequence polymorphisms in their DNA substrates. The naming convention for meganuclease is similar to the convention for other restriction endonuclease. Meganucleases are also characterized by prefix F-, I-, or PI- for enzymes encoded by free-standing ORFs, introns, and inteins, respectively. One step in the recombination process involves polynucleotide cleavage at or near the recognition site. This cleaving activity can be used to produce a double-strand break. For reviews of site-specific recombinases and their recognition sites, see, Sauer (1994) Curr Op Biotechnol
TAL effector nucleases are a new class of sequence-specific nucleases that can be used to make double-strand breaks at specific target sequences in the genome of a plant or other organism. (Miller et al. (2011) Nature Biotechnology 29:143-148). Zinc finger nucleases (ZFNs) include engineered double-strand break inducing agents comprised of a zinc finger DNA binding domain and a double-strand-break-inducing agent domain. Recognition site specificity is conferred by the zinc finger domain, which typically comprising two, three, or four zinc fingers, for example having a C2H2 structure, however other zinc finger structures are known and have been engineered. Zinc finger domains are amenable for designing polypeptides which specifically bind a selected polynucleotide recognition sequence. ZFNs consist of an engineered DNA-binding zinc finger domain linked to a non-specific endonuclease domain, for example nuclease domain from a Type II endonuclease such as FokI. Additional functionalities can be fused to the zinc-finger binding domain, including transcriptional activator domains, transcription repressor domains, and methylases. In some examples, dimerization of nuclease domain is required for cleavage activity. Each zinc finger recognizes three consecutive base pairs in the target DNA. For example, a 3 finger domain recognized a sequence of 9 contiguous nucleotides, with a dimerization requirement of the nuclease, two sets of zinc finger triplets are used to bind a 18 nucleotide recognition sequence.

In one embodiment of the disclosure, the composition comprises a plant or seed comprising a guide polynucleotide and a Cas9 endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said Cas9 endonuclease and guide polynucleotide are capable of forming a complex and creating a double strand break in a genomic target site of said plant.

Bacteria and archaea have evolved adaptive immune defenses termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems that use short RNA to direct degradation of foreign nucleic acids (WO2007/025097published March 1, 2007, and incorporated herein by
reference.) The type II CRISPR/Cas system from bacteria employs a crRNA and tracrRNA to guide the Cas endonuclease to its DNA target. The crRNA (CRISPR RNA) contains the region complementary to one strand of the double strand DNA target and base pairs with the tracrRNA (trans-activating CRISPR RNA) forming a RNA duplex that directs the Cas endonuclease to cleave the DNA target.

As used herein, the term "guide polynucleotide", relates to a polynucleotide sequence that can form a complex with a Cas endonuclease and enables the Cas endonuclease to recognize and optionally cleave a DNA target site. The guide polynucleotide can be a single molecule or a double molecule. The guide polynucleotide sequence can be a RNA sequence, a DNA sequence, or a combination thereof (a RNA-DNA combination sequence). Optionally, the guide polynucleotide can comprise at least one nucleotide, phosphodiester bond or linkage modification such as, but not limited, to Locked Nucleic Acid (LNA), 5-methyl dC, 2,6-Diaminopurine, 2'-Fluoro A, 2'-Fluoro U, 2'-O-Methyl RNA, Phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 (hexaethylene glycol chain) molecule, or 5' to 3' covalent linkage resulting in circularization. In some embodiment of this disclosure, the guide polynucleotide does not solely comprise ribonucleic acids (RNAs). A guide polynucleotide that solely comprises ribonucleic acids is also referred to as a "guide RNA".

The guide polynucleotide can be a double molecule (also referred to as duplex guide polynucleotide) comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA and a second nucleotide sequence domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide (Figure 1A). The CER domain of the double molecule guide polynucleotide comprises two separate molecules that are hybridized along a region of complementarity (Figure 1A). The two separate molecules can be RNA, DNA, and/or RNA-DNA-combination sequences. In one embodiment of this disclosure, the duplex guide polynucleotide does not solely comprise ribonucleic acids (RNAs) as show in, for example, but not limiting to, Figure 3A). In some embodiments, the first molecule of the duplex guide
polynucleotide comprising a VT domain linked to a CER domain (shown as "crNucleotide" in Figure 1A) is referred to as "crDNA" (when composed of a contiguous stretch of DNA nucleotides) or "crRNA" (when composed of a contiguous stretch of RNA nucleotides), or "crDNA-RNA" (when composed of a combination of DNA and RNA nucleotides). In some embodiments the second molecule of the duplex guide polynucleotide comprising a CER domain (shown as tracrNucleotide in Figure 1A) is referred to as "tracrRNA" (when composed of a contiguous stretch of RNA nucleotides) or "tracrDNA" (when composed of a contiguous stretch of DNA nucleotides) or "tracrDNA-RNA" (when composed of a combination of DNA and RNA nucleotides).

The guide polynucleotide can also be a single molecule comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that is complementary to a nucleotide sequence in a target DNA and a second nucleotide domain (referred to as Cas endonuclease recognition domain or CER domain) that interacts with a Cas endonuclease polypeptide (Figure 1B). By "domain" it is meant a contiguous stretch of nucleotides that can be RNA, DNA, and/or RNA-DNA-combination sequence. The VT domain and/or the CER domain of a single guide polynucleotide can comprise a RNA sequence, a DNA sequence, or a RNA-DNA-combination sequence. In some embodiments the single guide polynucleotide comprises a crNucleotide (comprising a VT domain linked to a CER domain) linked to a tracrNucleotide (comprising a CER domain), wherein the linkage is a nucleotide sequence comprising a RNA sequence, a DNA sequence, or a RNA-DNA combination sequence (Figure 1B). The single guide polynucleotide being comprised of sequences from the crNucleotide and tracrNucleotide may be referred to as "single guide RNA" (when composed of a contiguous stretch of RNA nucleotides) or "single guide DNA" (when composed of a contiguous stretch of DNA nucleotides) or "single guide RNA-DNA" (when composed of a combination of RNA and DNA nucleotides).

One advantage of using a single guide polynucleotide versus a duplex guide polynucleotide is that only one expression cassette needs to be made to express the single guide polynucleotide.
The term "variable targeting domain" or "VT domain" is used interchangeably herein and refers to a nucleotide sequence that is complementary to one strand (nucleotide sequence) of a double strand DNA target site. The % complementation between the first nucleotide sequence domain (VT domain) and the target sequence can be at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. The variable target domain can be at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. In some embodiments, the variable targeting domain comprises a contiguous stretch of 12 to 30 nucleotides.

The variable targeting domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence (see for example modifications described herein), or any combination thereof.

The term "Cas endonuclease recognition domain" or "CER domain" of a guide polynucleotide is used interchangeably herein and relates to a nucleotide sequence (such as a second nucleotide sequence domain of a guide polynucleotide), that interacts with a Cas endonuclease polypeptide. The CER domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence (see for example modifications described herein), or any combination thereof.

The nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide can comprise a RNA sequence, a DNA sequence, or a RNA-DNA combination sequence. In one embodiment, the nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide can be at least 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, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 nucleotides in length. In another embodiment, the nucleotide sequence linking the crNucleotide and the
tracrNucleotide of a single guide polynucleotide can comprise a tetraloop sequence, such as, but not limiting to a GAAA tetraloop sequence.

Nucleotide sequence modification of the guide polynucleotide, VT domain and/or CER domain can be selected from, but not limited to, the group consisting of a 5’ cap, a 3’ polyadenylated tail, a riboswitch sequence, a stability control sequence, a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2’-Fluoro A nucleotide, a 2’-Fluoro U nucleotide; a 2’-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5’ to 3’ covalent linkage, or any combination thereof. These modifications can result in at least one additional beneficial feature, wherein the additional beneficial feature is selected from the group of a modified or regulated stability, a subcellular targeting, tracking, a fluorescent label, a binding site for a protein or protein complex, modified binding affinity to complementary target sequence, modified resistance to cellular degradation, and increased cellular permeability.

In one embodiment of the disclosure, the composition comprises a guide polynucleotide comprising: (i) a first nucleotide sequence domain (VT domain) that is complementary to a nucleotide sequence in a target DNA; and, (ii) a second nucleotide sequence domain (CER domain) that interacts with a Cas endonuclease, wherein the first nucleotide sequence domain and the second nucleotide sequence domain are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids. The % complementation between the first nucleotide sequence domain (Variable Targeting domain) and the target sequence can be at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 63%, 65%, 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%, 99% or 100%.
In one embodiment of the disclosure, the first nucleotide sequence domain (VT domain) and the second nucleotide sequence domain (CER domain) of the guide polynucleotide are located on a single molecule. In another embodiment, the second nucleotide sequence domain (Cas Endonuclease Recognition domain) comprises two separate molecules that are capable of hybridizing along a region of complementarity.

In another embodiment, the composition comprises a guide polynucleotide, wherein the first nucleotide sequence domain is a DNA sequence and the second nucleotide sequence domain is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

In one embodiment, the composition comprises a guide polynucleotide, wherein the first nucleotide sequence domain (VT domain) is a DNA sequence and the second nucleotide sequence domain (CER domain) is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

The guide polynucleotide and Cas endonuclease are capable of forming a complex, referred to as the "guide polynucleotide/Cas endonuclease complex", that enables the Cas endonuclease to introduce a double strand break at a DNA target site.

In one embodiment, the composition comprises a guide polynucleotide/Cas endonuclease complex wherein the guide polynucleotide comprises (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

In another embodiment, the composition comprises a guide polynucleotide/Cas endonuclease complex, wherein the first nucleotide sequence domain (VT domain) and the second nucleotide sequence domain (CER domain) of the guide polynucleotide are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids.
In one embodiment the guide polynucleotide can be introduced into the plant cell directly using particle bombardment.

When the guide polynucleotide comprises solely of RNA sequences (also referred to as "guide RNA") it can be introduced indirectly by introducing a recombinant DNA molecule comprising the corresponding guide DNA sequence operably linked to a plant specific promoter that is capable of transcribing the guide polynucleotide in said plant cell. The term "corresponding guide DNA" refers to a DNA molecule that is identical to the RNA molecule but has a "T" substituted for each "U" of the RNA molecule.

In some embodiments, the guide polynucleotide is introduced via particle bombardment or Agrobacterium transformation of a recombinant DNA construct comprising the corresponding guide DNA operably linked to a plant U6 polymerase III promoter.

The terms "target site", "target sequence", "target DNA", "target locus", "genomic target site", "genomic target sequence", and "genomic target locus" are used interchangeably herein and refer to a polynucleotide sequence in the genome (including chloroplastic and mitochondrial DNA) of a cell at which a double-strand break is induced in the cell genome by a Cas endonuclease. The target site can be an endogenous site in the genome of an cell or organism, or alternatively, the target site can be heterologous to the cell or organism and thereby not be naturally occurring in the genome, or the target site can be found in a heterologous genomic location compared to where it occurs in nature. As used herein, terms "endogenous target sequence" and "native target sequence" are used interchangeable herein to refer to a target sequence that is endogenous or native to the genome of a cell or organism and is at the endogenous or native position of that target sequence in the genome of a cell or organism. Cells include, but are not limited to animal, bacterial, fungal, insect, yeast, and plant cells as well as plants and seeds produced by the methods described herein.

In one embodiments, the target site can be similar to a DNA recognition site or target site that that is specifically recognized and/or bound by a double-strand break inducing agent such as a LIG3-4 endonuclease (US patent publication 2009-

An "artificial target site" or "artificial target sequence" are used interchangeably herein and refer to a target sequence that has been introduced into the genome of a cell or organism, such as but not limiting to a plant or yeast. Such an artificial target sequence can be identical in sequence to an endogenous or native target sequence in the genome of a cell but be located in a different position (i.e., a non-endogenous or non-native position) in the genome of a cell or organism.

An "altered target site", "altered target sequence", "modified target site", "modified target sequence" are used interchangeably herein and refer to a target sequence as disclosed herein that comprises at least one alteration when compared to non-altered target sequence. Such "alterations" include, for example: (i) replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, or (iv) any combination of (i) - (iii).

Methods for modifying a genomic target site of an organism such as but not limiting to a plant or yeast are disclosed herein. In one embodiment, a method for modifying a target site in the genome of a plant cell comprises introducing a guide polynucleotide into a cell having a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site. This method can further comprise further comprising identifying at least one cell that has a modification at said target, wherein the modification at said target site is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii). This method can also further comprise introducing a donor DNA to said cell, wherein said donor DNA comprises a polynucleotide of interest.

Further provided is a method for modifying a target site in the genome of a cell, the method comprising introducing a guide polynucleotide and a Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas
endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site. This method can further comprise further comprising identifying at least one cell that has a modification at said target, wherein the modification at said target site is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii). This method can also further comprise introducing a donor DNA to said cell, wherein said donor DNA comprises a polynucleotide of interest.

Further provided is a method for modifying a target site in the genome of a cell, the method comprising: a) introducing into a cell a crNucleotide, a first recombinant DNA construct capable of expressing a tracrRNA, and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said crNucleotide is a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said crNucleotide, said tracrRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and, b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

Further provided is a method for modifying a target site in the genome of a cell, the method comprising: a) introducing into a cell a tracrNucleotide, a first recombinant DNA construct capable of expressing a crRNA and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said tracrNucleotide is selected a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said tracrNucleotide, said crRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and, b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).
The length of the target site can vary, and includes, for example, target sites that are at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides in length. It is further possible that the target site can be palindromic, that is, the sequence on one strand reads the same in the opposite direction on the complementary strand. The nick/cleavage site can be within the target sequence or the nick/cleavage site could be outside of the target sequence. In another variation, the cleavage could occur at nucleotide positions immediately opposite each other to produce a blunt end cut or, in other cases, the incisions could be staggered to produce single-stranded overhangs, also called "sticky ends", which can be either 5' overhangs, or 3' overhangs.

Active variants of genomic target sites can also be used. Such active variants can comprise at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the given target site, wherein the active variants retain biological activity and hence are capable of being recognized and cleaved by a Cas endonuclease. Assays to measure the double-strand break of a target site by an endonuclease are known in the art and generally measure the overall activity and specificity of the agent on DNA substrates containing recognition sites.

Various methods and compositions can be employed to obtain a cell or organism having a polynucleotide of interest inserted in a target site for a Cas endonuclease. Such methods can employ homologous recombination to provide integration of the polynucleotide of Interest at the target site. In one method provided, a polynucleotide of interest is provided to the cell in a donor DNA construct. As used herein, "donor DNA" is a DNA construct that comprises a polynucleotide of Interest to be inserted into the target site of a cas endonuclease. Optionally, the donor DNA construct can further comprise a first and a second region of homology that flank the polynucleotide of Interest. The first and second regions of homology of the donor DNA share homology to a first and a second genomic region, respectively, present in or flanking the target site of the plant genome. By "homology" is meant DNA sequences that are similar. For example, a "region of homology to a genomic region" that is found on the donor DNA is a region of DNA that has a similar sequence to a given "genomic region" in the plant
genome. A region of homology can be of any length that is sufficient to promote homologous recombination at the cleaved target site. For example, the region of 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, 5-1200, 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 in length such that the region of homology has sufficient homology to undergo homologous recombination with the corresponding genomic region. "Sufficient homology" indicates that two polynucleotide sequences have sufficient structural similarity to act as substrates for a homologous recombination reaction. The structural similarity includes overall length of each polynucleotide fragment, as well as the sequence similarity of the polynucleotides. Sequence similarity can be described by the percent sequence identity over the whole length of the sequences, and/or by conserved regions comprising localized similarities such as contiguous nucleotides having 100% sequence identity, and percent sequence identity over a portion of the length of the sequences.

The amount of homology or sequence identity shared by a target and a donor polynucleotide can vary and includes total lengths and/or regions having unit integral values in the ranges of about 1-20 bp, 20-50 bp, 50-100 bp, 75-150 bp, 100-250 bp, 150-300 bp, 200-400 bp, 250-500 bp, 300-600 bp, 350-750 bp, 400-800 bp, 450-900 bp, 500-1000 bp, 600-1250 bp, 700-1500 bp, 800-1750 bp, 900-2000 bp, 1-2.5 kb, 1.5-3 kb, 2-4 kb, 2.5-5 kb, 3-6 kb, 3.5-7 kb, 4-8 kb, 5-10 kb, or up to and including the total length of the target site. These ranges include every integer within the range, for example, the range of 1-20 bp includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 bp. The amount of homology can also be described by percent sequence identity over the full aligned length of the two polynucleotides which includes percent sequence identity of about at least 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%. Sufficient homology includes any combination of polynucleotide length, global percent sequence identity, and optionally conserved
regions of contiguous nucleotides or local percent sequence identity, for example sufficient homology can be described as a region of 75-150 bp having at least 80% sequence identity to a region of the target locus. Sufficient homology can also be described by the predicted ability of two polynucleotides to specifically hybridize under high stringency conditions, see, for example, Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, NY); Current Protocols in Molecular Biology, Ausubel et al., Eds (1994) Current Protocols, (Greene Publishing Associates, Inc. and John Wiley & Sons, Inc); and, Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, (Elsevier, New York).

As used herein, a "genomic region" is a segment of a chromosome in the genome of a plant cell that is present on either side of the target site or, alternatively, also comprises a portion of the target site. The genomic region 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'-1100, 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 such that the genomic region has sufficient homology to undergo homologous recombination with the corresponding region of homology.

The region of homology on the donor DNA can have homology to any sequence flanking the target site. While in some embodiments the regions of homology share significant sequence homology to the genomic sequence immediately flanking the target site, it is recognized that the regions of homology can be designed to have sufficient homology to regions that may be further 5' or 3' to the target site. In still other embodiments, the regions of homology can also have homology with a fragment of the target site along with downstream genomic regions. In one embodiment, the first region of homology further comprises a first fragment of the target site and the second region of homology comprises a second fragment of the target site, wherein the first and second fragments are dissimilar.

As used herein, "homologous recombination" refers to the exchange of DNA fragments between two DNA molecules at the sites of homology. The frequency of

Alteration of the genome of a plant cell, for example, through homologous recombination (HR), is a powerful tool for genetic engineering. Despite the low frequency of homologous recombination in higher plants, there are a few examples of successful homologous recombination of plant endogenous genes. The parameters for homologous recombination in plants have primarily been investigated by rescuing introduced truncated selectable marker genes. In these experiments, the homologous DNA fragments were typically between 0.3 kb to 2 kb. Observed frequencies for homologous recombination were on the order of 10^-4 to 10^-5. See, for example, Halfter et al., (1992) Mol Gen Genet 231:186-93; Offringa et al., (1990) EMBO J 9:3077-84; Offringa et al., (1993) Proc. Natl. Acad. Sci. USA 90:7346-50; Paszkowski et al., (1988) EMBO J 7:4021-6; Hourda and Paszkowski, (1994) Mol Gen Genet 243:106-11; and Risseeuw et al., (1995) Plant J 7:109-19.

Homologous recombination has been demonstrated in insects. In Drosophila, Dray and Gloor found that as little as 3 kb of total template:target homology sufficed to copy a large non-homologous segment of DNA into the target with reasonable efficiency (Dray and Gloor, (1997) Genetics 147:689-99). Using FLP-mediated DNA integration at a target FRT in Drosophila, Golic et al., showed integration was approximately 10-fold more efficient when the donor and target shared 4.1 kb of homology as compared to 1.1 kb of homology (Golic et al., (1997)

Homologous recombination has also been accomplished in other organisms. For example, at least 150-200 bp of homology was required for homologous recombination in the parasitic protozoan Leishmania (Papadopoulou and Dumas, (1997) Nucleic Acids Res 25:4278-86). In the filamentous fungus Aspergillus nidulans, gene replacement has been accomplished with as little as 50 bp flanking homology (Chaveroche et al., (2000) Nucleic Acids Res 28:e97). Targeted gene replacement has also been demonstrated in the ciliate Tetrahymena thermophila (Gaertig et al., (1994) Nucleic Acids Res 22:5391-8). In mammals, homologous recombination has been most successful in the mouse using pluripotent embryonic stem cell lines (ES) that can be grown in culture, transformed, selected and introduced into a mouse embryo. Embryos bearing inserted transgenic ES cells develop as genetically offspring. By interbreeding siblings, homozygous mice carrying the selected genes can be obtained. An overview of the process is provided in Watson et al., (1992) Recombinant DNA, 2nd Ed., (Scientific American Books distributed by WH Freeman & Co.); Capecchi, (1989) Trends Genet 5:70-6; and Bronson, (1994) J Biol Chem 269:271 55-8. Homologous recombination in mammals other than mouse has been limited by the lack of stem cells capable of being transplanted to oocytes or developing embryos. However, McCreath et al., Nature 405:1 066-9 (2000) reported successful homologous recombination in sheep by transformation and selection in primary embryo fibroblast cells.

Once a double-strand break is induced in the DNA, the cell's DNA repair mechanism is activated to repair the break. Error-prone DNA repair mechanisms can produce mutations at double-strand break sites. The most common repair mechanism to bring the broken ends together is the nonhomologous end-joining (NHEJ) pathway (Bleuyard et al., (2006) DNA Repair 5:1 -12). The structural integrity of chromosomes is typically preserved by the repair, but deletions, insertions, or other rearrangements are possible (Siebert and Puchta, (2002) Plant
Cell 14:1 121-31; Pacher et al., (2007) Genetics 175:21-9). The two ends of one
double-strand break are the most prevalent substrates of NHEJ (Kirik et al., (2000)
EMBO J 19:5562-6), however if two different double-strand breaks occur, the free
ends from different breaks can be ligated and result in chromosomal deletions
(Siebert and Puchta, (2002) Plant Cell 14:1 121-31), or chromosomal translocations

Episomal DNA molecules can also be ligated into the double-strand break,
for example, integration of T-DNAs into chromosomal double-strand breaks (Chilton
17:6086-95). Once the sequence around the double-strand breaks is altered, for
example, by exonuclease activities involved in the maturation of double-strand
breaks, gene conversion pathways can restore the original structure if a
homologous sequence is available, such as a homologous chromosome in non-
dividing somatic cells, or a sister chromatid after DNA replication (Molinier et al.,
(2004) Plant Cell 16:342-52). Ectopic and/or epigenic DNA sequences may also
serve as a DNA repair template for homologous recombination (Puchta, (1999)
Genetics 152:1 173-81).

Alternatively, the double-strand break can be repaired by homologous
recombination between homologous DNA sequences. Once the sequence around
the double-strand break is altered, for example, by exonuclease activities involved in
the maturation of double-strand breaks, gene conversion pathways can restore the
original structure if a homologous sequence is available, such as a homologous
chromosome in non-dividing somatic cells, or a sister chromatid after DNA
DNA sequences may also serve as a DNA repair template for homologous

DNA double-strand breaks appear to be an effective factor to stimulate
homologous recombination pathways (Puchta et al., (1995) Plant Mol Biol 28:281-
56:1-14). Using DNA-breaking agents, a two- to nine-fold increase of homologous
recombination was observed between artificially constructed homologous DNA

In some embodiments, the methods provided herein comprise contacting a cell with a donor DNA and a Cas endonuclease. Once a double-strand break is introduced in the target site by the Cas endonuclease, the first and second regions of homology of the donor DNA can undergo homologous recombination with their corresponding genomic regions of homology resulting in exchange of DNA between the donor and the genome.

As such, the provided methods result in the integration of the polynucleotide of interest of the donor DNA into the double-strand break in the target site in the genome of a cell or organism, thereby altering the original target site and producing an altered genomic target site.

In one embodiment of the disclosure, the method comprises a method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising: a) introducing a guide polynucleotide, a donor DNA and a Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and, c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target. The guide polynucleotide, Cas endonuclease and donor DNA can be introduced by any means known in the art. These means include, but are not limited to direct delivery of each component via particle bombardment, delivery through one or more recombinant DNA expression cassettes, or any combination thereof.

In some embodiment of the disclosure, the method comprises a method for introducing a polynucleotide of interest into a target site in the genome of a cell, wherein the donor DNA and Cas endonuclease are introduced into said cell using at least one recombinant DNA construct capable of expressing the donor DNA and/or
the Cas endonuclease; and/or, wherein the guide polynucleotide is introduced directly by particle bombardment.

In another embodiment of the disclosure, the method comprises method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising: a) introducing into a cell a first recombinant DNA construct capable of expressing a guide polynucleotide, and a second recombinant DNA construct capable of expressing a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and, c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.

The donor DNA may be introduced by any means known in the art. For example, a cell or organism, such as but not limiting to a plant or yeast having a target site is provided. The donor DNA may be provided by any transformation method known in the art including, for example, Agrobacterium-mediated transformation or biolistic particle bombardment. The donor DNA may be present transiently in the cell or it could be introduced via a viral replicon. In the presence of the Cas endonuclease and the target site, the donor DNA is inserted into the transformed genome.

Another approach uses protein engineering of existing homing endonucleases to alter their target specificities. Homing endonucleases, such as l-Scel or l-Crel, bind to and cleave relatively long DNA recognition sequences (18 bp and 22 bp, respectively). These sequences are predicted to naturally occur infrequently in a genome, typically only 1 or 2 sites/genome. The cleavage specificity of a homing endonuclease can be changed by rational design of amino acid substitutions at the DNA binding domain and/or combinatorial assembly and selection of mutated monomers (see, for example, Arnould et al., (2006) J Mol Biol 355:443-58; Ashworth et al., (2006) Nature 441:656-9; Doyon et al., (2006) J Am Chem Soc 128:2477-84; Rosen et al., (2006) Nucleic Acids Res 34:4791 -800; and Smith et al., (2006) Nucleic Acids Res 34:e149; Lyznik et al., (2009) U.S. Patent
Engineered meganucleases have been demonstrated that can cleave cognate mutant sites without broadening their specificity. An artificial recognition site specific to the wild type yeast l-Scel homing nuclease was introduced in maize genome and mutations of the recognition sequence were detected in 1% of analyzed F1 plants when a transgenic l-Scel was introduced by crossing and activated by gene excision (Yang et al., (2009) Plant Mol Biol 70:669-79). More practically, the maize liguleless locus was targeted using an engineered single-chain endonuclease designed based on the l-Crel meganuclease sequence. Mutations of the selected liguleless locus recognition sequence were detected in 3% of the T0 transgenic plants when the designed homing nuclease was introduced by Agrobacterium-mediated transformation of immature embryos (Gao et al., (2010) Plant J 61:176-87).

Polynucleotides of interest are further described herein and are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genetic engineering will change accordingly.

**Genome editing using the guide polynucleotide/Cas endonuclease system.**

As described herein, the guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide modification template to allow for editing of a genomic nucleotide sequence of interest. While numerous double-strand break-making systems exist, their practical applications for gene editing may be restricted due to the relatively low frequency of induced double-strand breaks (DSBs). To date, many genome modification methods rely on the homologous recombination system. Homologous recombination (HR) can provide molecular means for finding genomic DNA sequences of interest and modifying them according to the experimental specifications. Homologous recombination takes place in plant somatic cells at low frequency. The process can be enhanced to a practical level for genome engineering by introducing double-strand breaks.
(DSBs) at selected endonuclease target sites. The challenge has been to efficiently make DSBs at genomic sites of interest since there is a bias in the directionality of information transfer between two interacting DNA molecules (the broken one acts as an acceptor of genetic information). Described herein is the use of a guide polynucleotide/Cas system which provides flexible genome cleavage specificity and results in a high frequency of double-strand breaks at a DNA target site, thereby enabling efficient gene editing in a nucleotide sequence of interest, wherein the nucleotide sequence of interest to be edited can be located within or outside the target site recognized and cleaved by a Cas endonuclease.

A "modified nucleotide" or "edited nucleotide" refers to a nucleotide sequence of interest that comprises at least one alteration when compared to its non-modified nucleotide sequence. Such "alterations" include, for example: (i) replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, or (iv) any combination of (i) - (iii).

The term "polynucleotide modification template" refers to a polynucleotide that comprises at least one nucleotide modification when compared to the nucleotide sequence to be edited. A nucleotide modification can be at least one nucleotide substitution, addition or deletion. Optionally, the polynucleotide modification template can further comprise homologous nucleotide sequences flanking the at least one nucleotide modification, wherein the flanking homologous nucleotide sequences provide sufficient homology to the desired nucleotide sequence to be edited.

In one embodiment, the disclosure describes a method for editing a nucleotide sequence in the genome of a cell, the method comprising introducing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease introduces a double-strand break at a target site in the genome of said cell, wherein said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence. Cells include, but are not limited to, animal, bacterial, fungal, insect, yeast, and plant cells as well as plants and seeds produced by the methods described herein. The nucleotide to be edited can be located within or outside a
target site recognized and cleaved by a Cas endonuclease. In one embodiment, the
at least one nucleotide modification is not a modification at a target site recognized
and cleaved by a Cas endonuclease. In another embodiment, there are at least 1,
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, 30, 40, 50, 100, 200, 300, 400, 500, 600, 700, 900 or 1000 nucleotides between
the at least one nucleotide to be edited and the genomic target site.

The nucleotide sequence to be edited can be a sequence that is
endogenous, artificial, pre-existing, or transgenic to the cell that is being edited. For
example, the nucleotide sequence in the genome of a cell can be a transgene that is
stably incorporated into the genome of a cell. Editing of such transgene may result
in a further desired phenotype or genotype. The nucleotide sequence in the genome
of a cell can also be a mutated or pre-existing sequence that was either
endogenous or artificial from origin such as an endogenous gene or a mutated gene
of interest.

In one embodiment the nucleotide sequence can be a promoter wherein the
editing of the promoter results in any one of the following or any one combination of
the following: an increased promoter activity, an increased promoter tissue
specificity, a decreased promoter activity, a decreased promoter tissue specificity, a
mutation of DNA binding elements and / or a deletion or addition of DNA binding
elements.

In one embodiment the nucleotide sequence can be a regulatory sequence in
the genome of a cell. A regulatory sequence is a segment of a nucleic acid molecule
which is capable of increasing or decreasing the expression of specific genes within
an organism. Examples of regulatory sequences include, but are not limited to,
transcription activators, transcriptions repressors, and translational repressors,
splicing factors, miRNAs, siRNA, artificial miRNAs, a CAAT box, a CCAAT box, a
Prinbnow box, a TATA box, SECIS elements and polyadenylation signals. In some
embodiments the editing of a regulatory element results in altered protein
translation, RNA cleavage, RNA splicing or transcriptional termination.
Regulatory sequence modifications using the guide polynucleotide/Cas endonuclease system

In one embodiment the nucleotide sequence to be modified can be a regulatory sequence such as a promoter wherein the editing of the promoter comprises replacing the promoter (also referred to as a "promoter swap" or "promoter replacement") or promoter fragment with a different promoter (also referred to as replacement promoter) or promoter fragment (also referred to as replacement promoter fragment), wherein the promoter replacement results in any one of the following or any one combination of the following: an increased promoter activity, an increased promoter tissue specificity, a decreased promoter activity, a decreased promoter tissue specificity, a new promoter activity, an inducible promoter activity, an extended window of gene expression, a modification of the timing or developmental progress of gene expression in the same cell layer or other cell layer (such as but not limiting to extending the timing of gene expression in the tapetum of maize anthers (US 5,837,850 issued November 17, 1998), a mutation of DNA binding elements and/or a deletion or addition of DNA binding elements. The promoter (or promoter fragment) to be modified can be a promoter (or promoter fragment) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited. The replacement promoter (or replacement promoter fragment) can be a promoter (or promoter fragment) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

In one embodiment the nucleotide sequence can be a promoter wherein the editing of the promoter comprises replacing an ARGOS 8 promoter with a Zea mays GOS2 PRO:GOS2-intron promoter.

In one embodiment the nucleotide sequence can be a promoter wherein the editing of the promoter comprises replacing a native EPSPS1 promoter from with a plant ubiquitin promoter.

In one embodiment the nucleotide sequence can be a promoter wherein the editing of the promoter comprises replacing an endogenous maize NPK1 promoter with a stress inducible maize RAB17 promoter.

In one embodiment the nucleotide sequence can be a promoter wherein the promoter to be edited is selected from the group comprising Zea mays-PEPC1

In another embodiment, the guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide modification template or donor DNA sequence to allow for the insertion of a promoter or promoter element into a genomic nucleotide sequence of interest, wherein the promoter insertion (or promoter element insertion) results in any one of the following or any one combination of the following: an increased promoter activity (increased promoter strength), an increased promoter tissue specificity, a decreased promoter activity, a decreased promoter tissue specificity, a new promoter activity, an inducible promoter activity, an extended window of gene expression, a modification of the timing or developmental progress of gene expression a mutation of DNA binding elements and/or an addition of DNA binding elements. Promoter elements to be inserted can be, but are not limited to, promoter core elements (such as, but not limited to, a CAAT box, a CCAAT box, a Pribnow box, a and/or TATA box, translational regulation sequences and/or a repressor system for inducible expression (such as TET operator repressor/operator/inducer elements, or Sulphonylurea (Su) repressor/operator/inducer elements. The dehydration-responsive element (DRE) was first identified as a c/s-acting promoter element in the promoter of the drought-responsive gene rd29A, which contains a 9 bp conserved core sequence, TACCGACAT (YamaguchhShinozaki, K., and Shinozaki, K. (1994) Plant Cell 6, 251-264). Insertion of DRE into an endogenous promoter may confer a drought inducible expression of the downstream gene. Another example is ABA-responsive elements (ABREs) that contain a (C/T)ACGTGTC consensus sequence found to be present in numerous ABA and/or stress-regulated genes (Busk P. K., Pages M.(1 998) Plant Mol. Biol. 37:425-435). Insertion of 35S enhancer or MMV enhancer into an endogenous promoter region will increase gene
expression (US patent 5196525). The promoter (or promoter element) to be inserted can be a promoter (or promoter element) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to insert an enhancer element, such as but not limited to a Cauliflower Mosaic Virus 35S enhancer, in front of an endogenous FMT1 promoter to enhance expression of the FTM1.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to insert a component of the TET operator repressor/operator/inducer system, or a component of the sulphonylurea (Su) repressor/operator/inducer system into plant genomes to generate or control inducible expression systems.

In another embodiment, the guide polynucleotide/Cas endonuclease system can be used to allow for the deletion of a promoter or promoter element, wherein the promoter deletion (or promoter element deletion) results in any one of the following or any one combination of the following: a permanently inactivated gene locus, an increased promoter activity (increased promoter strength), an increased promoter tissue specificity, a decreased promoter activity, a decreased promoter tissue specificity, a new promoter activity, an inducible promoter activity, an extended window of gene expression, a modification of the timing or developmental progress of gene expression, a mutation of DNA binding elements and / or an addition of DNA binding elements. Promoter elements to be deleted can be, but are not limited to, promoter core elements, promoter enhancer elements or 35S enhancer elements (as described in Example 32) The promoter or promoter fragment to be deleted can be endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to delete the ARGOS 8 promoter present in a maize genome as described herein.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to delete a 35S enhancer element present in a plant genome as described herein.
Terminator modifications using the guide polynucleotide/Cas endonuclease system

In one embodiment the nucleotide sequence to be modified can be a terminator wherein the editing of the terminator comprises replacing the terminator (also referred to as a "terminator swap" or "terminator replacement") or terminator fragment with a different terminator (also referred to as replacement terminator) or terminator fragment (also referred to as replacement terminator fragment), wherein the terminator replacement results in any one of the following or any one combination of the following: an increased terminator activity, an increased terminator tissue specificity, a decreased terminator activity, a decreased terminator tissue specificity, a mutation of DNA binding elements and / or a deletion or addition of DNA binding elements."

The terminator (or terminator fragment) to be modified can be a terminator (or terminator fragment) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited. The replacement terminator (or replacement terminator fragment) can be a terminator (or terminator fragment) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

In one embodiment the nucleotide sequence to be modified can be a terminator wherein the terminator to be edited is selected from the group comprising terminators from maize Argos 8 or SRTF18 genes, or other terminators, such as potato Pinll terminator, sorghum actin terminator (SB-ACTIN TERM, WO 2013/184537 A1 published Dec 2013), sorghum SB-GKAF TERM (WO201 301 9461), rice T28 terminator (OS-T28 TERM, WO 2013/012729 A2), AT-T9 TERM (WO 2013/012729 A2) or GZ-W64A TERM (US7053282).

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide modification template or donor DNA sequence to allow for the insertion of a terminator or terminator element into a genomic nucleotide sequence of interest, wherein the terminator insertion (or terminator element insertion) results in any one of the following or any one combination of the following: an increased terminator activity (increased terminator strength), an increased terminator tissue specificity, a decreased terminator activity, a decreased terminator tissue specificity, a mutation of DNA binding elements and / or an addition of DNA binding elements.
The terminator (or terminator element) to be inserted can be a terminator (or terminator element) that is endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

In another embodiment, the guide polynucleotide/Cas endonuclease system can be used to allow for the deletion of a terminator or terminator element, wherein the terminator deletion (or terminator element deletion) results in any one of the following or any one combination of the following: an increased terminator activity (increased terminator strength), an increased terminator tissue specificity, a decreased terminator activity, a decreased terminator tissue specificity, a mutation of DNA binding elements and/or an addition of DNA binding elements. The terminator or terminator fragment to be deleted can be endogenous, artificial, pre-existing, or transgenic to the cell that is being edited.

**Additional regulatory sequence modifications using the guide polynucleotide/Cas endonuclease system**

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to modify or replace a regulatory sequence in the genome of a cell. A regulatory sequence is a segment of a nucleic acid molecule which is capable of increasing or decreasing the expression of specific genes within an organism and/or is capable of altering tissue specific expression of genes within an organism.

Examples of regulatory sequences include, but are not limited to, 3' UTR (untranslated region) region, 5' UTR region, transcription activators, transcriptional enhancers transcriptional repressors, translational repressors, splicing factors, miRNAs, siRNA, artificial miRNAs, promoter elements, CAMV 35 S enhancer, MMV enhancer elements (PCT/US1 4/23451 filed March 11, 2013), SECIS elements, polyadenylation signals, and polyubiquitination sites. In some embodiments the editing (modification) or replacement of a regulatory element results in altered protein translation, RNA cleavage, RNA splicing, transcriptional termination or post translational modification. In one embodiment, regulatory elements can be identified within a promoter and these regulatory elements can be edited or modified do to optimize these regulatory elements for up or down regulation of the promoter.

In one embodiment, the genomic sequence of interest to be modified is a polyubiquitination site, wherein the modification of the polyubiquitination sites results
in a modified rate of protein degradation. The ubiquitin tag condemns proteins to be
degraded by proteasomes or autophagy. Proteasome inhibitors are known to cause
a protein overproduction. Modifications made to a DNA sequence encoding a
protein of interest can result in at least one amino acid modification of the protein of
interest, wherein said modification allows for the polyubiquitination of the protein (a
post translational modification) resulting in a modification of the protein degradation

In one embodiment, the genomic sequence of interest to be modified is a
polyubiquitination site on a maize EPSPS gene, wherein the polyubiquitination site
modified resulting in an increased protein content due to a slower rate of EPSPS
protein degradation.

In one embodiment, the genomic sequence of interest to be modified is a an
intron site, wherein the modification consist of inserting an intron enhancing motif
into the intron which results in modulation of the transcriptional activity of the gene
comprising said intron.

In one embodiment, the genomic sequence of interest to be modified is a an
intron site, wherein the modification consist of replacing a soybean EPSP1 intron
with a soybean ubiquitin intron 1 as described herein (Example 25)

In one embodiment, the genomic sequence of interest to be modified is a an
intron or UTR site, wherein the modification consist of inserting at least one
microRNA into said intron or UTR site, wherein expression of the gene comprising
the intron or UTR site also results in expression of said microRNA, which in turn can
silence any gene targeted by the microRNA without disrupting the gene expression
of the native/transgene comprising said intron.

In one embodiment, the guide polynucleotide/Cas endonuclease system can
be used to allow for the deletion or mutation of a Zinc Finger transcription factor,
wherein the deletion or mutation of the Zinc Finger transcription factor results in or
allows for the creation of a dominant negative Zinc Finger transcription factor mutant
(Li et al 2013 Rice zinc finger protein DST enhances grain production through
controlling Gn1 a/OsCKX2 expression PNAS 110:31 67-31 72). Insertion of a single
base pair downstream zinc finger domain will result in a frame shift and produces a
new protein which still can bind to DNA without transcription activity. The mutant
protein will compete to bind to cytokinin oxidase gene promoters and block the
expression of cytokinin oxidase gene. Reduction of cytokinin oxidase gene expression will increase cytokinin level and promote panicle growth in rice and ear growth in maize, and increase yield under normal and stress conditions.

Modifications of splicing sites and/or introducing alternate splicing sites using the guide polynucleotide/Cas endonuclease system

Protein synthesis utilizes mRNA molecules that emerge from pre-mRNA molecules subjected to the maturation process. The pre-mRNA molecules are capped, spliced and stabilized by addition of polyA tails. Eukaryotic cells developed a complex process of splicing that result in alternative variants of the original pre-mRNA molecules. Some of them may not produce functional templates for protein synthesis. In maize cells, the splicing process is affected by splicing sites at the exon-intron junction sites. An example of a canonical splice site is AGGT. Gene coding sequences can contains a number of alternate splicing sites that may affect the overall efficiency of the pre-mRNA maturation process and as such may limit the protein accumulation in cells. The guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide modification template to edit a gene of interest to introduce a canonical splice site at a described junction or any variant of a splicing site that changes the splicing pattern of pre-mRNA molecules.

In one embodiment, the nucleotide sequence of interest to be modified is a maize EPSPS gene, wherein the modification of the gene consists of modifying alternative splicing sites resulting in enhanced production of the functional gene transcripts and gene products (proteins).

In one embodiment, the nucleotide sequence of interest to be modified is a gene, wherein the modification of the gene consists of editing the intron borders of alternatively spliced genes to alter the accumulation of splice variants.

Modifications of nucleotide sequences encoding a protein of interest using the guide polynucleotide/Cas endonuclease system

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used to modify or replace a coding sequence in the genome of a cell, wherein the modification or replacement results in any one of the following, or any one combination of the following: an increased protein (enzyme) activity, an increased
protein functionality, a decreased protein activity, a decreased protein functionality, a site specific mutation, a protein domain swap, a protein knock-out, a new protein functionality, a modified protein functionality,.

In one embodiment the protein knockout is due to the introduction of a stop codon into the coding sequence of interest.

In one embodiment the protein knockout is due to the deletion of a start codon into the coding sequence of interest.

Amino acid and/or protein fusions using the guide polynucleotide/ Cas endonuclease system

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used with or without a co-delivered polynucleotide sequence to fuse a first coding sequence encoding a first protein to a second coding sequence encoding a second protein in the genome of a cell, wherein the protein fusion results in any one of the following or any one combination of the following: an increased protein (enzyme) activity, an increased protein functionality, a decreased protein activity, a decreased protein functionality, a new protein functionality, a modified protein functionality, a new protein localization, a new timing of protein expression, a modified protein expression pattern, a chimeric protein, or a modified protein with dominant phenotype functionality.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used with or without a co-delivered polynucleotide sequence to fuse a first coding sequence encoding a chloroplast localization signal to a second coding sequence encoding a protein of interest, wherein the protein fusion results in targeting the protein of interest to the chloroplast.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used with or without a co-delivered polynucleotide sequence to fuse a first coding sequence encoding a chloroplast localization signal to a second coding sequence encoding a protein of interest, wherein the protein fusion results in targeting the protein of interest to the chloroplast.

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used with or without a co-delivered polynucleotide sequence to fuse a first coding sequence encoding a chloroplast localization signal (e.g., a chloroplast transit
peptide) to a second coding sequence, wherein the protein fusion results in a modified protein with dominant phenotype functionality.

*Gene silencing by expressing an inverted repeat into a gene of interest using the guide polynucleotide/Cas endonuclease system*

In one embodiment, the guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide sequence to insert an inverted gene fragment into a gene of interest in the genome of an organism, wherein the insertion of the inverted gene fragment can allow for an in-vivo creation of an inverted repeat (hairpin) and results in the silencing of said endogenous gene. In one embodiment the insertion of the inverted gene fragment can result in the formation of an in-vivo created inverted repeat (hairpin) in a native (or modified) promoter of a gene and/or in a native 5' end of the native gene. The inverted gene fragment can further comprise an intron which can result in an enhanced silencing of the targeted gene.

*Genome deletion for Trait Locus Characterization*

Trait mapping in plant breeding often results in the detection of chromosomal regions housing one or more genes controlling expression of a trait of interest. For a qualitative trait, the guide polynucleotide/Cas endonuclease system can be used to eliminate candidate genes in the identified chromosomal regions to determine if deletion of the gene affects expression of the trait. For quantitative traits, expression of a trait of interest is governed by multiple quantitative trait loci (QTL) of varying effect-size, complexity, and statistical significance across one or more chromosomes. In cases of negative effect or deleterious QTL regions affecting a complex trait, the guide polynucleotide/Cas endonuclease system can be used to eliminate whole regions delimited by marker-assisted fine mapping, and to target specific regions for their selective elimination or rearrangement. Similarly, presence/absence variation (PAV) or copy number variation (CNV) can be manipulated with selective genome deletion using the guide polynucleotide/Cas endonuclease system.

In one embodiment, the region of interest can be flanked by two independent guide polynucleotide/CAS endonuclease target sequences. Cutting would be done concurrently. The deletion event would be the repair of the two chromosomal ends...
without the region of interest. Alternative results would include inversions of the region of interest, mutations at the cut sites and duplication of the region of interest.

Methods for identifying at least one plant cell comprising in its genome a polynucleotide of Interest integrated at the target site.

Further provided, are methods for identifying at least one plant cell comprising in its genome a polynucleotide of Interest integrated at the target site. A variety of methods are available for identifying those plant cells with insertion into the genome at or near to the target site without using a screenable marker phenotype. Such methods can be viewed as directly analyzing a target sequence to detect any change in the target sequence, including but not limited to PCR methods, sequencing methods, nuclease digestion, Southern blots, and any combination thereof. See, for example, US Patent Application 12/147,834, herein incorporated to the extent necessary for the methods described herein.

The method also comprises recovering a plant from the plant cell comprising a polynucleotide of Interest integrated into its genome. The plant may be sterile or fertile. It is recognized that any polynucleotide of interest can be provided, integrated into the plant genome at the target site, and expressed in a plant.

Polynucleotides/polypeptides of interest include, but are not limited to, herbicide-tolerance coding sequences, insecticidal coding sequences, nematicidal coding sequences, antimicrobial coding sequences, antifungal coding sequences, antiviral coding sequences, abiotic and biotic stress tolerance coding sequences, or sequences modifying plant traits such as yield, grain quality, nutrient content, starch quality and quantity, nitrogen fixation and/or utilization, fatty acids, and oil content and/or composition. More specific polynucleotides of interest include, but are not limited to, genes that improve crop yield, polypeptides that improve desirability of crops, genes encoding proteins conferring resistance to abiotic stress, such as drought, nitrogen, temperature, salinity, toxic metals or trace elements, or those conferring resistance to toxins such as pesticides and herbicides, or to biotic stress, such as attacks by fungi, viruses, bacteria, insects, and nematodes, and development of diseases associated with these organisms. General categories of
genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, fertility or sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like that can be stacked or used in combination with other traits, such as but not limited to herbicide resistance, described herein.

Agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch. Hordeothionin protein modifications are described in U.S. Patent Nos. 5,703,049, 5,885,801, 5,885,802, and 5,990,389, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Patent No. 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson et al. (1987) Eur. J. Biochem. 165:99-106, the disclosures of which are herein incorporated by reference.

Commercial traits can also be encoded on a polynucleotide of interest that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxybutyrate synthase), and acetoacetyl-CoA reductase (see Schubert et al. (1988) J. Bacteriol. 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs).

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. Application Serial No. 08/740,682, filed November 1, 1996, and WO 98/20133, the disclosures of which
are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley et al. (1989) Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen et al. (1986) J. Biol. Chem. 261:6279; Kirihara et al. (1988) Gene 71:359; both of which are herein incorporated by reference); and rice (Musumura et al. (1989) Plant Mol. Biol. 12:1 23, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

Polynucleotides that improve crop yield include dwarfing genes, such as Rht1 and Rht2 (Peng et al. (1999) Nature 400:256-261), and those that increase plant growth, such as ammonium-inducible glutamate dehydrogenase. Polynucleotides that improve desirability of crops include, for example, those that allow plants to have reduced saturated fat content, those that boost the nutritional value of plants, and those that increase grain protein. Polynucleotides that improve salt tolerance are those that increase or allow plant growth in an environment of higher salinity than the native environment of the plant into which the salt-tolerant gene(s) has been introduced.

Polynucleotides/polypeptides that influence amino acid biosynthesis include, for example, anthranilate synthase (AS; EC 4.1.3.27) which catalyzes the first reaction branching from the aromatic amino acid pathway to the biosynthesis of tryptophan in plants, fungi, and bacteria. In plants, the chemical processes for the biosynthesis of tryptophan are compartmentalized in the chloroplast. See, for example, US Pub. 20080050506, herein incorporated by reference. Additional sequences of interest include Chorismate Pyruvate Lyase (CPL) which refers to a gene encoding an enzyme which catalyzes the conversion of chorismate to pyruvate and pHBA. The most well characterized CPL gene has been isolated from E. coli and bears the GenBank accession number M96268. See, US Patent No. 7,361,811, herein incorporated by reference.

These polynucleotide sequences of interest may encode proteins involved in providing disease or pest resistance. By "disease resistance" or "pest resistance" is intended that the plants avoid the harmful symptoms that are the outcome of the
plant-pathogen interactions. Pest resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Disease resistance and insect resistance genes such as lysozymes or cecropins for antibacterial protection, or proteins such as defensins, glucanases or chitinases for antifungal protection, or Bacillus thuringiensis endotoxins, protease inhibitors, collagenases, lectins, or glycosidases for controlling nematodes or insects are all examples of useful gene products. Genes encoding disease resistance traits include detoxification genes, such as against fumonisin (U.S. Patent No. 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones et al. (1994) Science 266:789; Martin et al. (1993) Science 262:1432; and Mindrinos et al. (1994) Cell 78:1 089); and the like. Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for example, Bacillus thuringiensis toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser et al. (1986) Gene 48:1 09); and the like.

An "herbicide resistance protein" or a protein resulting from expression of an "herbicide resistance-encoding nucleic acid molecule" includes proteins that confer upon a cell the ability to tolerate a higher concentration of an herbicide than cells that do not express the protein, or to tolerate a certain concentration of an herbicide for a longer period of time than cells that do not express the protein. Herbicide resistance traits may be introduced into plants by genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonyl urea-type herbicides, genes coding for resistance to herbicides that act to inhibit the action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), glyphosate (e.g., the EPSP synthase gene and the GAT gene), HPPD inhibitors (e.g., the HPPD gene) or other such genes known in the art. See, for example, US Patent Nos. 7,626,077, 5,310,667, 5,866,775, 6,225,114, 6,248,876, 7,169,970, 6,867,293, and US Provisional Application No. 61/401,456, each of which is herein incorporated by reference. The bar gene encodes resistance to the herbicide basta, the nptll gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.
Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male fertility genes such as MS26 (see for example U.S. Patents 7,098,388, 7,517,975, 7,612,251), MS45 (see for example U.S. Patents 5,478,369, 6,265,640) or MSCA1 (see for example U.S. Patent 7,919,676). Maize plants (Zea mays L.) can be bred by both self-pollination and cross-pollination techniques. Maize has male flowers, located on the tassel, and female flowers, located on the ear, on the same plant. It can self-pollinate ("selfing") or cross pollinate. Natural pollination occurs in maize when wind blows pollen from the tassels to the silks that protrude from the tops of the incipient ears. Pollination may be readily controlled by techniques known to those of skill in the art. The development of maize hybrids requires the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection are two of the breeding methods used to develop inbred lines from populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which new inbred lines are developed by selfing and selection of desired phenotypes. A hybrid maize variety is the cross of two such inbred lines, each of which may have one or more desirable characteristics lacked by the other or which complement the other. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential. The hybrid progeny of the first generation is designated $F_1$. The $F_1$ hybrid is more vigorous than its inbred parents. This hybrid vigor, or heterosis, can be manifested in many ways, including increased vegetative growth and increased yield.

Hybrid maize seed can be produced by a male sterility system incorporating manual detasseling. To produce hybrid seed, the male tassel is removed from the growing female inbred parent, which can be planted in various alternating row patterns with the male inbred parent. Consequently, providing that there is sufficient isolation from sources of foreign maize pollen, the ears of the female inbred will be fertilized only with pollen from the male inbred. The resulting seed is therefore hybrid ($F_1$) and will form hybrid plants.
Field variation impacting plant development can result in plants tasseling after manual detasseling of the female parent is completed. Or, a female inbred plant tassel may not be completely removed during the detasseling process. In any event, the result is that the female plant will successfully shed pollen and some female plants will be self-pollinated. This will result in seed of the female inbred being harvested along with the hybrid seed which is normally produced. Female inbred seed does not exhibit heterosis and therefore is not as productive as F1 seed. In addition, the presence of female inbred seed can represent a germplasm security risk for the company producing the hybrid.

Alternatively, the female inbred can be mechanically detasseled by machine. Mechanical detasseling is approximately as reliable as hand detasseling, but is faster and less costly. However, most detasseling machines produce more damage to the plants than hand detasseling. Thus, no form of detasseling is presently entirely satisfactory, and a need continues to exist for alternatives which further reduce production costs and to eliminate self-pollination of the female parent in the production of hybrid seed.

Mutations that cause male sterility in plants have the potential to be useful in methods for hybrid seed production for crop plants such as maize and can lower production costs by eliminating the need for the labor-intensive removal of male flowers (also known as de-tasseling) from the maternal parent plants used as a hybrid parent. Mutations that cause male sterility in maize have been produced by a variety of methods such as X-rays or UV-irradiations, chemical treatments, or transposable element insertions (ms23, ms25, ms26, ms32) (Chaubal et al. (2000) Am J Bot 87:1 193-1 201). Conditional regulation of fertility genes through fertility/sterility "molecular switches" could enhance the options for designing new male-sterility systems for crop improvement (Unger et al. (2002) Transgenic Res 11:455-465).

Besides identification of novel genes impacting male fertility, there remains a need to provide a reliable system of producing genetic male sterility.

In U.S. Patent No. 5,478,369, a method is described by which the Ms45 male fertility gene was tagged and cloned on maize chromosome 9. Previously, there had been described a male fertility gene on chromosome 9, ms2, which had never


Other known male fertility mutants or genes from Zea mays are listed in U.S. patent 7,919,676 incorporated herein by reference.

Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

Furthermore, it is recognized that the polynucleotide of interest may also comprise antisense sequences complementary to at least a portion of the messenger RNA (mRNA) for a targeted gene sequence of interest. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, 80%, or 85% sequence identity to the corresponding antisense sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene.
Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

In addition, the polynucleotide of interest may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using polynucleotides in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, generally greater than about 65% sequence identity, about 85% sequence identity, or greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The polynucleotide of interest can also be a phenotypic marker. A phenotypic marker is screenable or a selectable marker that includes visual markers and selectable markers whether it is a positive or negative selectable marker. Any phenotypic marker can be used. Specifically, a selectable or screenable marker comprises a DNA segment that allows one to identify, or select for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA, peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds or compositions and the like.

Examples of selectable markers include, but are not limited to, DNA segments that comprise restriction enzyme sites; DNA segments that encode products which provide resistance against otherwise toxic compounds including antibiotics, such as, spectinomycin, ampicillin, kanamycin, tetracycline, Basta, neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT); DNA segments that encode products which are otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); DNA segments that encode products which can be readily identified (e.g., phenotypic markers such as β-galactosidase, GUS; fluorescent proteins such as green fluorescent protein (GFP), cyan (CFP), yellow (YFP), red (RFP), and cell surface proteins); the generation of
new primer sites for PCR (e.g., the juxtaposition of two DNA sequence not previously juxtaposed), the inclusion of DNA sequences not acted upon or acted upon by a restriction endonuclease or other DNA modifying enzyme, chemical, etc.; and, the inclusion of a DNA sequences required for a specific modification (e.g., methylation) that allows its identification.

Exogenous products include plant enzymes and products as well as those from other sources including prokaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

The transgenes, recombinant DNA molecules, DNA sequences of interest, and polynucleotides of interest can be comprise one or more DNA sequences for gene silencing. Methods for gene silencing involving the expression of DNA sequences in plant are known in the art include, but are not limited to, cosuppression, antisense suppression, double-stranded RNA (dsRNA) interference, hairpin RNA (hpRNA) interference, intron-containing hairpin RNA (ihpRNA) interference, transcriptional gene silencing, and micro RNA (miRNA) interference.

As used herein, "nucleic acid" means a polynucleotide and includes a single or a double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence" and "nucleic acid fragment" are used interchangeably to denote a polymer of RNA and/or DNA that is single- or double-stranded, optionally containing synthetic, non-natural, or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenosine or deoxyadenosine (for RNA or DNA, respectively), "C" for cytosine or deoxycytosine, "G" for guanosine or deoxyguanosine, "U" for uridine, "T" for deoxythymidine, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

"Open reading frame" is abbreviated ORF.

The terms "subfragment that is functionally equivalent" and "functionally equivalent subfragment" are used interchangeably herein. These terms refer to a portion or subsequence of an isolated nucleic acid fragment in which the ability to alter gene expression or produce a certain phenotype is retained whether or not the fragment or subfragment encodes an active enzyme. For example, the fragment or subfragment can be used in the design of genes to produce the desired phenotype.
in a transformed plant. Genes can be designed for use in suppression by linking a nucleic acid fragment or subfragment thereof, whether or not it encodes an active enzyme, in the sense or antisense orientation relative to a plant promoter sequence.

The term "conserved domain" or "motif" means a set of amino acids conserved at specific positions along an aligned sequence of evolutionarily related proteins. While amino acids at other positions can vary between homologous proteins, amino acids that are highly conserved at specific positions indicate amino acids that are essential to the structure, the stability, or the activity of a protein. Because they are identified by their high degree of conservation in aligned sequences of a family of protein homologues, they can be used as identifiers, or "signatures", to determine if a protein with a newly determined sequence belongs to a previously identified protein family.

Polynucleotide and polypeptide sequences, variants thereof, and the structural relationships of these sequences can be described by the terms "homology", "homologous", "substantially identical", "substantially similar" and "corresponding substantially" which are used interchangeably herein. These refer to polypeptide or nucleic acid fragments wherein changes in one or more amino acids or nucleotide bases do not affect the function of the molecule, such as the ability to mediate gene expression or to produce a certain phenotype. These terms also refer to modification(s) of nucleic acid fragments that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. These modifications include deletion, substitution, and/or insertion of one or more nucleotides in the nucleic acid fragment.

Substantially similar nucleic acid sequences encompassed may be defined by their ability to hybridize (under moderately stringent conditions, e.g., 0.5X SSC, 0.1 % SDS, 60°C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions.
The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 90% sequence identity, up to and including 100% sequence identity (i.e., fully complementary) with each other.

The term "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will selectively hybridize to its target sequence in an in vitro hybridization assay. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salt(s)) at pH 7.0 to 8.3, and at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences
that are the same when aligned for maximum correspondence over a specified comparison window.

The term "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. Useful examples of percent sequence identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer percentage from 50% to 100%. These identities can be determined using any of the programs described herein.

Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

The "Clustal V method of alignment" corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, (1989) CABIOS 5:1 51-1 53; Higgins et ai, (1992) Comput Appl Biosci 8:189-191) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal
method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

The "Clustal W method of alignment" corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, 1989) CABIOS 5:1 51-153; Higgins et al, (1992) Comput Appl Biosci 8:189-191) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs (%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 (GCG, Accelrys, San Diego, CA) using the following parameters: % identity and % similarity for a nucleotide sequence using a gap creation penalty weight of 50 and a gap length extension penalty weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using a GAP creation penalty weight of 8 and a gap length extension penalty of 2, and the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1989) Proc. Natl. Acad. Sci. USA 89:1 091 5). GAP uses the algorithm of Needleman and Wunsch, (1970) J Mol Biol 48:443-53, to find an alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps, using a gap creation penalty and a gap extension penalty in units of matched bases.

"BLAST" is a searching algorithm provided by the National Center for Biotechnology Information (NCBI) used to find regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches to identify
sequences having sufficient similarity to a query sequence such that the similarity
would not be predicted to have occurred randomly. BLAST reports the identified
sequences and their local alignment to the query sequence.

It is well understood by one skilled in the art that many levels of sequence
identity are useful in identifying polypeptides from other species or modified
naturally or synthetically wherein such polypeptides have the same or similar
function or activity. Useful examples of percent identities include, but are not limited
to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer
percentage from 50% to 100%. Indeed, any integer amino acid identity from 50% to
100% may be useful in describing the present disclosure, such as 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%, 79%, 80%, 81%,
82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,
96%, 97%, 98% or 99%.

"Gene" includes a nucleic acid fragment that expresses a functional molecule
such as, but not limited to, a specific protein, including regulatory sequences
preceding (5' non-coding sequences) and following (3' non-coding sequences) the
coding sequence. "Native gene" refers to a gene as found in nature with its own
regulatory sequences.

A "mutated gene" is a gene that has been altered through human
intervention. Such a "mutated gene" has a sequence that differs from the sequence
of the corresponding non-mutated gene by at least one nucleotide addition, deletion,
or substitution. In certain embodiments of the disclosure, the mutated gene
comprises an alteration that results from a guide polynucleotide/Cas endonuclease
system as disclosed herein. A mutated plant is a plant comprising a mutated gene.

As used herein, a "targeted mutation" is a mutation in a native gene that was
made by altering a target sequence within the native gene using a method involving
a double-strand-break-inducing agent that is capable of inducing a double-strand
break in the DNA of the target sequence as disclosed herein or known in the art.

In one embodiment, the targeted mutation is the result of a guide
polynucleotide /Cas endonuclease induced gene editing as described herein. The
guide polynucleotide/Cas endonuclease induced targeted mutation can occur in a
nucleotide sequence that is located within or outside a genomic target site that is
recognized and cleaved by a Cas endonuclease.

The term "genome" as it applies to a plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondria, or plastid) of the cell.

A "codon-modified gene" or "codon-preferred gene" or "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell.

An "allele" is one of several alternative forms of a gene occupying a given locus on a chromosome. When all the alleles present at a given locus on a chromosome are the same, that plant is homozygous at that locus. If the alleles present at a given locus on a chromosome differ, that plant is heterozygous at that locus.

"Coding sequence" refers to a polynucleotide sequence which codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to: promoters, translation leader sequences, 5' untranslated sequences, 3' untranslated sequences, introns, polyadenylation target sequences, RNA processing sites, effector binding sites, and stem-loop structures.

"A plant-optimized nucleotide sequence" is nucleotide sequence that has been optimized for increased expression in plants, particularly for increased expression in plants or in one or more plants of interest. For example, a plant-optimized nucleotide sequence can be synthesized by modifying a nucleotide sequence encoding a protein such as, for example, double-strand-break-inducing agent (e.g., an endonuclease) as disclosed herein, using one or more plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:111 for a discussion of host-preferred codon usage.

Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray et al. (1989)
Nucleic Acids Res. 17:477-498, herein incorporated by reference. Additional sequence modifications are known to enhance gene expression in a plant host. These include, for example, elimination of: one or more sequences encoding spurious polyadenylation signals, one or more exon-intron splice site signals, one or more transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given plant host, as calculated by reference to known genes expressed in the host plant cell. When possible, the sequence is modified to avoid one or more predicted hairpin secondary mRNA structures. Thus, "a plant-optimized nucleotide sequence" of the present disclosure comprises one or more of such sequence modifications.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. An "enhancer" is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, and/or comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

It has been shown that certain promoters are able to direct RNA synthesis at a higher rate than others. These are called "strong promoters". Certain other promoters have been shown to direct RNA synthesis at higher levels only in particular types of cells or tissues and are often referred to as "tissue specific promoters", or "tissue-preferred promoters" if the promoters direct RNA synthesis preferably in certain tissues but also in other tissues at reduced levels. Since
patterns of expression of a chimeric gene (or genes) introduced into a plant are controlled using promoters, there is an ongoing interest in the isolation of novel promoters which are capable of controlling the expression of a chimeric gene or (genes) at certain levels in specific tissue types or at specific plant developmental stages.

New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) in The Biochemistry of Plants, Vol. 115, Stumpf and Conn, eds (New York, NY: Academic Press), pp. 1-82.

"Translation leader sequence" refers to a polynucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

Examples of translation leader sequences have been described (e.g., Turner and Foster, (1995) Mol Biotechnol 3:225-236).

"3' non-coding sequences", "transcription terminator" or "termination sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) Plant Cell 1:671 - 680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complimentary copy of the DNA sequence, it is referred to as the primary transcript. A RNA transcript is referred to as the mature RNA when it is a RNA sequence derived from post-transcriptional processing of the primary transcript. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to, and synthesized from, a mRNA template using the enzyme reverse transcriptase. The
cDNA can be single-stranded or converted into double-stranded form using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or in vitro. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that blocks the expression of a target gene (see, e.g., U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. In another example, the complementary RNA regions can be operably linked, either directly or indirectly, 5' to the target mRNA, or 3' to the target mRNA, or within the target mRNA, or a first complementary region is 5' and its complement is 3' to the target mRNA.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory*: Cold Spring Harbor, NY (1989). Transformation methods are well known to those skilled in the art and are described *infra*.

"PCR" or "polymerase chain reaction" is a technique for the synthesis of specific DNA segments and consists of a series of repetitive denaturation, annealing, and extension cycles. Typically, a double-stranded DNA is heat denatured, and two primers complementary to the 3' boundaries of the target segment are annealed to the DNA at low temperature, and then extended at an
intermediate temperature. One set of these three consecutive steps is referred to as a "cycle".

The term "recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis, or manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of the cell, and usually in the form of double-stranded DNA. Such elements may be autonomously replicating sequences, genome integrating sequences, phage, or nucleotide sequences, in linear or circular form, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a polynucleotides of interest into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for expression of that gene in a foreign host.

The terms "recombinant DNA molecule", "recombinant construct", "expression construct", "construct", "construct", and "recombinant DNA construct" are used interchangeably herein. A recombinant construct comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not all found together in nature. For example, a construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells. The skilled artisan will also recognize that different independent
transformation events may result in different levels and patterns of expression (Jones et al., 1985 EMBO J 4:241-2418; De Almeida et al., 1989 Mol Gen Genetics 218:78-86), and thus that multiple events are typically screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished standard molecular biological, biochemical, and other assays including Southern analysis of DNA, Northern analysis of mRNA expression, PCR, real time quantitative PCR (qPCR), reverse transcription PCR (RT-PCR), immunoblotting analysis of protein expression, enzyme or activity assays, and/or phenotypic analysis.

The term "expression", as used herein, refers to the production of a functional end-product (e.g., an mRNA, guide polynucleotide, or a protein) in either precursor or mature form.

The term "introduced" means providing a nucleic acid (e.g., expression construct) or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. Introduced includes reference to stable or transient transformation methods, as well as sexually crossing. Thus, "introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct/expression construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

"Mature" protein refers to a post-translationally processed polypeptide (i.e., one from which any pre- or propeptides present in the primary translation product have been removed). "Precursor" protein refers to the primary product of translation of mRNA (i.e., with pre- and propeptides still present). Pre- and propeptides may be but are not limited to intracellular localization signals.

"Stable transformation" refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes,
resulting in genetically stable inheritance. In contrast, "transient transformation" refers to the transfer of a nucleic acid fragment into the nucleus, or other DNA-containing organelle, of a host organism resulting in gene expression without integration or stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms.

The commercial development of genetically improved germplasm has also advanced to the stage of introducing multiple traits into crop plants, often referred to as a gene stacking approach. In this approach, multiple genes conferring different characteristics of interest can be introduced into a plant. Gene stacking can be accomplished by many means including but not limited to co-transformation, retransformation, and crossing lines with different genes of interest.

The term "plant" refers to whole plants, plant organs, plant tissues, seeds, plant cells, seeds and progeny of the same. Plant cells include, without limitation, cells from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores. Plant parts include differentiated and undifferentiated tissues including, but not limited to roots, stems, shoots, leaves, pollens, seeds, tumor tissue and various forms of cells and culture (e.g., single cells, protoplasts, embryos, and callus tissue). The plant tissue may be in plant or in a plant organ, tissue or cell culture. The term "plant organ" refers to plant tissue or a group of tissues that constitute a morphologically and functionally distinct part of a plant. The term "genome" refers to the entire complement of genetic material (genes and non-coding sequences) that is present in each cell of an organism, or virus or organelle; and/or a complete set of chromosomes inherited as a (haploid) unit from one parent. "Progeny" comprises any subsequent generation of a plant.

A transgenic plant includes, for example, a plant which comprises within its genome a heterologous polynucleotide introduced by a transformation step. The heterologous polynucleotide can be stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct. A transgenic plant can also comprise more than one heterologous polynucleotide within its genome. Each heterologous polynucleotide may confer a
different trait to the transgenic plant. A heterologous polynucleotide can include a
sequence that originates from a foreign species, or, if from the same species, can
be substantially modified from its native form. Transgenic can include any cell, cell
line, callus, tissue, plant part or plant, the genotype of which has been altered by the
presence of heterologous nucleic acid including those transgenics initially so altered
as well as those created by sexual crosses or asexual propagation from the initial
transgenic. The alterations of the genome (chromosomal or extra-chromosomal) by
conventional plant breeding methods, by the genome editing procedure described
herein that does not result in an insertion of a foreign polynucleotide, or by naturally
occurring events such as random cross-fertilization, non-recombinant viral infection,
non-recombinant bacterial transformation, non-recombinant transposition, or
spontaneous mutation are not intended to be regarded as transgenic.

In one embodiment of the disclosure, the composition comprises a plant or
seed comprising a recombinant DNA construct and a guide polynucleotide, wherein
said guide polynucleotide does not solely comprise ribonucleic acids, wherein said
recombinant DNA construct comprises a promoter operably linked to a nucleotide
sequence encoding a plant optimized Cas endonuclease, wherein said plant
optimized Cas endonuclease and guide polynucleotide are capable of forming a
complex and creating a double strand break in a genomic target site of said plant.

In another embodiment of the disclosure, the composition further comprising
a polynucleotide of interest integrated into a genomic target site of said plant.

In another embodiment of the disclosure, the composition further comprising
a modification at a genomic target site, wherein the modification is selected from the
group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at
least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any
combination of (i) - (iii).

In another embodiment of the disclosure, the composition comprises a plant
or seed comprising at least one altered target sequence, wherein the at least one
altered target sequence originated from a corresponding target sequence that was
recognized and cleaved by a guide polynucleotide /Cas endonuclease complex,
wherein the Cas endonuclease is capable of introducing a double-strand break at
said target site in the plant genome, wherein said guide polynucleotide does not solely comprise ribonucleic acids.

In another embodiment of the disclosure, the composition comprises a plant or seed comprising a modified nucleotide sequence, wherein the modified nucleotide sequence was produced by providing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease is capable of introducing a double-strand break at a target site in the plant genome, wherein said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence.

In certain embodiments of the disclosure, a fertile plant is a plant that produces viable male and female gametes and is self-fertile. Such a self-fertile plant can produce a progeny plant without the contribution from any other plant of a gamete and the genetic material contained therein. Other embodiments of the disclosure can involve the use of a plant that is not self-fertile because the plant does not produce male gametes, or female gametes, or both, that are viable or otherwise capable of fertilization. As used herein, a "male sterile plant" is a plant that does not produce male gametes that are viable or otherwise capable of fertilization. As used herein, a "female sterile plant" is a plant that does not produce female gametes that are viable or otherwise capable of fertilization. It is recognized that male-sterile and female-sterile plants can be female-fertile and male-fertile, respectively. It is further recognized that a male fertile (but female sterile) plant can produce viable progeny when crossed with a female fertile plant and that a female fertile (but male sterile) plant can produce viable progeny when crossed with a male fertile plant.

A "centimorgan" (cM) or "map unit" is the distance between two linked genes, markers, target sites, loci, or any pair thereof, wherein 1% of the products of meiosis are recombinant. Thus, a centimorgan is equivalent to a distance equal to a 1% average recombination frequency between the two linked genes, markers, target sites, loci, or any pair thereof.
Breeding methods and methods for selecting plants utilizing a two component RNA guide and Cas endonuclease system.

The present disclosure finds use in the breeding of plants comprising one or more transgenic traits. Most commonly, transgenic traits are randomly inserted throughout the plant genome as a consequence of transformation systems based on Agrobacterium, biolistics, or other commonly used procedures. More recently, gene targeting protocols have been developed that enable directed transgene insertion. One important technology, site-specific integration (SSI) enables the targeting of a transgene to the same chromosomal location as a previously inserted transgene.

Custom-designed meganucleases and custom-designed zinc finger meganucleases allow researchers to design nucleases to target specific chromosomal locations, and these reagents allow the targeting of transgenes at the chromosomal site cleaved by these nucleases.

The currently used systems for precision genetic engineering of eukaryotic genomes, e.g. plant genomes, rely upon homing endonucleases, meganucleases, zinc finger nucleases, and transcription activator-like effector nucleases (TALENs), which require de novo protein engineering for every new target locus.

The highly specific, guide polynucleotide/ Cas9 endonuclease system described herein, is more easily customizable and therefore more useful when modification of many different target sequences is the goal. In one embodiment, the disclosure takes further advantage of the multiple component nature of the guide polynucleotide/ Cas system, with its constant protein component, the Cas endonuclease, and its variable and easily reprogrammable targeting component, the guide polynucleotide. As described herein, the guide polynucleotide can comprise a DNA, RNA or DNA-RNA combination sequence making it very customizable and therefore more useful for when modification of one or many different target sequences is the goal.

The guide polynucleotide/Cas system described herein is especially useful for genome engineering, especially plant genome engineering, in circumstances where nuclease off-target cutting can be toxic to the targeted cells. In one embodiment of the guide polynucleotide/Cas system described herein, the constant component, in the form of an expression-optimized Cas9 gene, is stably integrated into the target
genome, e.g. plant genome. Expression of the Cas9 gene is under control of a promoter, e.g. plant promoter, which can be a constitutive promoter, tissue-specific promoter or inducible promoter, e.g. temperature-inducible, stress-inducible, developmental stage inducible, or chemically inducible promoter. In the absence of the variable targeting domain, of the guide polynucleotide, the Cas protein is not able to recognize and cut DNA and therefore its presence in the plant cell should have little or no consequence. Hence a key advantage of the guide polynucleotide/Cas system described herein is the ability to create and maintain a cell line or transgenic organism capable of efficient expression of the Cas protein with little or no consequence to cell viability. In order to induce cutting at desired genomic sites to achieve targeted genetic modifications, guide polynucleotides can be introduced by a variety of methods into cells containing the stably-integrated and expressed Cas gene. For example, guide polynucleotides can be chemically or enzymatically synthesized, and introduced into the Cas expressing cells via direct delivery methods such a particle bombardment or electroporation.

Alternatively, genes capable of efficiently expressing guide polynucleotides in the target cells can be synthesized chemically, enzymatically or in a biological system, and these genes can be introduced into the Cas expressing cells via direct delivery methods such a particle bombardment, electroporation or biological delivery methods such as Agrobacterium mediated DNA delivery.

One embodiment of the disclosure is a method for selecting a plant comprising an altered target site in its plant genome, the method comprising: a) obtaining a first plant comprising at least one Cas endonuclease capable of introducing a double strand break at a target site in the plant genome; b) obtaining a second plant comprising a guide polynucleotide that is capable of forming a complex with the Cas endonuclease of (a), wherein the guide polynucleotide does not solely comprise ribonucleic acids, c) crossing the first plant of (a) with the second plant of (b); d) evaluating the progeny of (c) for an alteration in the target site and e) selecting a progeny plant that possesses the desired alteration of said target site.

Another embodiment of the disclosure is a method for selecting a plant comprising an altered target site in its plant genome, the method comprising: a) obtaining a first plant comprising at least one Cas endonuclease capable of
introducing a double strand break at a target site in the plant genome; b) obtaining a second plant comprising a guide polynucleotide and a donor DNA, wherein the guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide is capable of forming a complex with the Cas endonuclease of (a), wherein said donor DNA comprises a polynucleotide of interest; c) crossing the first plant of (a) with the second plant of (b); d) evaluating the progeny of (c) for an alteration in the target site and e) selecting a progeny plant that comprises the polynucleotide of interest inserted at said target site.

Another embodiment of the disclosure is a method for selecting a plant comprising an altered target site in its plant genome, the method comprising selecting at least one progeny plant that comprises an alteration at a target site in its plant genome, wherein said progeny plant was obtained by crossing a first plant expressing at least one Cas endonuclease to a second plant comprising a guide polynucleotide and a donor DNA, wherein the guide polynucleotide does not solely comprise ribonucleic acids,, wherein said Cas endonuclease is capable of introducing a double strand break at said target site, wherein said donor DNA comprises a polynucleotide of interest.

As disclosed herein, a guide polynucleotide/Cas system mediating gene targeting can be used in methods for directing transgene insertion and/or for producing complex transgenic trait loci comprising multiple transgenes in a fashion similar as disclosed in WO2013/0198888 (published August 1, 2013) where instead of using a double strand break inducing agent to introduce a gene of interest, a guide polynucleotide/Cas system as disclosed herein is used. In one embodiment, a complex transgenic trait locus is a genomic locus that has multiple transgenes genetically linked to each other. By inserting independent transgenes within 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, or even 5 centimorgans (cM) from each other, the transgenes can be bred as a single genetic locus (see, for example, U.S. patent application 13/427,138) or PCT application PCT/US2014/030061. After selecting a plant comprising a transgene, plants containing (at least) one transgene can be crossed to form an F1 that contains both transgenes. In progeny from these F1 (F2 or BC1) 1/500 progeny would have the two different transgenes recombined onto the same chromosome. The complex locus can then be bred as single genetic locus with
both transgene traits. This process can be repeated to stack as many traits as desired.

Proteins may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known. For example, amino acid sequence variants of the protein(s) can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations include, for example, Kunkel, (1985) Proc. Natl. Acad. Sci. USA 82:488-92; Kunkel et al., (1987) Meth EnzymoM 54:367-82; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) Techniques in Molecular Biology (MacMillan Publishing Company, New York) and the references cited therein. Guidance regarding amino acid substitutions not likely to affect biological activity of the protein is found, for example, in the model of Dayhoff et ai, (1978) Atlas of Protein Sequence and Structure (Natl Biomed Res Found, Washington, D.C.). Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferable. Conservative deletions, insertions, and amino acid substitutions are not expected to produce radical changes in the characteristics of the protein, and the effect of any substitution, deletion, insertion, or combination thereof can be evaluated by routine screening assays. Assays for double-strand-break-inducing activity are known and generally measure the overall activity and specificity of the agent on DNA substrates containing target sites.

A variety of methods are known for the introduction of nucleotide sequences and polypeptides into an organism, including, for example, transformation, sexual crossing, and the introduction of the polypeptide, DNA, or mRNA into the cell.

Methods for contacting, providing, and/or introducing a composition into various organisms are known and include but are not limited to, stable transformation methods, transient transformation methods, virus-mediated methods, and sexual breeding. Stable transformation indicates that the introduced polynucleotide integrates into the genome of the organism and is capable of being inherited by progeny thereof. Transient transformation indicates that the introduced composition is only temporarily expressed or present in the organism.

Protocols for introducing polynucleotides and polypeptides into plants may vary depending on the type of plant or plant cell targeted for transformation, such as

Alternatively, polynucleotides may be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a polynucleotide within a viral DNA or RNA molecule. In some examples a polypeptide of interest may be initially synthesized as part of a viral
polyprotein, which is later processed by proteolysis \textit{in vivo} or \textit{in vitro} to produce the desired recombinant protein. Methods for introducing polynucleotides into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known, see, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931. Transient transformation methods include, but are not limited to, the introduction of polypeptides, such as a double-strand break inducing agent, directly into the organism, the introduction of polynucleotides such as DNA and/or RNA polynucleotides, and the introduction of the RNA transcript, such as an mRNA encoding a double-strand break inducing agent, into the organism. Such methods include, for example, microinjection or particle bombardment. See, for example Crossway et al., (1986) Mol Gen Genet 202:79-85; Nomura et al., (1986) Plant Sci 44:53-8; Hepler et al., (1994) Proc. Natl. Acad. Sci. USA 91:2176-80; and, Hush et al., (1994) J Cell Sci 107:775-84.

The term "dicot" refers to the subclass of angiosperm plants also knows as "dicotyledoneae" and includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of the same. Plant cell, as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

The term "crossed" or "cross" or "crossing" in the context of this disclosure means the fusion of gametes via pollination to produce progeny (i.e., cells, seeds, or plants). The term encompasses both sexual crosses (the pollination of one plant by another) and selfing (self-pollination, i.e., when the pollen and ovule are from the same plant or genetically identical plants).

The term "introgression" refers to the transmission of a desired allele of a genetic locus from one genetic background to another. For example, introgression of a desired allele at a specified locus can be transmitted to at least one progeny plant via a sexual cross between two parent plants, where at least one of the parent plants has the desired allele within its genome. Alternatively, for example, transmission of an allele can occur by recombination between two donor genomes, e.g., in a fused protoplast, where at least one of the donor protoplasts has the
desired allele in its genome. The desired allele can be, e.g., a transgene or a selected allele of a marker or QTL.

Standard DNA isolation, purification, molecular cloning, vector construction, and verification/characterization methods are well established, see, for example Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, NY). Vectors and constructs include circular plasmids, and linear polynucleotides, comprising a polynucleotide of interest and optionally other components including linkers, adapters, regulatory regions, introns, restriction sites, enhancers, insulators, selectable markers, nucleotide sequences of interest, promoters, and/or other sites that aid in vector construction or analysis. In some examples a recognition site and/or target site can be contained within an intron, coding sequence, 5' UTRs, 3' UTRs, and/or regulatory regions.

The present disclosure further provides expression constructs for expressing in a yeast or plant, plant cell, or plant part a guide polynucleotide/Cas system that is capable of binding to and creating a double strand break in a target site. In one embodiment, the expression constructs of the disclosure comprise a promoter operably linked to a nucleotide sequence encoding a Cas gene and a promoter operably linked to a guide polynucleotide of the present disclosure. The promoter is capable of driving expression of an operably linked nucleotide sequence in a plant cell.

5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142 and 6,177,611. In some examples an inducible promoter may be used. Pathogen-inducible promoters induced following infection by a pathogen include, but are not limited to those regulating expression of PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. The promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters include, but are not limited to, the maize ln2-2 promoter, activated by benzene sulfonamide herbicide safeners (De Veylder et al., (1997) Plant Cell Physiol 38:568-77), the maize GST promoter (GST-II-27, WO93/01 294), activated by hydrophobic electrophilic compounds used as pre-emergent herbicides, and the tobacco PR-1 a promoter (Ono et al., (2004) Biosci Biotechnol Biochem 68:803-7) activated by salicylic acid. Other chemical-regulated promoters include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter (Schena et al., (1991) Proc. Natl. Acad. Sci. USA 88:1 0421 -5; McNellis et al., (1998) Plant J 14:247-257); tetracycline-inducible and tetracycline-repressible promoters (Gatz et al., (1991) Mol Gen Genet 227:229-37; U.S. Patent Nos. 5,814,618 and 5,789,156).

5 5,459,252; 5,401,836; 5,110,732 and 5,023,179.

Seed-preferred promoters include both seed-specific promoters active during seed development, as well as seed-germinating promoters active during seed germination. See, Thompson et al., (1989) BioEssays 10:1 08. Seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ1 9B1 (maize 19 kDa zein); and milps (myo-inositol-1 phosphate synthase); (WO00/1 1177; and U.S. Patent 6,225,529). For dicots, seed-preferred promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-preferred promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa gamma zein, waxy, shrunken 1, shrunken 2, globulin 1, oleosin, and nuc1. See also, WO00/1 2733, where seed-preferred promoters from END1 and END2 genes are disclosed.

A phenotypic marker is a screenable or selectable marker that includes visual markers and selectable markers whether it is a positive or negative selectable marker. Any phenotypic marker can be used. Specifically, a selectable or screenable marker comprises a DNA segment that allows one to identify, or select for or against a molecule or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of
RNA, peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds or compositions and the like.

Examples of selectable markers include, but are not limited to, DNA segments that comprise restriction enzyme sites; DNA segments that encode products which provide resistance against otherwise toxic compounds including antibiotics, such as, spectinomycin, ampicillin, kanamycin, tetracycline, Basta, neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT)); DNA segments that encode products which are otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); DNA segments that encode products which can be readily identified (e.g., phenotypic markers such as β-galactosidase, GUS; fluorescent proteins such as green fluorescent protein (GFP), cyan (CFP), yellow (YFP), red (RFP), and cell surface proteins); the generation of new primer sites for PCR (e.g., the juxtaposition of two DNA sequence not previously juxtaposed), the inclusion of DNA sequences not acted upon or acted upon by a restriction endonuclease or other DNA modifying enzyme, chemical, etc.; and, the inclusion of a DNA sequences required for a specific modification (e.g., methylation) that allows its identification.


The cells having the introduced sequence may be grown or regenerated into plants using conventional conditions, see for example, McCormick et al., (1986) Plant Cell Rep 5:81 -4. These plants may then be grown, and either pollinated with the same transformed strain or with a different transformed or untransformed strain, and the resulting progeny having the desired characteristic and/or comprising the introduced polynucleotide or polypeptide identified. Two or more generations may be grown to ensure that the polynucleotide is stably maintained and inherited, and seeds harvested.

Any plant can be used, including monocot and dicot plants. Examples of monocot plants that can be used include, but are not limited to, corn (Zea mays), rice (Oryza sativa), rye (Secale cereale), sorghum (Sorghum bicolor, Sorghum vulgare), millet (e.g., pearl millet (Pennisetum glaucum), proso millet (Panicum miliaceum), foxtail millet (Setaria italica), finger millet (Eleusine coracana)), wheat (Triticum aestivum), sugarcane (Saccharum spp.), oats (Avena), barley (Hordeum), switchgrass (Panicum virgatum), pineapple (Ananas comosus), banana (Musa spp.), palm, ornamentals, turfgrasses, and other grasses. Examples of dicot plants that can be used include, but are not limited to, soybean (Glycine max), canola (Brassica napus and B. campestris), alfalfa (Medicago sativa), tobacco (Nicotiana tabacum), Arabidopsis (Arabidopsis thaliana), sunflower (Helianthus annuus), cotton (Gossypium arboreum), and peanut (Arachis hypogaea), tomato (Solanum lycopersicum), potato (Solanum tuberosum) etc.

The transgenes, recombinant DNA molecules, DNA sequences of interest, and polynucleotides of interest can comprise one or more genes of interest. Such genes of interest can encode, for example, a protein that provides agronomic advantage to the plant.

**Marker Assisted Selection and Breeding of Plants**

A primary motivation for development of molecular markers in crop species is the potential for increased efficiency in plant breeding through marker assisted
selection (MAS). Genetic marker alleles, or alternatively, quantitative trait loci (QTL alleles, are used to identify plants that contain a desired genotype at one or more loci, and that are expected to transfer the desired genotype, along with a desired phenotype to their progeny. Genetic marker alleles (or QTL alleles) can be used to identify plants that contain a desired genotype at one locus, or at several unlinked or linked loci (e.g., a haplotype), and that would be expected to transfer the desired genotype, along with a desired phenotype to their progeny. It will be appreciated that for the purposes of MAS, the term marker can encompass both marker and QTL loci.

After a desired phenotype and a polymorphic chromosomal locus, e.g., a marker locus or QTL, are determined to segregate together, it is possible to use those polymorphic loci to select for alleles corresponding to the desired phenotype — a process called marker-assisted selection (MAS). In brief, a nucleic acid corresponding to the marker nucleic acid is detected in a biological sample from a plant to be selected. This detection can take the form of hybridization of a probe nucleic acid to 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. A variety of procedures for detecting markers are well known in the art. After the presence (or absence) of a particular marker in the biological sample is verified, the plant is selected, i.e., used to make progeny plants by selective breeding.

Plant breeders need to combine traits of interest with genes for high yield and other desirable traits to develop improved plant varieties. Screening for large numbers of samples can be expensive, time consuming, and unreliable. Use of markers, and/or genetically-linked nucleic acids is an effective method for selecting plant having the desired traits in breeding programs. For example, one advantage of marker-assisted selection over field evaluations is that MAS can be done at any time of year regardless of the growing season. Moreover, environmental effects are irrelevant to marker-assisted selection.

When a population is segregating for multiple loci affecting one or multiple traits, the efficiency of MAS compared to phenotypic screening becomes even
greater because all the loci can be processed in the lab together from a single sample of DNA.

The DNA repair mechanisms of cells are the basis to introduce extraneous DNA or induce mutations on endogenous genes. DNA homologous recombination is a specialized way of DNA repair that the cells repair DNA damages using a homologous sequence. In plants, DNA homologous recombination happens at frequencies too low to be routinely used in gene targeting or gene editing until it has been found that the process can be stimulated by DNA double-strand breaks (Bibikova et al., (2001) Mol. Cell Biol. 21:289-297; Puchta and Baltimore, (2003) Science 300:763; Wright et al., (2005) Plant J. 44:693-705).

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmol" means micromole(s), "g" means gram(s), "µg" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s) and "kb" means kilobase(s).

Non-limiting examples of compositions and methods disclosed herein are as follows:

1. A guide polynucleotide comprising:
   (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and,
   (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein the first nucleotide sequence domain and the second nucleotide sequence domain are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids.

2. The guide polynucleotide of embodiment 1 wherein the first nucleotide sequence domain and the second nucleotide sequence domain are located on a single molecule.
3. The guide polynucleotide of embodiment 1 wherein the second nucleotide sequence domain comprises two separate molecules that are capable of hybridizing along a region of complementarity.

4. The guide polynucleotide of any one of embodiments 1-3, wherein the first nucleotide sequence domain is a DNA sequence and the second nucleotide sequence domain is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

5. The guide polynucleotide of embodiment 1 wherein the first nucleotide sequence domain and the second nucleotide sequence domain are DNA sequences.

6. The guide polynucleotide of embodiment 1, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain comprises at least one modification, wherein said at least one modification is selected from the group consisting of a 5′ cap, a 3′ polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2′-Fluoro A nucleotide, a 2′-Fluoro U nucleotide; a 2′-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5′ to 3′ covalent linkage, or any combination thereof.

7. The guide polynucleotide of embodiment 1, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain comprises at least one modification that provides for an additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5′ cap, a 3′ polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-
Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5' to 3' covalent linkage, or any combination thereof.

8. The guide polynucleotide of embodiment 7, wherein the additional beneficial feature is selected from the group of a modified or regulated stability, a subcellular targeting, tracking, a fluorescent label, a binding site for a protein or protein complex, modified binding affinity to complementary target sequence, modified resistance to cellular degradation, and increased cellular permeability.

9. A plant or seed comprising the guide polynucleotide of any one of embodiments 1-8.

10. A guide polynucleotide/Cas endonuclease complex wherein the guide polynucleotide comprises (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

11. The guide polynucleotide/Cas endonuclease complex of embodiment 10, wherein the first nucleotide sequence domain and the second nucleotide sequence domain of the guide polynucleotide are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids.

12. The guide polynucleotide/Cas endonuclease complex of embodiment 10, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain of said guide polynucleotide comprises at least one modification that provides for an additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence
that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5' to 3' covalent linkage, or any combination thereof.

13. The guide polynucleotide/Cas endonuclease complex of any one of embodiments 10-12, wherein the Cas endonuclease is a Cas9 endonuclease.

14. A plant or seed comprising the guide polynucleotide/Cas endonuclease complex of any one of embodiments 10-13.

15. A method for modifying a target site in the genome of a cell, the method comprising introducing a guide polynucleotide into a cell having a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

16. A method for modifying a target site in the genome of a cell, the method comprising introducing a guide polynucleotide and a Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

17. The method of any one of embodiments 15-16, further comprising introducing a donor DNA to said cell, wherein said donor DNA comprises a polynucleotide of interest.

18. The method of any one of embodiments 15-17, further comprising identifying at least one cell that has a modification at said target, wherein the modification at said target site is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

19. A method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising:
a) introducing a guide polynucleotide, a donor DNA and a Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site;
b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and,
c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.

20. The method of embodiment 19, wherein the donor DNA and Cas endonuclease are introduced into said cell using at least one recombinant DNA construct capable of expressing the donor DNA and/or the Cas endonuclease.

21. The method of any one of embodiments 15-20, wherein the guide polynucleotide is introduced directly by particle bombardment.

22. The method of any one of embodiments 15-20, wherein the guide polynucleotide is introduced via particle bombardment or Agrobacterium transformation of a recombinant DNA construct comprising a U6 polymerase III

23. The method of any one of embodiments 15-20, wherein the guide polynucleotide is a single guide polynucleotide comprising a variable targeting domain and a cas endonuclease recognition domain.

24. The method of any one of embodiments 15-20, wherein the guide polynucleotide is a duplex guide polynucleotide comprising a crNucleotide molecule and a tracrNucleotide molecule.

25. A method for modifying a target site in the genome of a cell, the method comprising:
   a) introducing into a cell a crNucleotide, a first recombinant DNA construct capable of expressing a tracrRNA, and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said crNucleotide is a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said crNucleotide, said tracrRNA and said Cas endonuclease are capable of forming a complex that enables
the Cas endonuclease to introduce a double strand break at said target site; and,

b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

26. A method for modifying a target site in the genome of a cell, the method comprising:

10 a) introducing into a cell a tracrNucleotide, a first recombinant DNA construct capable of expressing a crRNA and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said tracrNucleotide is selected a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said tracrNucleotide, said crRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and,

b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

27. A method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising:

25 a) introducing into a cell a first recombinant DNA construct capable of expressing a guide polynucleotide, and a second recombinant DNA construct capable of expressing a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site;
b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and,
c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.

28. A method for editing a nucleotide sequence in the genome of a cell, the method comprising introducing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease introduces a double-strand break at a target site in the genome of said cell, wherein said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence.

29. The method of any one of embodiments 15-28, wherein the cell is selected from the group consisting of a non-human animal, bacterial, fungal, insect, yeast, and a plant cell.

30. The method of embodiment 29, wherein the plant cell is selected from the group consisting of a monocot and dicot cell.

31. The method of embodiment 29, wherein the plant cell is selected from the group consisting of maize, rice, sorghum, rye, barley, wheat, millet, oats, sugarcane, turfgrass, or switchgrass, soybean, canola, alfalfa, sunflower, cotton, tobacco, peanut, potato, tobacco, Arabidopsis, and safflower cell.

32. A plant or seed comprising a guide polynucleotide and a Cas9 endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said Cas9 endonuclease and guide polynucleotide are capable of forming a complex and creating a double strand break in a genomic target site of said plant.

33. A plant or seed comprising a recombinant DNA construct and a guide polynucleotide, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said recombinant DNA construct comprises a promoter operably linked to a nucleotide sequence encoding a plant optimized Cas endonuclease, wherein said plant optimized Cas endonuclease and guide polynucleotide are capable of forming a complex and creating a double strand break in a genomic target site of said plant.
34. The plant of any one of embodiments 32-33, further comprising a polynucleotide of interest integrated into said genomic target site of said plant.

35. The plant or seed of any one of embodiments 32-33 further comprising a modification at said genomic target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

36. A plant or seed comprising at least one altered target sequence, wherein the at least one altered target sequence originated from a corresponding target sequence that was recognized and cleaved by a guide polynucleotide /Cas endonuclease complex, wherein the Cas endonuclease is capable of introducing a double-strand break at said target site in the plant genome, wherein said guide polynucleotide does not solely comprise ribonucleic acids.

37. A plant or seed comprising a modified nucleotide sequence, wherein the modified nucleotide sequence was produced by providing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease is capable of introducing a double-strand break at a target site in the plant genome, wherein said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence.

38. The plant or plant cell of embodiment 29 wherein the at least one nucleotide modification is not a modification at said target site.

39. The plant of any one of embodiments 32-38, wherein the plant is a monocot or a dicot.

40. The plant of embodiment 39, wherein the monocot is selected from the group consisting of maize, rice, sorghum, rye, barley, wheat, millet, oats, sugarcane, turfgrass, or switchgrass.

41. The plant of embodiment 39, wherein the dicot is selected from the group consisting of soybean, canola, alfalfa, sunflower, cotton, tobacco, peanut, potato, tobacco, Arabidopsis, or safflower.
42. A method for selecting a plant comprising an altered target site in its plant genome, the method comprising: a) obtaining a first plant comprising at least one Cas endonuclease capable of introducing a double strand break at a target site in the plant genome; b) obtaining a second plant comprising a guide polynucleotide that is capable of forming a complex with the Cas endonuclease of (a), wherein the guide polynucleotide does not solely comprise ribonucleic acids, c) crossing the first plant of (a) with the second plant of (b); d) evaluating the progeny of (c) for an alteration in the target site and e) selecting a progeny plant that possesses the desired alteration of said target site.

EXAMPLES

The present disclosure is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating embodiments of the disclosure, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications of the disclosure to adapt it to various usages and conditions. Such modifications are also intended to fall within the scope of the appended embodiments.
EXAMPLE 1

Maize optimized expression cassettes for a duplex guide polynucleotide/Cas endonuclease system for genome modification in maize plants

In this example, expression cassettes for the Cas9 endonuclease and the mature fully processed naturally occurring CRISPR-RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) belonging to the type II adaptive viral immune system from *S. pyogenes* as described in Deltcheva *et al.* (2011) *Nature* 471:602-7 and Jinek *et al.* (2012) *Science* 337:816-21 are maize optimized to examine their use as genome engineering tools in maize.

As shown in Figure 1A, the crNucleotide and tracrNucleotide molecules composed entirely of RNA nucleotides in this example, form a duplex comprised of a first nucleotide sequence domain referred to as the "variable targeting" (VT) domain, and a second nucleotide sequence domain, referred to as the "Cas endonuclease recognition" (CER) domain. The CER domain of a crRNA and tracrRNA polynucleotide duplex comprises two separate molecules (a nucleotide sequence 3’ of the VT domain located on the crNucleotide and a tracrNucleotide) that are hybridized along a region of complementarity (Figure 1A). The VT domain helps facilitate DNA target site recognition while the CER domain promotes recognition by the Cas9 protein. Along with the required protospacer adjacent motif (PAM) sequence, both domains of the crRNA/tracrRNA polynucleotide duplex function to guide Cas endonuclease DNA target site cleavage and will herein be referred to as a "duplex guide polynucleotide", "duplex guide RNA" or "crRNA/tracrRNA duplex" as described in Example 1 of US provisional application 61/868706, filed August 22, 2013.

To test a duplex guide polynucleotide/Cas endonuclease system in maize, the Cas9 gene from *Streptococcus pyogenes* M1 GAS (SF370) (SEQ ID NO: 1) was maize codon optimized per standard techniques known in the art and the potato ST-LS1 intron (SEQ ID NO: 2) was introduced in order to eliminate its expression in *E.coli* and *Agrobacterium* (Figure 2A). To facilitate nuclear localization of the Cas9 protein in maize cells, *Simian virus 40* (SV40) monopartite (MAPKKKRKV, SEQ ID NO: 3) and *Agrobacterium tumefaciens* bipartite VirD2 T-DNA border endonuclease (KRPRDRHDGELGGRKRAR, SEQ ID NO: 4) nuclear localization signals were
incorporated at the amino and carboxyl-termini of the Cas9 open reading frame, respectively (Figure 2A). The maize optimized Cas9 gene was operably linked to a maize constitutive or regulated promoter by standard molecular biological techniques. An example of the maize optimized Cas9 expression cassette (SEQ ID NO: 5) is illustrated in Figure 2A containing a maize optimized Cas9 gene with a ST-LS1 intron, SV40 amino terminal nuclear localization signal (NLS) and VirD2 carboxyl terminal NLS driven by a plant Ubiquitin promoter.

To confer efficient crRNA and tracrRNA expression in maize cells so that crRNA/tracrRNA polynucleotide duplexes may guide the Cas9 protein to cleave DNA target sites in vivo, the maize U6 polymerase III promoter (SEQ ID NO: 6) and maize U6 polymerase III terminator (TTTTTTTT) residing on chromosome 8 were isolated and operably fused as 5' and 3' terminal fusions, respectively, to both the crRNA and tracrRNA DNA coding sequences using standard molecular biology techniques generating expression cassettes as illustrated in Figure 2B and Figure 2C. Sequences of the resulting maize optimized crRNA and tracrRNA expression cassettes may be found in SEQ ID NO: 8 (crRNA expression cassette with a VT domain targeting the LIGCas-3 target site (Table 1) and SEQ ID NO: 9 (tracrRNA expression cassette).

As shown in Figure 3A, the crRNA molecule requires a region of complementarity to the DNA target (VT domain) that is approximately 12-30 nucleotides in length and upstream of a PAM sequence for target site recognition and cleavage (Gasiunas et al. (2012) Proc. Natl. Acad. Sci. USA 109:E2579-86, Jinek et al. (2012) Science 337:81 6-21 , Mali et al. (2013) Science 339:823-26, and Cong et al. (2013) Science 339:81 9-23). To facilitate the rapid introduction of maize genomic DNA target sequences into the crRNA expression construct, two Type IIS BbsI restriction endonuclease target sites were introduced in an inverted tandem orientation with cleavage orientated in an outward direction as described in Cong et al. (2013) Science 339:81 9-23. Upon cleavage, the Type IIS restriction endonuclease excises its target sites from the crRNA expression plasmid, generating overhangs allowing for the in-frame directional cloning of duplexed oligos containing the desired maize genomic DNA target site into the VT domain. In the
example shown, only target sequences starting with a G nucleotide were used to promote favorable polymerase III expression of the crRNA.

Expression of both the Cas endonuclease gene and the crRNA and tracrRNA molecules then allows for the formation of the duplex guide RNA/Cas endonuclease system (also referred to as crRNA/tracrRNA/Cas endonuclease complex) depicted in Figure 3A (SEQ ID NOs: 10-11).

EXAMPLE 2

The duplex guide RNA/Cas endonuclease system cleaves chromosomal DNA in maize and introduces mutations by imperfect non-homologous end-joining.

To test whether the maize optimized duplex guide RNA/Cas endonuclease system described in Example 1 could recognize, cleave, and mutate maize chromosomal DNA through imprecise non-homologous end-joining (NHEJ) repair pathways, three different genomic target sequences were targeted for cleavage (see Table 1) and examined by deep sequencing for the presence of NHEJ mutations.

Table 1. Maize genomic target sequences introduced into the crRNA expression cassette.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Cas RNA System Used</th>
<th>Target Site Designation</th>
<th>Maize Genomic Target Site Sequence</th>
<th>PAM Sequence</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIG</td>
<td>Chr. 2: 28.45cM</td>
<td>crRNA/tracrRNA</td>
<td>LIGCas-1</td>
<td>GTACCGTACGTGCCCCGCGG</td>
<td>AGG</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>crRNA/tracrRNA</td>
<td>LIGCas-2</td>
<td>GGAATTGTACCGTACGTGCCC</td>
<td>CGG</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>crRNA/tracrRNA</td>
<td>LIGCas-3</td>
<td>GCCGTACGCGTACGTTG</td>
<td>AGG</td>
<td>14</td>
</tr>
</tbody>
</table>

LIG=approximately 600bp upstream of the Liguleless 1 gene start codon

The maize optimized Cas9 endonuclease expression cassette, crRNA expression cassettes containing the specific maize VT domains complementary to the antisense strand of the maize genomic target sequences listed in Table 1 and tracrRNA expression cassette were co-delivered to 60-90 Hi-II immature maize embryos by particle-mediated delivery (see Example 7) in the presence of BBM and WUS2 genes (see Example 8). Hi-II maize embryos transformed with the Cas9 and long guide RNA expression cassettes (as described in US provisional patent application 61/868706, filed on August 22, 2013) targeting the LIGCas-3 genomic
target site for cleavage served as a positive control and embryos transformed with only the Cas9 expression cassette served as a negative control. After 7 days, the 20-30 most uniformly transformed embryos from each treatment were pooled and total genomic DNA was extracted. The region surrounding the intended target site was PCR amplified with Phusion® HighFidelity PCR Master Mix (New England Biolabs, M0531 L) adding on the sequences necessary for amplicon-specific barcodes and Illumina sequencing using "tailed" primers through two rounds of PCR. The primers used in the primary PCR reaction are shown in Table 2 and the primers used in the secondary PCR reaction were

AATGATACGGCGACACCCAAGATCTACACTCTTTTACACG
(forward, SEQ ID NO: 21) and CAAGCAGAAGACGCGATA
(reverse, SEQ ID NO: 22).

Table 2. PCR primer sequences

<table>
<thead>
<tr>
<th>Target Site</th>
<th>Cas RNA System Used</th>
<th>Primer Orientation</th>
<th>Primary PCR Primer Sequence</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIGCas-1</td>
<td>crRNA/ tracrRNA</td>
<td>Forward</td>
<td>CTACACTCTTTCCCTACACGACGCCTCTTACGAT</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTCCCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-1</td>
<td>crRNA/ tracrRNA</td>
<td>Reverse</td>
<td>CAAGCAGAAGACGCGACACCCAAGATCTACACTCTTACGAT</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-2</td>
<td>crRNA/ tracrRNA</td>
<td>Forward</td>
<td>CTACACTCTTTCCCTACACGACGCCTCTTACGAT</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-2</td>
<td>crRNA/ tracrRNA</td>
<td>Reverse</td>
<td>CAAGCAGAAGACGCGACACCCAAGATCTACACTCTTACGAT</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-3</td>
<td>crRNA/ tracrRNA</td>
<td>Forward</td>
<td>CTACACTCTTTCCCTACACGACGCCTCTTACGAT</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-3</td>
<td>crRNA/ tracrRNA</td>
<td>Reverse</td>
<td>CAAGCAGAAGACGCGACACCCAAGATCTACACTCTTACGAT</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-3</td>
<td>Long guide RNA</td>
<td>Forward</td>
<td>CTACACTCTTTCCCTACACGACGCCTCTTACGAT</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
<tr>
<td>LIGCas-3</td>
<td>Long guide RNA</td>
<td>Reverse</td>
<td>CAAGCAGAAGACGCGACACCCAAGATCTACACTCTTACGAT</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCTTCTTGTAACGATTTACGACCTTGCT</td>
<td></td>
</tr>
</tbody>
</table>

The resulting PCR amplifications were purified with a Qiagen PCR purification spin column, concentration measured with a Hoechst dye-based fluorometric assay, combined in an equimolar ratio, and single read 100 nucleotide-length deep sequencing was performed on Illumina's MiSeq Personal Sequencer.
with a 30-40% (v/v) spike of PhiX control v3 (Illumina, FC-1 10-3001) to off-set sequence bias. Only those reads with a >1 nucleotide indel arising within the 10 nucleotide window centered over the expected site of cleavage and not found in a similar level in the negative control were classified as NHEJ mutations. NHEJ mutant reads with the same mutation were counted and collapsed into a single read and the top 10 most prevalent mutations were visually confirmed as arising within the expected site of cleavage. The total numbers of visually confirmed NHEJ mutations were then used to calculate the % mutant reads based on the total number of reads of an appropriate length containing a perfect match to the barcode and forward primer.

The frequency of NHEJ mutations recovered by deep sequencing for the duplex guide RNA/Cas endonuclease system targeting the three LIGCas targets (SEQ ID NOs: 12-14) compared to the single long guide RNA/Cas endonuclease system targeting the same locus is shown in Table 3. The ten most prevalent types of NHEJ mutations recovered based on the duplex guide RNA/Cas endonuclease system are shown in Figure 4A (corresponding to SEQ ID NOs: 24-33 wherein SEQ ID NO: 23 is the reference maize sequence comprising the LIGCas-1 target site), Figure 4B (corresponding to SEQ ID NOs: 34-43 wherein SEQ ID NO: 23 is the reference maize sequence comprising the LIGCas-2 target site) and Figure 4C (corresponding to SEQ ID NOs: 45-54, wherein SEQ ID NO: 44 is the reference maize sequence comprising the LIGCas-3 target site).

Taken together, this data indicates that the maize optimized duplex guide RNA/Cas endonuclease system described herein cleaves maize chromosomal DNA and generates imperfect NHEJ mutations.

Table 3. Percent (%) mutant reads at maize Liqueless 1 target locus produced by duplex guide RNA/Cas endonuclease system compared to the long guide RNA/Cas endonuclease system

<table>
<thead>
<tr>
<th>System</th>
<th>Total Number of Reads</th>
<th>Number of Mutant Reads</th>
<th>% Mutant Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas9 Only Control</td>
<td>1,744,427</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>LIGCas-3 long guide RNA</td>
<td>1,596,955</td>
<td>35,300</td>
<td>2.21%</td>
</tr>
<tr>
<td>LIGCas-1 crRNA/tracrRNA</td>
<td>1,803,163</td>
<td>4,331</td>
<td>0.24%</td>
</tr>
</tbody>
</table>
EXAMPLE 3

Deoxyribonucleic acid (DNA) can be used to guide the Cas9 protein to cleave maize chromosomal DNA and introduce mutations by imperfect non-homologous end-joining.

As previously described in Gasiunas et al. (2012) Proc. Natl. Acad. Sci. USA 109:E2579-86, Jinek et al. (2012) Science 337:81 6-21, Mali et al. (2013) Science 339:823-26 and Cong et al. (2013) Science 339:819-23, ribonucleic acids or RNA have been the only molecules described to guide a Cas9 endonuclease to recognize and cleave a specific DNA target site. In this example, we provide evidence that a new class of molecules, deoxyribonucleic acids (DNA), can also be used to guide a Cas endonuclease to recognize and cleave chromosomal DNA target sites resulting in the recovery of imperfect NHEJ mutations.

In this example, we used a duplex guide polynucleotide comprising of a first nucleotide sequence domain, referred to as the "variable targeting" (VT) domain, and a second nucleotide sequence domain, referred to as the "Cas endonuclease recognition" (CER) domain, wherein the variable targeting domain is a contiguous stretch of deoxyribonucleic acids (DNA). The CER domain of the duplex guide polynucleotide comprised two separate molecules, one DNA molecule that was linked to the VT domain of the crNucleotide molecule (Figure 1B) and hybridized along a region of complementarity to a second molecule (the tracrNucleotide, Figure 1A) consisting of a contiguous stretch of ribonucleic acids (RNA) nucleotides (referred to as tracrRNA). In this example the crNucleotide of the duplex guide polynucleotide (Figure 1A) consisted solely of DNA nucleotides and is herein referred to as crDNA.

The crDNA sequence containing VT domain targeting the LIGCas-3 target site (Table 1)(SEQ ID No: 55) was synthesized at Integrated DNA Technologies, Inc. with a 5′ phosphate group and purified by PAGE and then used to test if a duplex guide crDNA-tracrRNA polynucleotide/Cas endonuclease complex as
illustrated in Figure 5 may recognize and cleave maize chromosomal DNA target sites resulting in the recovery of NHEJ mutations.

To determine the optimal delivery concentration for the synthetic crDNA molecules, different concentrations of crDNA (20 ng, 50 ng, 100 ng, 1 μg and 5 μg) were co-delivered along with a maize optimized tracrRNA and Cas9 expression cassettes to 60-90 Hi-II immature maize embryos and assayed for the presence of NHEJ mutations as described in Example 2. Embryos transformed with only the Cas9 and tracrRNA expression cassettes served as a negative control. As shown in Table 4, NHEJ mutations were detected with an optimal crDNA delivery concentration near 50 ng.

To compare the NHEJ mutational activity of the duplex guide crDNA-tracrRNA polynucleotide/Cas endonuclease complex (Figure 5) with a duplex guide RNA (crRNA-tracrRNA)/Cas endonuclease complex (Figure 3A), a crRNA comprising a VT domain targeting the LIGCas-3 target site (Table 1) was synthesized at Bio-Synthesis, Inc. with a 5’ phosphate group and purified by PAGE (SEQ ID NO: 10). 50 ng of both the synthetic crDNA and crRNA were then independently co-delivered along with the maize optimized tracrRNA and Cas9 DNA expression cassettes and assayed for NHEJ mutations as described previously. The transformation experiment was performed twice to demonstrate reproducibility.

Negative controls consisted of Hi-II maize embryos transformed with 50 ng of crDNA, the Cas9 expression cassette or 50 ng of crDNA plus the tracrRNA expression cassette.

As shown in Tables 4 and 5, NHEJ mutations resulting from the duplex guide crDNA-tracRNA polynucleotide/Cas endonuclease system were identified when the crDNA was delivered in combination with a tracrRNA and Cas9 DNA expression cassettes compared to the absence of NHEJ mutations in the Cas9 only, crDNA only, crDNA plus tracrRNA only and tracrRNA plus Cas9 only controls. The top 3 most abundant crRNA NHEJ mutations are shown in Figure 6 A (SEQ ID NO:56, 57, 58, wherein SEQ ID NO:44 is the unmodified reference sequence for LIGCas-3 locus) and the top 3 most abundant crDNA NHEJ mutations are shown in FIGURE 6 B (SEQ ID NO: 59, 60, 61, wherein SEQ ID NO:44 is the unmodified reference...
sequence for LIGCas-3 locus) identified are compared and shown in Figure 6 (SEQ ID NOs: 44, 56-61).

Taken together, this data indicates that deoxyribonucleic acids (DNA) may also be used to guide Cas endonucleases in a duplex guide crDNA-tracrRNA polynucleotide/Cas endonuclease complex (Figure 4) to cleave maize chromosomal DNA resulting in imprecise NHEJ mutations.

Table 4. Percent (%) mutant reads at maize Liqueless 1 target locus produced by different concentrations of transiently delivered crDNA molecules co-delivered with tracrRNA and Cas9 DNA expression cassettes

<table>
<thead>
<tr>
<th>Amount of crDNA Delivered</th>
<th>DNA Expression Cassettes Delivered</th>
<th>Total Number of Reads</th>
<th>Number of Mutant Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Cas9, tracrRNA</td>
<td>1,046,553</td>
<td>0</td>
</tr>
<tr>
<td>20 ng</td>
<td>Cas9, tracrRNA</td>
<td>926,915</td>
<td>0</td>
</tr>
<tr>
<td>50 ng</td>
<td>Cas9, tracrRNA</td>
<td>1,032,080</td>
<td>18</td>
</tr>
<tr>
<td>100 ng</td>
<td>Cas9, tracrRNA</td>
<td>860,565</td>
<td>8</td>
</tr>
<tr>
<td>1 µg</td>
<td>Cas9, tracrRNA</td>
<td>398,996</td>
<td>0</td>
</tr>
<tr>
<td>5 µg</td>
<td>Cas9, tracrRNA</td>
<td>394,959</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5. Comparison of percent (%) mutant reads at maize Liqueless 1 target locus produced by transiently delivered crDNA or crRNA molecules co-delivered with tracrRNA and Cas9 DNA expression cassettes

<table>
<thead>
<tr>
<th>Synthetic crDNA or crRNA Delivered</th>
<th>Transformation Replicate</th>
<th>DNA Expression Cassettes Delivered</th>
<th>Total Number of Reads</th>
<th>Number of Mutant Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>Cas9</td>
<td>1,151,532</td>
<td>0</td>
</tr>
<tr>
<td>crDNA</td>
<td>1</td>
<td>-</td>
<td>1,234,489</td>
<td>0</td>
</tr>
<tr>
<td>crDNA</td>
<td>1</td>
<td>tracrRNA</td>
<td>666,151</td>
<td>0</td>
</tr>
<tr>
<td>crDNA</td>
<td>1</td>
<td>tracrRNA, Cas9</td>
<td>1,046,189</td>
<td>40</td>
</tr>
<tr>
<td>crDNA</td>
<td>2</td>
<td>tracrRNA, Cas9</td>
<td>913,430</td>
<td>39</td>
</tr>
<tr>
<td>crRNA</td>
<td>1</td>
<td>tracrRNA, Cas9</td>
<td>1,217,740</td>
<td>136</td>
</tr>
<tr>
<td>crRNA</td>
<td>2</td>
<td>tracrRNA, Cas9</td>
<td>1,028,995</td>
<td>281</td>
</tr>
</tbody>
</table>

**EXAMPLE 4**

Modifying nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system to increase cleavage activity and specificity.

In this example, modifying the nucleotide base, phosphodiester bond linkage or molecular topography of the guiding nucleic acid component(s) of the guide
polynucleotide/Cas endonuclease system is described for increasing cleavage activity and specificity.

As shown in Figure 1A and Figure 1B, the nucleic acid component(s) of the guide polynucleotide include a variable targeting (VT) domain and a Cas endonuclease recognition (CER) domain. The VT domain is responsible for interacting with the DNA target site through direct nucleotide-nucleotide base pairings while the CER domain is required for proper Cas endonuclease recognition (Figure 3A and Figure 3B). Along with the required PAM sequence, both domains of the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system function to link DNA target site recognition with Cas endonuclease target site cleavage (Figure 3A and Figure 3B).

Given the direct interaction of the VT domain with the DNA target site, nucleotide base modifications within the VT domain can be utilized to alter the nucleotide-nucleotide base pairing relationships facilitating Cas endonuclease target site recognition. Such modifications can be used to strengthen the binding affinity to the complementary DNA target sequence enhancing guide polynucleotide/Cas endonuclease target site recognition and/or specificity. Non-limiting examples of nucleotide base modifications that can enhance target site binding affinity and/or specificity when introduced into the VT domain of a guide polynucleotide are listed in Table 6. These modifications can be used individually or in combination within the VT domain.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyribonucleic Acid</td>
<td>More specific hybridization to complementary DNA target sequence¹</td>
</tr>
<tr>
<td>Locked Nucleic Acid</td>
<td>Increased binding affinity and more specific hybridization to complementary DNA target sequence²</td>
</tr>
<tr>
<td>5-methyl dC</td>
<td>Increase binding affinity to complementary DNA target sequence³</td>
</tr>
<tr>
<td>Nucleotide Modification</td>
<td>Effect on DNA Binding</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>2,6-Diaminopurine</td>
<td>Increase affinity</td>
</tr>
<tr>
<td>2'-Fluoro A or U</td>
<td>Increase affinity</td>
</tr>
</tbody>
</table>

Nucleotide modifications similar to those shown in Table 6 can also be made in the CER domain of the single or duplex guide polynucleotide (Figure 1A and Figure 1B). These modifications may act to strengthen or stabilize inter-molecular interactions in the CER domain of a duplexed crNucleotide molecule (for example, but not limiting to crRNA, crDNA or a combination thereof) and tracrNucleotide molecule (for example tracrRNA, tracrDNA, or a combination thereof)(Figure 3A). These modifications can also help recapitulate crNucleotide and tracrNucleotide (such as for example but not limiting to crRNA/tracrRNA; crDNA/tracrRNA; crRNA/tracrDNA, crDNA/tracrDNA) structures required for proper Cas endonuclease recognition in the secondary structure of guide polynucleotides being comprised of a single molecule (Figure 3A and Figure 3B).

Nucleic acids expressed or delivered transiently to cells are subject to turnover or degradation. To increase the effective lifespan or stability of the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system in vivo, nucleotide and/or phosphodiester bond modifications may be introduced to reduce unwanted degradation. Examples of nuclease resistant nucleotide and phosphodiester bond modifications are shown in Table 7 and may be introduced in any one of the VT and/or CER domains of the guide polynucleotide. Modifications may be introduced at the 5’ and 3’ ends of any one of the nucleic acid residues comprising the VT or CER domains to inhibit exonuclease cleavage activity, can be introduced in the middle of the nucleic acid sequence comprising the VT or CER domains to slow endonuclease cleavage activity or can be introduced throughout the nucleic acid sequences comprising the VT or CER domains to provide protection from both exo- and endo-nucleases.
Table 7. Nucleotide base and phosphodiester bond modifications to decrease unwanted nuclease degradation.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyribonucleic Acid</td>
<td>Less susceptible to nuclease degradation than RNA(^1)</td>
</tr>
<tr>
<td>Locked Nucleic Acid</td>
<td>Very resistant to nuclease cleavage(^2)</td>
</tr>
<tr>
<td>2'-Fluoro A or U</td>
<td>Increased resistance to nuclease cleavage(^3)</td>
</tr>
<tr>
<td>2'-O-Methyl RNA Bases</td>
<td>Resistant to ribonucleases and 5-10 fold more resistant to DNases than DNA(^4)</td>
</tr>
<tr>
<td>Phosphorothioate bond</td>
<td>Very resistant to nuclease cleavage(^5)</td>
</tr>
</tbody>
</table>

To provide resistance against turnover or degradation in cells, the nucleic acid component(s) of the guide polynucleotide may also be circularized where the 5’ and 3’ ends are covalently joined together. Circular RNA can be more resistant to nuclease degradation than linear RNA and can persist in cells long after corresponding linear transcripts (Jeck et al. (2013) RNA 19:141-157).

Modifications to any one of the guide polynucleotide nucleic acid components may also be introduced to increase their permeability or delivery into cells. Such modifications would include, but not be limited to, linkage to cholesterol, polyethylene glycol and spacer 18 (hexaethylene glycol chain).

Many of the above mentioned modified guide polynucleotides can be synthesized and delivered transiently by biolistic particle-mediated transformation, transfection or electroporation. The remaining components of the guide polynucleotide/Cas endonuclease system needed to form a functional complex capable of binding and/or cleaving a chromosomal DNA target site may be co-delivered as any combination of DNA expression cassettes, RNA, mRNA (5’-capped and polyadenylated) or protein. Cell lines or transformants may also be established stably expressing all but one or two of the components needed to form a functional guide polynucleotide/Cas endonuclease complex so that upon transient delivery of the above mentioned modified nucleic acid guide(s) a functional guide polynucleotide/Cas endonuclease complex may form. Modified guide
polynucleotides described above may also be delivered simultaneously in multiplex to target multiple chromosomal DNA sequences for cleavage or nicking.

The above mentioned modified guide polynucleotides may be used in plants, animals, yeast and bacteria or in any organism subject to genome modification with the guide polynucleotide/Cas endonuclease system and be used to introduce imprecise NHEJ mutations into chromosomal DNA, excise chromosomal DNA fragments comprised of either transgenic or endogenous DNA, edit codon composition of native or transgenic genes by homologous recombination repair with a donor DNA repair template(s) and site-specifically insert transgenic or endogenous DNA sequences by homologous recombination repair with a donor DNA repair template(s).

**EXAMPLE 5**

Examining the effect of nucleotide base and phosphodiester bond modifications to the guide polynucleotide component of the guide polynucleotide/Cas endonuclease system in maize

In this example, some of the nucleotide base and phosphodiester bond modifications described in Example 4 are introduced into the VT domain and/or CER domain of a crNucleotide and methods for evaluating the impact of these modifications on the ability of a duplexed guide polynucleotide/Cas endonuclease system to cleave maize chromosomal DNA will be discussed.

As illustrated in Table 8, nucleotide base and phosphodiester linkage modifications were introduced individually or in combination into the VT domain and the CER domain of the crNucleotide (crRNA or crDNA) component of the duplexed guide polynucleotide/Cas endonuclease system targeting the LIGCas-3 site (see Table 1) for cleavage. Although a number of different nucleotide base and phosphodiester linkage modifications are examined in combination here, other possible combinations may be envisioned.

Locked Nucleic Acid (+), 5-Methyl dC (iMe-dC) and 2,6-Diaminopurine (i6diPr) nucleotide base modifications made in the VT domain are introduced to increase the binding affinity to the complementary DNA target sequence and in the case of the Locked Nucleic Acid modifications to also increase resistance to in vivo nucleases. All other modifications designed in both the VT and CER domains at the
5' and 3' ends or throughout the crRNA or crDNA sequence are introduced to decrease the effect of in vivo nucleases and increase the effective lifespan of the crRNA or crDNA component.

To examine the effect that the modified crRNA or crDNA components described in Table 8 have on the ability of their associated modified guide polynucleotide/Cas endonuclease complex to recognize and cleave the LIGCas-3 site (see Table 1), the modified crRNA and crDNA molecules are co-delivered to Hill immature maize embryos with tracrRNA and Cas9 expression cassettes as described in Example 3. Unmodified crRNA or crDNA molecules co-delivered with tracrRNA and Cas9 expression cassettes serve as comparators. Negative controls consist of immature maize embryos transformed with only the corresponding modified crRNA or crDNA or Cas9 expression cassette. Frequencies of imperfect NHEJ mutations, assayed as described in Example 2, are used to evaluate the effect of each crRNA or crDNA modification on Cas endonuclease cleavage activity relative to the comparable unmodified crRNA or crDNA experiments.

Table 8. crRNA and crDNA nucleotide base and phosphodiester linkage modifications.

<table>
<thead>
<tr>
<th>Nucleic Acid Type</th>
<th>Modification</th>
<th>crRNA or crDNA Sequence and Corresponding Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VT Domain</td>
</tr>
<tr>
<td>crRNA</td>
<td>None</td>
<td>GCGUACGCGUACGUGUG (SEQ ID NO: 62)</td>
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<td>GUUUAGAGCUAGCUGUUUUG (SEQ ID NO: 63)</td>
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<td>crRNA</td>
<td>Phosphorothioate bonds near ends</td>
<td>G<em>C</em>G*UACGCGUACGUGUG (SEQ ID NO: 64)</td>
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<td>GUUUAGAGCUAGCUGU<em>U</em>U*G (SEQ ID NO: 65)</td>
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<td>crRNA</td>
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<td>mGmCmGUACGCUGCUAGUG (SEQ ID NO: 66)</td>
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<td></td>
<td></td>
<td>GUUUAGAGCUAGCUGUUmUmG (SEQ ID NO: 67)</td>
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| crRNA            | 2'-O-Methyl RNA nucleotides for each nucleotide | mGmGmUmAmAmGmGmCmGmUmAmAmGmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGmUmUmGm Um

<p>| crDNA            | None         | GCGTACGCCTACGTGTG (SEQ ID NO: 70)                     |
|                  |              | GTTTTAGAGCTATGCTGTTTTG (SEQ ID NO: 71)                |
| crDNA            | 1 Locked Nucleic Acid nucleotide in the | GCGTACGCCTACGTGTG+TG (SEQ ID NO: 72) |
|                  |              | GTTTTAGAGCTATGCTGTTTTG (SEQ ID NO: 71)                |</p>
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<th>3 Locked Nucleic Acid nucleotides in the variable targeting domain</th>
<th>GCGTACGCCTA+CG+TG+TG (SEQ ID NO: 73)</th>
<th>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</th>
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<tr>
<td>crDNA</td>
<td>6 Locked Nucleic Acid nucleotides in the variable targeting domain</td>
<td>GCGTA+CG+CG+TA+CG+TG+TG (SEQ ID NO: 74)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</td>
</tr>
<tr>
<td>crDNA</td>
<td>3 Locked Nucleic Acid nucleotides in the variable targeting domain plus Phosphorothioate bonds near ends</td>
<td>G˘C˘G˘TACCGCTA+CG+TG+TG (SEQ ID NO: 75)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 76)</td>
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<tr>
<td>crDNA</td>
<td>One 5-Methyl dC nucleotide in variable targeting domain</td>
<td>GCGTACGCCTA/iMe-dC/GTGTG (SEQ ID NO: 75)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</td>
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<tr>
<td>crDNA</td>
<td>Three 5-Methyl dC nucleotides in the variable targeting domain</td>
<td>GCGTA/iMe-dC/G/iMe-dC/GTGTG (SEQ ID NO: 78)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</td>
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<tr>
<td>crDNA</td>
<td>One 2,6-Diaminopurine nucleotide in the variable targeting domain</td>
<td>GCGTACCGCTA/i6diPr/GTGTG (SEQ ID NO: 79)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</td>
</tr>
<tr>
<td>crDNA</td>
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<td>GCGT/i6diPr/CGCGT/i6diPr/GTGTG (SEQ ID NO: 80)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 71)</td>
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<tr>
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<td>Locked Nucleic Acid nucleotides at ends</td>
<td>+G+C+GTACCGCTACGTGTTG (SEQ ID NO: 81)</td>
<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 82)</td>
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<td>crDNA</td>
<td>Phosphorothioate bonds near ends</td>
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<td>GTTTTAGAGCTATGCTGTTTTTGT (SEQ ID NO: 76)</td>
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<td>crDNA</td>
<td>2'-O-Methyl RNA nucleotides at ends</td>
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<td>GTTTTAGAGCTATGCTGTTTTTGTmUmUmG (SEQ ID NO: 85)</td>
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<tr>
<td>crDNA</td>
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<td>mGmGmGmGmGmGmGmGmGmGmGTACCGCTACGTGTTG (SEQ ID NO: 87)</td>
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</table>

1: "+" before nucleotide denotes lock nucleic acid base modification, "*" after nucleotide denotes Phosphorothioate bond backbone modification, "m" before nucleotide denotes 2'-O-Methyl RNA base modification, "iMe-dC" denotes 5-Methyl dC base modification and "i6diPr" denotes 2,6-Diaminopurine base modification.
EXAMPLE 6

Methods to examine the effect of modifications to the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system in yeast

In this example, yeast screening methods are devised to identify optimal modifications to the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system that result in enhanced cleavage activity.

A. ADE:URA3:DE2 yeast screening strain

To identify optimal modification(s) or combinations thereof to the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system outlined in Example 4, a *Saccharomyces cerevisiae* strain is developed to carefully monitor the cleavage activity of the guide polynucleotide/Cas endonuclease system. This is accomplished by replacing the native ADE2 gene on chromosome 15 of yeast strain BY4247 with a non-functional partially duplicated ADE2 gene disrupted by the yeast URA3 gene (ADE:URA3:DE2) as shown in Figure 7. A guide polynucleotide/Cas endonuclease target site adjacent to the appropriate PAM sequence is then designed against the implanted URA3 gene so that upon cleavage the disrupted ADE2 gene containing 305 bp of duplicated overlapping sequence can be repaired by intramolecular homologous recombination pathways resulting in the loss of the URA3 gene and the gain of a functional ADE2 gene as shown in Figure 8. Media containing 5-Fluoroorotic Acid (5-FOA) or media deficient in adenine can then be used to select for cells where cleavage has occurred. The frequency of yeast cells recovered after selection can then be used to quantify the cleavage efficiency of the guide polynucleotide/Cas endonuclease system when examining different modifications to the nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system.

Yeast cells containing a functional ADE2 gene as a result of cleavage and repair of the ADE:URA3:DE2 locus can also be subject to a visual phenotypic screen for cleavage activity. In the absence of 5-FOA or adenine minus selection, functional ADE2 gene products result in a white phenotype while non-functional products result in a red phenotype (Ugolini *et al.* (1996) *Curr. Genet.* 30:485-492). To visualize the white or red phenotype, individual yeast cell transformants can be
plated on solid media and allowed to grow into a colony large enough to inspect visually. The amount of white to red sectoring provides an indication as to the amount of cleavage activity. Since the sectoring phenotype is a qualitative measure, a 0-4 numerical scoring system can be implemented. As shown in Figure 9, a score of 0 indicates that no white sectors (no target site cleavage) were observed; a score of 4 indicates completely white colonies (complete cutting of the recognition site); scores of 1-3 indicate intermediate white sectoring phenotypes (and intermediate degrees of target site cleavage).

B. Cas9 component of the guide polynucleotide/Cas endonuclease system

To stably express the Cas endonuclease for pairing with the transiently delivered modified nucleic acid component(s) described in Example 4, the Cas9 gene from *Streptococcus pyogenes* M1 GAS (SF370) can be S. cerevisiae codon optimized per standard techniques known in the art (SEQ ID NO: 88) and a SV40 (Simian virus 40) nuclear localization signal (SRADPKKKRKV, SEQ ID NO: 7) can be incorporated at the carboxyl terminal to facilitate nuclear localization. The resulting Cas9 open reading frame will then be operably fused to the yeast inducible GAL1 promoter and CYC1 terminator. The resulting Cas9 expression cassette will then be placed into a CEN6 autonomously replicating yeast vector containing a LEU2 selectable marker.

To be able to test transient delivery of both the Cas9 component and the modified nucleic acid component(s) described in Example 4, the Cas9 gene can also be delivered as mRNA. To generate S. cerevisiae optimized Cas9 mRNA, PCR can be used to amplify the S. cerevisiae optimized Cas9 open reading frame and associated nuclear localization signal tailing on the required T7 promoter sequence (TAATACTGACTCAGCTAGG, SEQ ID NO: 89) just 5' of the translation ATG start site. The resulting linear template containing the T7 promoter can then be used to transcribe uncapped or capped Cas9 mRNA with or without polyadenylation in vitro.

The Cas9 component can also be delivered transiently as protein and paired with the modified nucleic acid component(s). Cas9 protein with associated carboxyl-terminal nuclear localization signal can be expressed and purified per
standard techniques similar to that described by Fonfara et al. (2013) Nucl. Acids Res. doi:10.1093/nar/gkt1074 or by other methods.

C. Nucleic acid component(s) of the guide polynucleotide/Cas endonuclease system

It can be advantageous to pair the transient delivery of modified crRNA or tracrRNA components with the corresponding stably expressed unmodified crRNA or tracrRNA. To facilitate stable expression of crRNA and tracrRNA in yeast, S. cerevisiae optimized crRNA and tracrRNA expression cassettes can be generated. The yeast RNA polymerase III SNR52 promoter and SUP4 terminator can be operably fused to the ends of DNA fragments encoding the appropriate crRNA and tracrRNA sequences required for recognition by the S. pyogenes Cas9 protein. All crRNA expression cassettes will contain the ADE:URA3:DE2 target sequence (GCAGACATTACGAATGCACA, SEQ ID NO: 90) in the VT domain and target the ADE:URA3:DE2 locus for cleavage. The resulting expression cassettes will then be placed into a CEN6 autonomously replicating yeast vector containing a HIS3 selectable marker.

To deliver unmodified crRNA, tracrRNA or guide RNA transiently, PCR can be used to amplify the corresponding crRNA, tracrRNA or guide RNA sequence tailing on the required T7 promoter sequence (TAATAC GACTC ACTATAG GG, SEQ ID NO: 89) just 5' of the transcriptional start site. The resulting linear template containing the T7 promoter can then be used to transcribe the corresponding crRNA, tracrRNA or long guide RNA.

Modified nucleic acid components(s) of the guide polynucleotide/Cas endonuclease system as outlined in Example 4 will also be transiently delivered. Nucleotide base and/or phosphodiester bond modifications similar to those illustrated in Example 5 Table 8 can be introduced individually or in combination into the crRNA, crDNA, tracrRNA, tracrDNA, long guide RNA or long guide DNA nucleic acid components of the guide polynucleotide/Cas endonuclease system and synthesized per standard techniques.

Circular RNAs, also discussed in Example 4, containing the necessary VT and CER domains capable of forming a functional complex with the Cas
endonuclease can be generated in vitro as described by Diegleman et al. (1998) Nucl. Acids Res. 26:3235-3241 and delivered transiently to yeast cells.

D. Transformation of guide polynucleotide/Cas endonuclease components into the ADE:URA3:DE2 yeast strain

Components of the guide polynucleotide/Cas endonuclease system can be delivered to ADE:URA3:DE2 yeast cells using standard lithium acetate, polyethylene glycol (PEG), electroporation or biolistic transformation methods and monitored for their ability to cleave the ADE:URA3:DE2 target. The yeast optimized guide polynucleotide/Cas endonuclease components discussed in Example 6 sections B and C can be delivered as expression cassettes on low copy autonomously replicating plasmid DNA vectors, as non-replicating transient molecules (such as mRNA, protein, RNA or modified guide nucleic acids) or in any combination of plasmid DNA vector expression cassette(s) and transient molecule(s).

EXAMPLE 7
Transformation of Maize Immature Embryos

Transformation can be accomplished by various methods known to be effective in plants, including particle-mediated delivery, Agrobacterium-mediated transformation, PEG-mediated delivery, and electroporation.

a. Particle-mediated delivery

Transformation of maize immature embryos using particle delivery is performed as follows. Media recipes follow below.

The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are isolated and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment. Alternatively, isolated embryos are placed on 560L (Initiation medium) and placed in the dark at temperatures ranging from 26°C to 37°C for 8 to 24 hours prior to placing on 560Y for 4 hours at 26°C prior to bombardment as described above.
Plasmids containing the double strand brake inducing agent and donor DNA are constructed using standard molecular biology techniques and co-bombarded with plasmids containing the developmental genes ODP2 (AP2 domain transcription factor ODP2 (Ovule development protein 2); US20090328252 A1) and Wushel (US201 1/0167516).

The plasmids and DNA of interest are precipitated onto 0.6 μm (average diameter) gold pellets using a water-soluble cationic lipid transfection reagent as follows. DNA solution is prepared on ice using 1 μg of plasmid DNA and optionally other constructs for co-bombardment such as 50 ng (0.5 μl) of each plasmid containing the developmental genes ODP2 (AP2 domain transcription factor ODP2 (Ovule development protein 2); US20090328252 A1) and Wushel. To the pre-mixed DNA, 20 μl of prepared gold particles (15 mg/ml) and 5 μl of the a water-soluble cationic lipid transfection reagent is added in water and mixed carefully. Gold particles are pelleted in a microfuge at 10,000 rpm for 1 min and supernatant is removed. The resulting pellet is carefully rinsed with 100 ml of 100% EtOH without resuspending the pellet and the EtOH rinse is carefully removed. 105 μl of 100% EtOH is added and the particles are resuspended by brief sonication. Then, 10 μl is spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Alternatively, the plasmids and DNA of interest are precipitated onto 1.1 μm (average diameter) tungsten pellets using a calcium chloride (CaCl^2) precipitation procedure by mixing 100 μl prepared tungsten particles in water, 10 μl (1 μg) DNA in Tris EDTA buffer (1 μg total DNA), 100 μl 2.5 M CaCl2, and 10 μl 0.1 M spermidine. Each reagent is added sequentially to the tungsten particle suspension, with mixing. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid is removed, and the particles are washed with 500 ml 100% ethanol, followed by a 30 second centrifugation. Again, the liquid is removed, and 105 μl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated. 10 μl of the tungsten/DNA particles is spotted onto the center of each macrocarrier, after which the spotted particles are allowed to dry about 2 minutes before bombardment.
The sample plates are bombarded at level #4 with a Biorad Helium Gun. All samples receive a single shot at 450 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Following bombardment, the embryos are incubated on 560P (maintenance medium) for 12 to 48 hours at temperatures ranging from 26C to 37C, and then placed at 26C. After 5 to 7 days the embryos are transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks at 26C. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to a lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to a 2.5” pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to Classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for transformation efficiency, and/or modification of regenerative capabilities.

Initiation medium (560L) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson’s Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 20.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-l H2O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-l H2O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature).

Maintenance medium (560P) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson’s Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, 2.0 mg/l 2,4-D, and 0.69 g/l L-proline (brought to volume with D-l H2O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-l H2O); and 0.85 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature).

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson’s Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl,
120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-l H2O following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-l H2O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature).

Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-l H2O following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-l H2O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.1 0 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-l H2O) (Murashige and Skoog (1962) Physiol. Plant. 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-l H2O after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-l H2O); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C).

Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.1 0 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-l H2O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-l H2O after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-l H2O), sterilized and cooled to 60°C.

b. **Agrobacterium-mediated** transformation

Agrobacterium-mediated transformation was performed essentially as described in Djukanovic *et al.* (2006) *Plant Biotech* J 4:345-57. Briefly, 10-12 day old immature embryos (0.8 -2.5 mm in size) were dissected from sterilized kernels and placed into liquid medium (4.0 g/l N6 Basal Salts (Sigma C-141 6), 1.0 ml/L Eriksson's Vitamin Mix (Sigma E-1511), 1.0 mg/L thiamine HCl, 1.5 mg/L 2, 4-D, 0.690 g/L L-proline, 68.5 g/L sucrose, 36.0 g/L glucose, pH 5.2). After embryo collection, the medium was replaced with 1 ml Agrobacterium at a concentration of
0.35-0.45 OD550. Maize embryos were incubated with Agrobacterium for 5 min at room temperature, then the mixture was poured onto a media plate containing 4.0 g/L N6 Basal Salts (Sigma C-1416), 1.0 ml/L Eriksson’s Vitamin Mix (Sigma E-1511), 1.0 mg/L thiamine HCl, 1.5 mg/L 2, 4-D, 0.690 g/L L-proline, 30.0 g/L sucrose, 0.85 mg/L silver nitrate, 0.1 nM acetosyringone, and 3.0 g/L Gelrite, pH 5.8. Embryos were incubated axis down, in the dark for 3 days at 20°C, then incubated 4 days in the dark at 28°C, then transferred onto new media plates containing 4.0 g/L N6 Basal Salts (Sigma C-1416), 1.0 ml/L Eriksson’s Vitamin Mix (Sigma E-1511), 1.0 mg/L thiamine HCl, 1.5 mg/L 2, 4-D, 0.69 g/L L-proline, 30.0 g/L sucrose, 0.5 g/L MES buffer, 0.85 mg/L silver nitrate, 3.0 mg/L Bialaphos, 100 mg/L carbenicillin, and 6.0 g/L agar, pH 5.8. Embryos were subcultured every three weeks until transgenic events were identified. Somatic embryogenesis was induced by transferring a small amount of tissue onto regeneration medium (4.3 g/L MS salts (Gibco 11117), 5.0 ml/L MS Vitamins Stock Solution, 100 mg/L myo-inositol, 0.1 μM ABA, 1 mg/L IAA, 0.5 mg/L zeatin, 60.0 g/L sucrose, 1.5 mg/L Bialaphos, 100 mg/L carbenicillin, 3.0 g/L Gelrite, pH 5.6) and incubation in the dark for two weeks at 28°C. All material with visible shoots and roots were transferred onto media containing 4.3 g/L MS salts (Gibco 11117), 5.0 ml/L MS Vitamins Stock Solution, 100 mg/L myo-inositol, 40.0 g/L sucrose, 1.5 g/L Gelrite, pH 5.6, and incubated under artificial light at 28°C. One week later, plantlets were moved into glass tubes containing the same medium and grown until they were sampled and/or transplanted into soil.

**EXAMPLE 8**

Transient Expression of BBM Enhances Transformation

Parameters of the transformation protocol can be modified to ensure that the BBM activity is transient. One such method involves precipitating the BBM-containing plasmid in a manner that allows for transcription and expression, but precludes subsequent release of the DNA, for example, by using the chemical PEL in one example, the BBM plasmid is precipitated onto gold particles with PEI, while the transgenic expression cassette (UBI::moPAT::GFPm::Pinll; moPAT is the maize optimized PAT gene) to be integrated is precipitated onto gold particles using the standard calcium chloride method.
Briefly, gold particles were coated with PEI as follows. First, the gold particles were washed. Thirty-five mg of gold particles, 1.0 in average diameter (A.S.I. #162-001 0), were weighed out in a microcentrifuge tube, and 1.2 ml absolute EtOH was added and vortexed for one minute. The tube was incubated for 15 minutes at room temperature and then centrifuged at high speed using a microfuge for 15 minutes at 4oC. The supernatant was discarded and a fresh 1.2 ml aliquot of ethanol (EtOH) was added, vortexed for one minute, centrifuged for one minute, and the supernatant again discarded (this is repeated twice). A fresh 1.2 ml aliquot of EtOH was added, and this suspension (gold particles in EtOH) was stored at -20oC for weeks. To coat particles with polyethyylimine (PEI; Sigma #P3143), 250 µl of the washed gold particle/EtOH mix was centrifuged and the EtOH discarded. The particles were washed once in 100 µl ddH2O to remove residual ethanol, 250 µl of 0.25 mM PEI was added, followed by a pulse-sonication to suspend the particles and then the tube was plunged into a dry ice/EtOH bath to flash-freeze the suspension, which was then lyophilized overnight. At this point, dry, coated particles could be stored at -80oC for at least 3 weeks. Before use, the particles were rinsed 3 times with 250 µl aliquots of 2.5 mM HEPES buffer, pH 7.1 , with 1x pulse-sonication, and then a quick vortex before each centrifugation. The particles were then suspended in a final volume of 250 µl HEPES buffer. A 25 µl aliquot of the particles was added to fresh tubes before attaching DNA. To attach uncoated DNA, the particles were pulse-sonicated, then 1 µg of DNA (in 5 µl water) was added, followed by mixing by pipetting up and down a few times with a Pipetteman and incubated for 10 minutes. The particles were spun briefly (i.e. 10 seconds), the supernatant removed, and 60 µl EtOH added. The particles with PEI-precipitated DNA-1 were washed twice in 60 µl of EtOH. The particles were centrifuged, the supernatant discarded, and the particles were resuspended in 45 µl water. To attach the second DNA (DNA-2), precipitation using a water-soluble cationic lipid transfection reagent was used. The 45 µl of particles/DNA-1 suspension was briefly sonicated, and then 5 µl of 100 ng/µl of DNA-2 and 2.5 µl of the water-soluble cationic lipid transfection reagent were added. The solution was placed on a rotary shaker for 10 minutes, centrifuged at 10,000g for 1 minute. The supernatant was removed, and the particles resuspended in 60 µl of EtOH. The solution was spotted...
onto macrocarriers and the gold particles onto which DNA-1 and DNA-2 had been sequentially attached were delivered into scutellar cells of 10 DAP Hi-II immature embryos using a standard protocol for the PDS-1 000. For this experiment, the DNA-1 plasmid contained a UBI::RFP::pinll expression cassette, and DNA-2 contained a UBI::CFP::pinll expression cassette. Two days after bombardment, transient expression of both the CFP and RFP fluorescent markers was observed as numerous red & blue cells on the surface of the immature embryo. The embryos were then placed on non-selective culture medium and allowed to grow for 3 weeks before scoring for stable colonies. After this 3-week period, 10 multicellular, stably-expressing blue colonies were observed, in comparison to only one red colony. This demonstrated that PEI-precipitation could be used to effectively introduce DNA for transient expression while dramatically reducing integration of the PEI-introduced DNA and thus reducing the recovery of RFP-expressing transgenic events. In this manner, PEI-precipitation can be used to deliver transient expression of BBM and/or WUS2.

For example, the particles are first coated with UBI::BBM::pinll using PEI, then coated with UBI::moPAT~YFP using a water-soluble cationic lipid transfection reagent, and then bombarded into scutellar cells on the surface of immature embryos. PEI-mediated precipitation results in a high frequency of transiently expressing cells on the surface of the immature embryo and extremely low frequencies of recovery of stable transformants. Thus, it is expected that the PEI-precipitated BBM cassette expresses transiently and stimulates a burst of embryogenic growth on the bombarded surface of the tissue (i.e. the scutellar surface), but this plasmid will not integrate. The PAT-GFP plasmid released from the Ca++/gold particles is expected to integrate and express the selectable marker at a frequency that results in substantially improved recovery of transgenic events. As a control treatment, PEI-precipitated particles containing a UBI::GUS::pinll (instead of BBM) are mixed with the PAT~GFP/Ca++ particles. Immature embryos from both treatments are moved onto culture medium containing 3mg/l bialaphos. After 6-8 weeks, it is expected that GFP+, bialaphos-resistant calli will be observed in the PEI/BBM treatment at a much higher frequency relative to the control treatment (PEI/GUS).
As an alternative method, the BBM plasmid is precipitated onto gold particles with PEI, and then introduced into scutellar cells on the surface of immature embryos, and subsequent transient expression of the BBM gene elicits a rapid proliferation of embryogenic growth. During this period of induced growth, the explants are treated with Agrobacterium using standard methods for maize (see Example 1), with T-DNA delivery into the cell introducing a transgenic expression cassette such as UBI::moPAT~GFPm::pinll. After co-cultivation, explants are allowed to recover on normal culture medium, and then are moved onto culture medium containing 3 mg/l bialaphos. After 6-8 weeks, it is expected that GFP+, bialaphos-resistant calli will be observed in the PEI/BBM treatment at a much higher frequency relative to the control treatment (PEI/GUS).

It may be desirable to "kick start" callus growth by transiently expressing the BBM and/or WUS2 polynucleotide products. This can be done by delivering BBM and WUS2 5'-capped polyadenylated RNA, expression cassettes containing BBM and WUS2 DNA, or BBM and/or WUS2 proteins. All of these molecules can be delivered using a biolistics particle gun. For example 5'-capped polyadenylated BBM and/or WUS2 RNA can easily be made in vitro using Ambion's mMMessage mMachine kit. RNA is co-delivered along with DNA containing a polynucleotide of interest and a marker used for selection/screening such as Ubi::moPAT~GFPm::Pinll. It is expected that the cells receiving the RNA will immediately begin dividing more rapidly and a large portion of these will have integrated the agronomic gene. These events can further be validated as being transgenic clonal colonies because they will also express the PAT-GFP fusion protein (and thus will display green fluorescence under appropriate illumination).

Plants regenerated from these embryos can then be screened for the presence of the polynucleotide of interest.
THAT WHICH IS CLAIMED:

1. A guide polynucleotide comprising:

   (i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and,
   
   (ii) a second nucleotide sequence domain that interacts with a Cas endonuclease,

   wherein the first nucleotide sequence domain and the second nucleotide sequence domain are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids.

2. The guide polynucleotide of claim 1, wherein the first nucleotide sequence domain and the second nucleotide sequence domain are located on a single molecule.

3. The guide polynucleotide of claim 1, wherein the second nucleotide sequence domain comprises two separate molecules that are capable of hybridizing along a region of complementarity.

4. The guide polynucleotide of claim 1, wherein the first nucleotide sequence domain is a DNA sequence and the second nucleotide sequence domain is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

5. The guide polynucleotide of claim 1, wherein the first nucleotide sequence domain and the second nucleotide sequence domain are DNA sequences.

6. The guide polynucleotide of claim 1, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain comprises at least one modification, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability
control sequence, a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer molecule, a 5' to 3' covalent linkage, and any combination thereof.

7. The guide polynucleotide of claim 1, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain comprises at least one modification that provides for an additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide poly nucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer molecule, a 5' to 3' covalent linkage, and any combination thereof.

8. The guide polynucleotide of claim 7, wherein the additional beneficial feature is selected from the group consisting of a modified or regulated stability, a subcellular targeting, tracking, a fluorescent label, a binding site for a protein or protein complex, modified binding affinity to complementary target sequence, modified resistance to cellular degradation, and increased cellular permeability.

9. A plant or seed comprising the guide polynucleotide of claim 1.

10. A guide polynucleotide/Cas endonuclease complex wherein the guide polynucleotide comprises:
(i) a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA; and,

(ii) a second nucleotide sequence domain that interacts with a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

11. The guide polynucleotide/Cas endonuclease complex of claim 10, wherein the first nucleotide sequence domain and the second nucleotide sequence domain of the guide polynucleotide are composed of deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or a combination thereof, wherein the guide polynucleotide does not solely comprise ribonucleic acids.

12. The guide polynucleotide/Cas endonuclease complex of claim 10, wherein the first nucleotide sequence domain and/or the second nucleotide sequence domain of said guide polynucleotide comprises at least one modification that provides for an additional beneficial feature, wherein said at least one modification is selected from the group consisting of a 5' cap, a 3' polyadenylated tail, a riboswitch sequence, a stability control sequence; a sequence that forms a dsRNA duplex, a modification or sequence that targets the guide polynucleotide to a subcellular location, a modification or sequence that provides for tracking, a modification or sequence that provides a binding site for proteins, a Locked Nucleic Acid (LNA), a 5-methyl dC nucleotide, a 2,6-Diaminopurine nucleotide, a 2'-Fluoro A nucleotide, a 2'-Fluoro U nucleotide; a 2'-O-Methyl RNA nucleotide, a phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 molecule, a 5' to 3' covalent linkage, and any combination thereof.

13. The guide polynucleotide/Cas endonuclease complex of claim 10, wherein the Cas endonuclease is a Cas9 endonuclease.
14. A plant or seed comprising the guide polynucleotide/Cas endonuclease complex of claims 10.

15. A method for modifying a target site in the genome of a cell, the method comprising providing a guide polynucleotide to a cell having a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

16. A method for modifying a target site in the genome of a cell, the method comprising providing a guide polynucleotide and a Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site.

17. The method of claim 16, further comprising providing a donor DNA to said cell, wherein said donor DNA comprises a polynucleotide of interest.

18. The method of any one of claims 16, further comprising identifying at least one cell that has a modification at said target, wherein the modification at said target site is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

19. A method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising:

a) providing a guide polynucleotide, a donor DNA and a Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site;
b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and,
c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.

20. The method of claim 19, wherein the donor DNA and Cas endonuclease are introduced into said cell using at least one recombinant DNA construct capable of expressing the donor DNA and/or the Cas endonuclease.

21. The method of claims 16, wherein the guide polynucleotide is provided directly by particle bombardment.

22. The method of claims 16, wherein the guide polynucleotide is provided via particle bombardment or *Agrobacterium* transformation of a recombinant DNA construct comprising a U6 polymerase III promoter.

23. The method of claims 16, wherein the guide polynucleotide is a single guide polynucleotide comprising a variable targeting domain and a cas endonuclease recognition domain.

24. The method of claims 16, wherein the guide polynucleotide is a duplex guide polynucleotide comprising a crNucleotide molecule and a tracrNucleotide molecule.

25. A method for modifying a target site in the genome of a cell, the method comprising:
a) providing to a cell a crNucleotide, a first recombinant DNA construct capable of expressing a tracrRNA, and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said crNucleotide is a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said crNucleotide, said tracrRNA and said Cas endonuclease are capable of forming a complex that enables
the Cas endonuclease to introduce a double strand break at said target site; and,

b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

26. A method for modifying a target site in the genome of a cell, the method comprising:

   a) providing to a cell a tracrNucleotide, a first recombinant DNA construct capable of expressing a crRNA and a second recombinant DNA capable of expressing a Cas endonuclease, wherein said tracrNucleotide is selected a deoxyribonucleotide sequence or a combination of a deoxyribonucleotide and ribonucleotide sequence, wherein said tracrNucleotide, said crRNA and said Cas endonuclease are capable of forming a complex that enables the Cas endonuclease to introduce a double strand break at said target site; and,

   b) identifying at least one cell that has a modification at said target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

27. A method for introducing a polynucleotide of interest into a target site in the genome of a cell, the method comprising:

   a) providing to a cell a first recombinant DNA construct capable of expressing a guide polynucleotide, and a second recombinant DNA construct capable of expressing a Cas endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide and Cas endonuclease are capable of forming a
complex that enables the Cas endonuclease to introduce a double strand break at said target site;

b) contacting the cell of (a) with a donor DNA comprising a polynucleotide of interest; and,

c) identifying at least one cell from (b) comprising in its genome the polynucleotide of interest integrated at said target site.

28. A method for editing a nucleotide sequence in the genome of a cell, the method comprising introducing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease into a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease introduces a double-strand break at a target site in the genome of said cell, wherein said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence.

29. The method of claim 16, wherein the cell is selected from the group consisting of a non-human animal, bacterial, fungal, insect, yeast, and a plant cell.

30. The method of claim 29, wherein the plant cell is selected from the group consisting of a monocot and dicot cell.

31. The method of claim 29, wherein the plant cell is selected from the group consisting of maize, rice, sorghum, rye, barley, wheat, millet, oats, sugarcane, turfgrass, or switchgrass, soybean, canola, alfalfa, sunflower, cotton, tobacco, peanut, potato, tobacco, Arabidopsis, and safflower cell.

32. A plant or seed comprising a guide polynucleotide and a Cas9 endonuclease, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said Cas9 endonuclease and guide polynucleotide are capable of forming a complex and creating a double strand break in a genomic target site of said plant.
33. A plant or seed comprising a recombinant DNA construct and a guide polynucleotide, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein said recombinant DNA construct comprises a promoter operably linked to a nucleotide sequence encoding a plant optimized Cas endonuclease, wherein said plant optimized Cas endonuclease and guide polynucleotide are capable of forming a complex and creating a double strand break in a genomic target site of said plant.

34. The plant of claim 33, further comprising a polynucleotide of interest integrated into said genomic target site of said plant.

35. The plant or seed of claim 33 further comprising a modification at said genomic target site, wherein the modification is selected from the group consisting of (i) a replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, and (iv) any combination of (i) - (iii).

36. A plant or seed comprising at least one altered target sequence, wherein the at least one altered target sequence originated from a corresponding target sequence that was recognized and cleaved by a guide polynucleotide /Cas endonuclease complex, wherein the Cas endonuclease is capable of introducing a double-strand break at said target site in the plant genome, wherein said guide polynucleotide does not solely comprise ribonucleic acids.

37. A plant or seed comprising a modified nucleotide sequence, wherein the modified nucleotide sequence was produced by providing a guide polynucleotide, a polynucleotide modification template and at least one Cas endonuclease to a cell, wherein said guide polynucleotide does not solely comprise ribonucleic acids, wherein the Cas endonuclease is capable of introducing a double-strand break at a target site in the plant genome, wherein
said polynucleotide modification template comprises at least one nucleotide modification of said nucleotide sequence.

38. The plant or plant cell of claim 29 wherein the at least one nucleotide modification is not a modification at said target site.

39. The plant of claim 32, wherein the plant is a monocot or a dicot.

40. The plant of claim 39, wherein the monocot is selected from the group consisting of maize, rice, sorghum, rye, barley, wheat, millet, oats, sugarcane, turfgrass, or switchgrass.

41. The plant of claim 39, wherein the dicot is selected from the group consisting of soybean, canola, alfalfa, sunflower, cotton, tobacco, peanut, potato, tobacco, Arabidopsis, or safflower.

42. A method for selecting a plant comprising an altered target site in its plant genome, the method comprising: a) obtaining a first plant comprising at least one Cas endonuclease capable of introducing a double strand break at a target site in the plant genome; b) obtaining a second plant comprising a guide polynucleotide that is capable of forming a complex with the Cas endonuclease of (a), wherein the guide polynucleotide does not solely comprise ribonucleic acids, c) crossing the first plant of (a) with the second plant of (b); d) evaluating the progeny of (c) for an alteration in the target site and e) selecting a progeny plant that possesses the desired alteration of said target site.

43. A method for selecting a plant comprising an altered target site in its plant genome, the method comprising: a) obtaining a first plant comprising at least one Cas endonuclease capable of introducing a double strand break at a target site in the plant genome; b) obtaining a second plant comprising a guide polynucleotide and a donor DNA, wherein the guide polynucleotide does not solely comprise ribonucleic acids, wherein said guide polynucleotide is capable
of forming a complex with the Cas endonuclease of (a), wherein said donor DNA comprises a polynucleotide of interest; c) crossing the first plant of (a) with the second plant of (b); d) evaluating the progeny of (c) for an alteration in the target site and e) selecting a progeny plant that comprises the polynucleotide of interest inserted at said target site.
Figure 1A

Duplex Guide Polynucleotide

Cas Endonuclease Recognition (CER) Domain
Variable Targeting (VT) Domain

5' crNucleotide
5' tracrNucleotide

3'
FIGURE 1B

Single Guide Polynucleotide

Cas Endonuclease Recognition (CER) Domain  Variable Targeting (VT) Domain

3' → 5'  \{ \text{crNucleotide} \}  \{ \text{tracrNucleotide} \}
Maize codon optimized Streptococcus pyogenes Cas9 (N & C Terminal NLSs)

SV40 NLS

ST-L51 intron

Ubi Pro

Maize U6 PolIII snRNA promoter driving crRNA

Maize U6 PolIII Pro (Chr8)

Maize U6 PolIII Terminator

KrRNA

Maize U6 PolIII snRNA promoter driving tracrRNA

Maize U6 PolIII Pro (Chr8)

Maize U6 PolIII Terminator

tracrRNA
FIGURE 3A

Cas Endonuclease Recognition (CER) Domain

Region Complementing the DNA Target (Variable Targeting (VT) Domain)

crRNA

tracrRNA

5' GATATATATA C
3' CTATATATAT

PAM

Cas9 Endonuclease

5' GATATATATA C
3' CTATATATAT

G A

GTGTGCGATGCGCATGCG CAT

GTA TATATACGTG 3'
ATATATGCAC 5'
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FIGURE 7

Guide Polynucleotide/Cas Endonuclease
Target Site

ADE → URA3 → DE2

Sequence Duplication
Sequence Duplication
A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/113 C12N15/115 C07K14/315 C12N15/82

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

B. FIELD SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>M. JINEK ET AL: &quot;A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacteriaal Immunity&quot;</td>
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<td>SCI ENCE, vol. 337, no. 6096, 17 August 2012 (2012-08-17), pages 816-821, XP05567740, ISSN: 0036-8075, DOI: 10.1126/science.1225829</td>
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* Special categories of cited documents:

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

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

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

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

Date of the actual completion of the international search

26 November 2014

Date of mailing of the international search report

09/12/2014

Name and mailing address of the ISA/Authorized officer

European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel.: (+31-70) 340-2040, Fax: (+31-70) 340-3016

Bilang, Jurg
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<td>PRASHANT MALI ET AL: &quot;CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering&quot; , NATURE BIOTECHNOLOGY, vol. 31, no. 9, 1 August 2013 (2013-08-01) , pages 833-838, XP055149275 , ISSN: 1087-0156, DOI: 10.1038/nbt.2675</td>
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<td>ZHENGYAN FENG ET AL: &quot;Efficient genome editing in plants using a CRISPR/Cas system&quot; , CELL RESEARCH, vol. 23, no. 10, 20 August 2013 (2013-08-20) , pages 1229-1232, XP055153531, ISSN: 1001-0602, DOI: 10.1038/cr.2013.114</td>
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