protoplasts. The method requires transformation of protoplasts with T-MLO plus *GFP* donor plasmids (promoter-less *GFP* coding sequence and CaMV 35S terminator, flanked by T-MLO recognition sites).

[0004] Zhang et al., *Nat. Commun.*, **7***:*12617 (2016), describes a method of introducing a targeted indel into a locus within the wheat genome. The method requires the use of plasmids encoding or expressing CRISPR/Cas9 RNA and guide RNA sequences and the insertion of those sequences into wheat callus cells.

[0005] Liang, Z. et al., *Nat. Commun*., **8**:14261 (2017) describes a method for introducing targeted indels into homologs of a wheat gene (*TaGW2*). The method requires the delivery of Cas9/sgRNA RNPs into embryonic wheat calli using particle bombardment or PEG-mediated transformation.

[0006] PCT application WO2016007948, published January 14, 2016, and titled "Agronomic trait modification using guide RNA/cas endonuclease systems and methods of use," describes methods for introducing targeted modifications to the maize genome. The methods require homologous recombination and/or the transformation of maize embryos with plasmids, and/or the use of selectable markers.

SUMMARY

[0007] Disclosed herein are methods for providing novel plant cells or plant protoplasts, plant callus, tissues or parts, whole plants, and seeds having one or more altered genetic sequences. Among other features, the methods and compositions described herein enable the stacking of preferred alleles without introducing unwanted genetic or epigenetic variation in the modified plants or plant cells. The efficiency and reliability of these targeted modification methods are significantly improved relative to traditional plant breeding, and can be used not only to augment traditional breeding techniques but also as a substitute for them.

[0008] Disclosed herein are methods for providing novel plant cells or plant protoplasts, plant callus, tissues or parts, whole plants, and seeds having one or more altered genetic sequences.

[0008a] In one aspect, the present disclosure refers to a method of modifying a diploid, tetraploid, or hexaploid plant cell by creating a plurality of targeted insertions in the genome of the plant cell, comprising: contacting the genome with one or more effector molecules comprising a site-specific double stranded break (DSB)-inducing agent which introduces a DSB at two or more predetermined target sites within the genome of the plant cell and one or more single-stranded DNA polynucleotide donor molecules, wherein each of at least two of the targeted insertions comprises integration of a predetermined sequence encoded by the single-stranded DNA polynucleotide donor molecule into the genome at the position of at least one DSB or between two DSBs, wherein at least one of the polynucleotide donor molecules lacks homology to the genome sequences adjacent to the site of insertion at the DSB(s), thereby resulting in a modified plant cell;

wherein the plurality of targeted insertions occurs without an intervening step of separately identifying an individual insertion and without a step of separately selecting for the occurrence of

an individual insertion among the plurality of targeted insertions; and wherein the targeted insertions alter at least one trait of the modified plant cell, or at least one trait of a plant comprising the modified plant cell, or at least one trait of a plant grown from the modified plant cell, or result in a detectable phenotype in the modified plant cell.

[0008b] In another example, the present disclosure refers to a modified plant or plant part derived from the modified plant cell resulting from the method as disclosed herein, or a seed of the modified plant.

[0008c] In another example, the present disclosure refers to a method of manufacturing a plant comprising a modified cell, wherein the modified cell is generated by the method as disclosed herein.

[0008d] In another example, there is provided a method of manufacturing a plant comprising growing a plant from a modified plant cell, wherein the modified plant cell is generated by the method as disclosed herein.

[0008e] In another example, the present disclosure refers to a modified diploid, tetraploid, or hexaploid plant cell, wherein the modified plant cell comprises at least 2 separately targeted modifications in its genome, wherein at least two of the targeted modifications are insertions of predetermined sequences encoded by one or more polynucleotide donor molecules, wherein at least one of the polynucleotide donor molecules lacks homology to the genome sequences adjacent to the site of insertion, wherein the targeted modifications are determined relative to an original plant cell, and wherein the modified plant cell is genetically identical to the original plant cell with the exception of the targeted modifications and any changes as a consequence of multiplying the modified plant cell.

[0008f] In another example, there present disclosure refers to a modified plant regenerated or grown from the modified plant cell as disclosed herein, comprising at least some cells that comprise in their genome the at least 2 separately targeted modifications and that are genetically identical to the original plant cell with the exception of the targeted modifications and any changes as a consequence of regenerating or growing the modified plant from the modified plant cell as disclosed herein, and – optionally – further propagating the modified plant.

[0009] In one example, the disclosure provides a method of changing expression of a sequence of interest in a genome, including integrating a sequence encoded by a polynucleotide, such as double-stranded or single-stranded polynucleotides including DNA, RNA, or a combination of DNA and RNA, at the site of at least one double-strand break (DSB) in a genome, which can be the genome of a eukaryotic nucleus (*e. g.*, the nuclear genome of a plant cell) or a genome of an organelle (*e. g.*, a mitochondrion or a plastid in a plant cell). Effector molecules (or targeting agents) for site-specific introduction of a DSB into a genome include various endonucleases (*e. g.*, RNA-guided nucleases such as a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, or a C2c3) and guide RNAs that direct cleavage by an RNA-guided nuclease. Examples include those where the DSB is introduced into a genome by a

sequence of interest. In examples, the two or more loci in a genome are in different genes or sequences of interest. In examples, the two or more loci are alleles of a given sequence of interest; when all alleles of a given gene or sequence of interest are modified in the same way, the result is homozygous modification of the gene. For example, examples of the method enable targeted modification of both alleles of a gene in a diploid (2n ploidy, where n = 1) plant, or targeted modification of all three alleles in a triploid (2n ploidy, where n = 1.5) plant, or targeted modification of all six alleles of a gene in a hexaploid (2n ploidy, where n =3) plant.

[0012] In another example, the disclosure provides a polynucleotide for disrupting gene expression, wherein the polynucleotide is double-stranded and includes at least 18 contiguous base-pairs and encoding at least one stop codon in each possible reading frame on each strand, or is single-stranded and includes at least 11 contiguous nucleotides; and wherein the polynucleotide encodes at least one stop codon in each possible reading frame on each strand. In examples, the polynucleotide is a double-stranded DNA (dsDNA) or a double-stranded DNA/RNA hybrid molecule including at least 18 contiguous base-pairs and encoding at least one stop codon in each possible reading frame on either strand. In examples, the polynucleotide is a single-stranded DNA or a single-stranded DNA/RNA hybrid molecule including at least 11 contiguous nucleotides and encoding at least one stop codon in each possible reading frame on the strand. Such a polynucleotide is especially useful in methods disclosed herein, wherein, when a sequence encoded by the polynucleotide is integrated or inserted into a genome at the site of a DSB in a sequence of interest (such as a protein-coding gene), the sequence of the heterologously inserted polynucleotide serves to stop translation of the transcript containing the sequence of interest and the heterologously inserted polynucleotide sequence. Examples of the polynucleotide include those wherein the polynucleotide includes one or more chemical modifications or labels, *e. g.*, at least one phosphorothioate modification.

[0013] In another example, the disclosure provides a method of identifying the locus of at least one double-stranded break (DSB) in genomic DNA in a cell (such as a plant cell) including the genomic DNA, wherein the method includes the steps of: (a) contacting the genomic DNA having a DSB with a polynucleotide (such as a double-stranded DNA, a single-stranded DNA, a single-stranded DNA/RNA hybrid, or a double-stranded DNA/RNA hybrid) donor molecule, wherein the polynucleotide donor molecule is capable of being integrated (or having its sequence integrated) at the DSB (preferably by non-homologous end-joining (NHEJ)) and has a length of between about 18 to about 300 base-pairs (or nucleotides, if single-stranded), or between about 30 to about 100 base-pairs (or nucleotides, if single-stranded); wherein a sequence encoded by the polynucleotide donor molecule, if integrated at the DSB, forms a heterologous insertion; and (b) using at least part of the sequence of the polynucleotide molecule as a target for PCR primers to allow amplification of DNA in the locus of the DSB. In a related example, the disclosure provides a method of identifying the locus of double-stranded breaks (DSBs) in genomic DNA in a pool of cells (such as

CLAIMS:

1. A method of modifying a diploid, tetraploid, or hexaploid plant cell by creating a plurality of targeted insertions in the genome of the plant cell, comprising:

contacting the genome with one or more effector molecules comprising a site-specific double stranded break (DSB)-inducing agent which introduces a DSB at two or more predetermined target sites within the genome of the plant cell and one or more single-stranded DNA polynucleotide donor molecules, wherein each of at least two of the targeted insertions comprises integration of a predetermined sequence encoded by the single-stranded DNA polynucleotide donor molecule into the genome at the position of at least one DSB or between two DSBs, wherein at least one of the polynucleotide donor molecules lacks homology to the genome sequences adjacent to the site of insertion at the DSB(s), thereby resulting in a modified plant cell;

wherein the plurality of targeted insertions occurs without an intervening step of separately identifying an individual insertion and without a step of separately selecting for the occurrence of an individual insertion among the plurality of targeted insertions;

and wherein the targeted insertions alter at least one trait of the modified plant cell, or at least one trait of a plant comprising the modified plant cell, or at least one trait of a plant grown from the modified plant cell, or result in a detectable phenotype in the modified plant cell.

2. The method of claim 1, wherein the insertions are between 10 and 150 nucleotides in length.

3. The method of claim 1 or 2, wherein the insertions comprise or create at least one transcription factor binding site.

4. The method of claim 1 or 2, wherein the targeted insertions independently up- or down-regulate the expression of two or more distinct genes.

5. The method of claim 1, wherein the method results in a non-transgenic plant cell containing homozygous edits, without an intervening chromosome segregation event.

6. The method of claim 1 or 2, wherein repetition of the method results in an efficiency of at least 1%, wherein said efficiency is determined by dividing the number of successfully targeted cells by the total number of cells targeted.

7. The method of claim 1 or 2, wherein the donor polynucleotide is tethered to a crRNA by a covalent bond, a non-covalent bond, or a combination of covalent and non-covalent bonds.

8. The method of claim 1 or 2, wherein the site-specific double stranded break (DSB)-inducing agent is selected from the group consisting of:

(a) a nuclease selected from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TAL-effector nuclease), an Argonaute, a meganuclease, an engineered meganuclease;

(b) a polynucleotide encoding one or more RNA-guided nucleases; and

(c) a guide RNA (gRNA) for an RNA-guided nuclease, or a polynucleotide encoding a gRNA for an RNA-guided nuclease.

9. The method of claim 1 or 2, wherein the one or more effector molecules are introduced into the genome by at least one treatment selected from the group consisting of:

(a) bacterially mediated transfection;

(b) biolistics or particle bombardment;

(c) treatment with at least one chemical, enzymatic, or physical agent; and

(d) application of heat or cold, ultrasonication, centrifugation, positive or negative pressure, cell wall or membrane disruption or deformation, or electroporation.

10. The method of claim 1 or 2, wherein each of the predetermined sequences encoded by a polynucleotide donor molecule is integrated in the genome: (a) within a sequence of interest, (b) upstream of a sequence of interest, or (c) downstream of a sequence of interest.

11. The method of claim 1 or 2, wherein the predetermined sequence encoded by a polynucleotide donor molecule, when integrated into the genome, is functionally or operably linked to the sequence of interest.

12. The method of claim 11, wherein the polynucleotide donor molecule comprises at least one of the nucleotide sequences selected from the group consisting of:

(a) DNA or RNA encoding at least one stop codon, or at least one stop codon on each strand, or at least one stop codon within each reading frame on each strand;

(b) DNA or RNA encoding heterologous primer sequence;

(c) DNA or RNA encoding a unique identifier sequence;

(d) DNA or RNA encoding a transcript-stabilizing sequence;

(e) DNA or RNA encoding a transcript-destabilizing sequence;

(f) a DNA or RNA aptamer, or DNA encoding an RNA aptamer, or DNA or RNA encoding an amino acid aptamer; and

(g) DNA or RNA encoding a sequence recognizable by a specific binding agent.

13. The method of claim 1 or 2, further comprising obtaining a modified plant from the modified plant cell.

14. The method of claim 13, wherein the genome of the modified plant is identical to that of the original plant cell but for (i) the targeted insertions, (ii) mutations arising naturally during mitotic propagation, and optionally, (iii) any off-target mutations.