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(54) **Title:** METHODS AND COMPOSITIONS FOR CO-DELIVERY OF T-DNAS EXPRESSING MULTIPLE GUIDE POLYNUCLEOTIDES INTO PLANTS

(57) **Abstract:** The present disclosure relates to methods and compositions for co-delivery of multiple T-DNAs expressing multiple guide polynucleotides to a plant cell. In one aspect the optical density of Agrobacteria for transformation is from about 0.8 to about 2.0. In a further aspect U6 promoters having at least 95% sequence identity to Seq. ID Nos: 4-7,86-91, 96-109 and 113-121 are used.

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# METHODS AND COMPOSITIONS FOR CO-DELIVERY OF T-DNAS EXPRESSING MULTIPLE GUIDE POLYNUCLEOTIDES INTO PLANTS

## CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to US provisional patent application no. 63/386,240, filed December 6, 2022, and US provisional application no. 63/595,496, filed November 2, 2023, both of which are incorporated by reference herein in their entireties.

## REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] The official copy of the sequence listing is submitted electronically as an XML formatted sequence listing with a file named "108430-WO-SEC-1_Sequence_Listing_ST26" created on December 4, 2023 and having a size of 113 kilobytes and is filed concurrently with the specification. The sequence listing comprised in this XML formatted document is part of the specification and is herein incorporated by reference in its entirety.

## FIELD OF THE INVENTION

[0003] The disclosure relates to the field of plant molecular biology, in particular, to compositions and methods for altering the genome of a cell.

## BACKGROUND

[0004] Polygenic traits like yield involve the coordinated effect of tens, hundreds, or even thousands of genes. To improve these traits using multiplex genome editing, approaches that enable simultaneous delivery of tens, hundreds, or thousands of guide polynucleotides are needed.

[0005] The present disclosure relates to methods and compositions for co-delivery of guide polynucleotides into a plant cell.

## SUMMARY OF DISCLOSURE

[0006] In a first aspect, the disclosure provides a method of delivering guide polynucleotides to a plant cell, the method comprising: transforming the plant cell with a first T-DNA, the first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the plant cell; and transforming the plant cell with a second T-

DNA, the second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell, wherein the optical density of the first and second T-DNAs ranges from about 0.2 to about 3.0.

[0007] In some aspects of the method, the first and second T-DNAs are *Agrobacterium.*

[0008] In some aspects of the method, the first and second T-DNAs are *Ochrobacterium*.

[0009] In some aspects of the method, the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.

[0010] In some aspects of the method, the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the first and second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0011] In some aspects of the method, the method further comprises pre-treating the plant cell prior to transforming with the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell for about 2 minutes to about 10 minutes at a temperature above about 28°C. In some aspects of the method, the pre-treatment temperature ranges from about 28°C to about 50°C.

[0012] In some aspects of the method, the method further comprises pre-treating the plant cell prior to transforming with the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell at about 45°C for about 5 minutes.

[0013] In some aspects of the method, the method further comprises inhibiting an immune response in the plant cell. In some aspects of the method, inhibiting cyclic adenine diphosphate ribose, or a variant or isomer thereof, in the plant cell. In some aspects of the method, inhibiting cyclic adenine diphosphate ribose, or the variant or isomer thereof, comprises providing to the plant cell a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide. In some aspects of the method, the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell prior to transforming the plant cell with the first and second

T-DNAs. In some aspects of the method, the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell simultaneously with the first and second T-DNAs.

[0014] In some aspects of the method, the method further comprises transforming the plant cell with a third T-DNA, the third T-DNA expressing a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0015] In some aspects of the method, the method further comprises transforming the plant cell with a fourth T-DNA, the fourth T-DNA expressing a fourth plurality of guide polynucleotides for multiplexed genome editing of a fourth set of target nucleotide sequences in the plant cell, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0016] In some aspects of the method, the method further comprises transforming the plant cell with a fifth T-DNA, the fifth T-DNA expressing a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide

polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0017] In some aspects of the method, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0018] In some aspects of the method, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0019] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.

[0020] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.

[0021] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.

[0022] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.

[0023] In some aspects of the method, each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.

[0024] In some aspects of the method, each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.

[0025] In some aspects of the method, each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0026] In some aspects of the method, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (d) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0027] In some aspects of the method, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos:

4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (e) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0028] In some aspects of the method, each guide polynucleotide is expressed by a separate promoter. For example, the separate promoter of each guide polynucleotide is a U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0029] In some aspects of the method, the method further comprises providing one or more developmental genes to the plant cell. In some aspects of the method, the one or more developmental genes is BBM and/or WUS.

[0030] In some aspects of the method, the first *Agrobacterium* T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.

[0031] In some aspects of the method, the second *Agrobacterium* T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.

[0032] In some aspects of the method, the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0033] In some aspects of the method, the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0034] In another aspect, the disclosure provides a composition for delivering guide polynucleotides for multiplexed genome editing in a plant cell, the composition comprising: (a) a first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of

the first plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; (b) a second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; and (c) optionally an inhibitor of cyclic adenine diphosphate ribose, or a variant or isomer thereof.

[0035] In some aspects of the composition, the inhibitor is a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide.

[0036] In some aspects of the composition, the first and second T-DNAs are *Agrobacterium.*

[0037] In some aspects of the composition, the first and second T-DNAs are *Ochrobacterium.*

[0038] In some aspects of the composition, the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.

[0039] In some aspects of the composition, the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides.

[0040] In some aspects of the composition, the composition further comprises a third T-DNA comprising a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0041] In some aspects of the composition, the composition further comprises a fourth T-DNA comprising a fourth plurality of guide polynucleotides for multiplexed genome editing of a fourth set of target nucleotide sequences in the plant cell, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0042] In some aspects of the composition, the composition further comprises a fifth T-DNA comprising a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0043] In some aspects of the composition, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0044] In some aspects of the composition, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide

polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0045] In some aspects of the composition, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.

[0046] In some aspects of the composition, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.

[0047] In some aspects of the composition, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.

[0048] In some aspects of the composition, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.

[0049] In some aspects of the composition, each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.

[0050] In some aspects of the composition, each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.

[0051] In some aspects of the composition, each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0052] In some aspects of the composition, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (d) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0053] In some aspects of the composition, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (e) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0054] In some aspects of the composition, each guide polynucleotide is expressed by a separate promoter. For example, the separate promoter of each guide polynucleotide is a U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0055] In some aspects of the composition, the composition further comprises a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide.

[0056] In some aspects of the composition, the Cas polypeptide has endonuclease activity. In some aspects of the composition, the composition further comprises a donor DNA or a polynucleotide modification template. In some aspects of the composition, the Cas polypeptide is Cas12f or Cas9.

[0057] In some aspects of the composition, the Cas polypeptide comprises a deactivated Cas endonuclease (dCas). In some aspects of the composition, the dCas is dCas12f or dCas9. In some aspects of the composition, the dCas endonuclease is operably associated with a deaminase. In some aspects of the composition, the deaminase is a cytosine deaminase or an adenine deaminase.

[0058] In some aspects of the composition, the Cas polypeptide has nickase activity and is operably associated with or co-expressed with a reverse transcriptase.

[0059] In some aspects of the composition, the composition further comprises an expression cassette comprising one or more developmental genes. In some aspects of the composition, the one or more developmental genes is BBM and/or WUS.

[0060] In some aspects of the method, the expression cassette comprising the one or more developmental genes is expressed by the first T-DNA and/or the second T-DNA.

[0061] In some aspects of the composition, the first T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.

[0062] In some aspects of the composition, the second T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.

[0063] In some aspects of the composition, the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0064] In some aspects of the composition, the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0065] In yet another aspect, the disclosure provides a method for editing a plant genome at a plurality of target sites in the genome, the method comprising: providing a plant cell with: (a) a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide; (b) a first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the first

plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; and (c) a second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence, wherein the optical density of the first and second T-DNAs ranges from about 0.2 to about 3.0; introducing site-specific modifications in the first set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the first plurality of guide polynucleotides; and introducing site-specific modifications in the second set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the second plurality of guide polynucleotides.

[0066] In some aspects of the method, the first and second T-DNAs are *Agrobacterium.*

[0067] In some aspects of the method, the first and second T-DNAs are *Ochrobacterium*.

[0068] In some aspects of the method, the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.

[0069] In some aspects of the method, the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides.

[0070] In some aspects of the method, the method further comprises pre-treating the plant cell prior to providing the Cas polypeptide and the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell for about 2 minutes to about 10 minutes at a temperature above about 28°C. In some aspects of the method, the method of claim 32, wherein the pre-treatment temperature ranges from about 28°C to about 50°C.

[0071] In some aspects of the method, the method further comprises pre-treating the plant cell prior to providing the Cas polypeptide and the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell at about 45°C for about 5 minutes.

[0072] In some aspects of the method, the method further comprises inhibiting an immune response in the plant cell. In some aspects of the method, the method further comprises inhibiting cyclic adenine diphosphate ribose, or a variant or isomer thereof, in the plant cell. In some aspects of the method, inhibiting cyclic adenine diphosphate ribose, or the variant or isomer thereof, comprises providing to the plant cell a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide. In some aspects of the method, the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell simultaneously with the first and second T-DNAs.

[0073] In some aspects of the method, the method further comprises providing the plant cell with a third T-DNA, the third T-DNA expressing a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the third set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the third plurality of guide polynucleotides, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0074] In some aspects of the method, the method further comprises providing the plant cell with a fourth T-DNA, the fourth T-DNA expressing a fourth plurality of guide polynucleotides for multiplexed genome editing of a fourth set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the fourth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fourth plurality of guide polynucleotides, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at

least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0075] In some aspects of the method, the method further comprises providing the plant cell with a fifth T-DNA, the fifth T-DNA expressing a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the fifth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fifth plurality of guide polynucleotides, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

[0076] In some aspects of the method, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0077] In some aspects of the method, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0078]

[0079] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.

[0080] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.

[0081] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.

[0082] In some aspects of the method, each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.

[0083] In some aspects of the method, each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.

[0084] In some aspects of the method, each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.

[0085] In some aspects of the method, each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0086] In some aspects of the method, the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ

ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (d) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0087] In some aspects of the method, the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises: (a) a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (b) a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (c) a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; (d) a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and (e) a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91, wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

[0088] In some aspects of the method, each guide polynucleotide is expressed by a separate promoter. For example, the separate promoter of each guide polynucleotide is a U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; or (e) at least 95% sequence identity to SEQ ID Nos: 113-121.

[0089] In some aspects of the method, the Cas polypeptide has endonuclease activity. In some aspects of the method, the method further comprises providing the plant cell with a donor DNA or a polynucleotide modification template. In some aspects of the method, the Cas polypeptide is Cas12f or Cas9.

[0090] In some aspects of the method, the Cas polypeptide comprises a deactivated Cas endonuclease (dCas). In some aspects of the method, the dCas is dCas12f or dCas9. In some

aspects of the method, the dCas endonuclease is operably associated with a deaminase. In some aspects of the method, the deaminase is a cytosine deaminase or an adenine deaminase.

[0091] In some aspects of the method, the method further comprises providing one or more developmental genes to the plant cell. In some aspects of the method, the one or more developmental genes is BBM and/or WUS.

[0092] In some aspects of the method, the first T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.

[0093] In some aspects of the method, the second T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.

[0094] In some aspects of the method, the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0095] In some aspects of the method, the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

[0096] In some aspects of the method, introducing site-specific modifications in the first, second, third, fourth, and/or fifth target nucleotide sequences in the plant cell comprises bi-allelic site-specific modification of the target nucleotide sequences.

[0097] In yet a further aspect, the disclosure provides a recombinant DNA construct comprising a U6 promoter, wherein the U6 promoter comprises: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; (c) at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91; (d) at least 95% sequence identity to SEQ ID Nos: 96-109; (e) at least 95% sequence identity to SEQ ID Nos: 113-121; (f) a polynucleotide sequence of SEQ ID Nos: 4-7 or 86-91; (g) a polynucleotide sequence of SEQ ID Nos: 96-109; or (h) a polynucleotide sequence of SEQ ID Nos: 113-121. The recombinant DNA construct can further comprise a guide polynucleotide operably linked to the U6 promoter.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0098] 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.

[0099] **FIGS. 1A – 1B** are alignments of *Zea mays*-derived U6 promoters i) SEQ ID NO: 85; ii) SEQ ID NO: 3; iii) SEQ ID NO: 4; iv) SEQ ID NO: 86; v) SEQ ID NO: 5; vi) SEQ ID NO: 87; vii) SEQ ID NO: 88; viii) SEQ ID NO: 6; ix) SEQ ID NO: 89; x) SEQ ID NO: 7; xi) SEQ ID NO: 90; and xii) SEQ ID NO: 91. Conserved regions included Motif 1 (SEQ ID NO: 92); Motif 2 (SEQ ID NO: 93); and Motif 3 (SEQ ID NO: 94). N = A, C, G, or T; B = C, G, or T; D = A, G, or T; V = A, C, or G; H = A, C, or T; Y = C or T; M = C or A; R = A or G; and S = C or G. Gaps in alignment are indicated by "-". Conserved base pairs are indicated by "*". Positions having only two base pairs are indicated by ":".

[0100] **FIGS. 2A – 2E** are alignments of *Glycine max*- derived U6 promoters i) SEQ ID NO: 95; ii) SEQ ID NO: 96; iii) SEQ ID NO: 97; iv) SEQ ID NO: 98; v) SEQ ID NO: 99; vi) SEQ ID NO: 100; vii) SEQ ID NO: 101; viii) SEQ ID NO: 102; ix) SEQ ID NO: 103; x) SEQ ID NO: 104; xi) SEQ ID NO: 105; xii) SEQ ID NO: 106; xiii) SEQ ID NO: 107; xiv) SEQ ID NO: 108; and xv) SEQ ID NO: 109. Conserved regions included Motif 1 (SEQ ID NO: 110); Motif 2 (SEQ ID NO: 111); and Motif 3 (SEQ ID NO: 112). N = A, C, G, or T; B = C, G, or T; D = A, G, or T; V = A, C, or G; H = A, C, or T; Y = C or T; M = C or A; R = A or G; and S = C or G. Gaps in alignment are indicated by "-". Conserved base pairs are indicated by "*". Positions having only two base pairs are indicated by ":".

[0101] **FIG. 3** illustrates a T-DNA containing multiple sgRNA expression cassettes according to Example 1.

[0102] **FIG. 4** illustrates a method for delivering multiple T-DNAs to a plant cell.

[0103] **FIG. 5** illustrates a method for delivering multiple T-DNAs to a plant cell for multiplex genome modification.

[0104] **FIG. 6** is a graph illustrating the number of sgRNA expression cassettes in T0 plants according to Example 2.

[0105] **FIG. 7** is a graph illustrating the number of sites with targeted modification in T0 plants according to Example 2.

[0106] **FIG. 8** is a graph illustrating the percent of targeted modification in a T0 plant according to Example 2.

[0107] 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.

[0108] **SEQ ID NO: 1** is a Zea mays U6 promoter 1 DNA sequence.

[0109] **SEQ ID NO: 2** is a SpyCas9 PRT sequence from Streptococcus pyogenes.

[0110] **SEQ ID NO: 3** is a DNA sequence for truncated Zea mays U6 promoter 1.

[0111] **SEQ ID NO: 4** is a DNA sequence for truncated Zea mays U6 promoter 2.

[0112] **SEQ ID NO: 5** is a DNA sequence for truncated Zea mays U6 promoter 3.

[0113] **SEQ ID NO: 6** is a DNA sequence for truncated Zea mays U6 promoter 4.

[0114] **SEQ ID NO: 7** is a DNA sequence for truncated Zea mays U6 promoter 5 DNA.

[0115] **SEQ ID NO: 8** is an artificial RNA sequence of a single guide RNA backbone engineered from a SpyCas9 CRISPR system.

[0116] **SEQ ID NO: 9** is an artificial RNA sequence of a single guide RNA backbone engineered from a SmaCas9 CRISPR system.

[0117] **SEQ ID NO: 10** is an artificial RNA sequence of a single guide RNA backbone engineered from a SorCas9 CRISPR system.

[0118] **SEQ ID NO: 11** is an artificial RNA sequence of a single guide RNA backbone engineered from a DpiCas9 CRISPR system.

[0119] **SEQ ID NO: 12** is an artificial RNA sequence of a single guide RNA backbone engineered from a FhoCas9 CRISPR system.

[0120] **SEQ ID NO: 13** is an artificial RNA sequence of a single guide RNA backbone engineered from a ScaCas9 CRISPR system.

[0121] **SEQ ID NO: 14** is a Zea mays NAC7 genomic SpyCas9 target site DNA sequence.

[0122] **SEQ ID NO: 15** is a Zea mays MS26 genomic SpyCas9 target site DNA sequence.

[0123] **SEQ ID NO: 16** is a Zea mays MS45 genomic SpyCas9 target site DNA sequence.

[0124] **SEQ ID NO: 17** is a Zea mays TS26 genomic SpyCas9 target site DNA sequence.

[0125] **SEQ ID NO: 18** is a Zea mays WXY genomic SpyCas9 target site DNA sequence.

[0126] **SEQ ID NO: 19** is an artificial RNA sequence of a SpyCas9 single guide targeting the NAC7 site.

[0127] **SEQ ID NO: 20** is an artificial RNA sequence of a SpyCas9 single guide targeting the MS26 site.

[0128] **SEQ ID NO: 21** is an artificial RNA sequence of a SpyCas9 single guide targeting the MS45 site.

[0129] **SEQ ID NO: 22** is an artificial RNA sequence of a SpyCas9 single guide targeting the TS45 site.

[0130] **SEQ ID NO: 23** is an artificial RNA sequence of a SpyCas9 single guide targeting the WXY site.

[0131] **SEQ ID NO: 24** is an artificial RNA sequence of a SmaCas9 single guide RNA targeting the MS26 site.

[0132] **SEQ ID NO: 25** is an artificial RNA sequence of a SorCas9 single guide RNA targeting the MS26 site.

[0133] **SEQ ID NO: 26** is an artificial RNA sequence of a DpiCas9 single guide RNA targeting the MS26 site.

[0134] **SEQ ID NO: 27** is an artificial RNA sequence of a FhoCas9 single guide RNA targeting the MS26 site.

[0135] **SEQ ID NO: 28** is an artificial RNA sequence of a ScaCas9 single guide RNA targeting the MS26 site.

[0136] **SEQ ID NO: 29** is an artificial RNA sequence of a SmaCas9 single guide RNA targeting the WXY site.

[0137] **SEQ ID NO: 30** is an artificial RNA sequence of a SorCas9 single guide RNA targeting the WXY site.

[0138] **SEQ ID NO: 31** is an artificial RNA sequence of a DpiCas9 single guide RNA targeting the WXY site.

[0139] **SEQ ID NO: 32** is an artificial RNA sequence of a FhoCas9 single guide RNA targeting the WXY site.

[0140] **SEQ ID NO: 33** is an artificial RNA sequence of a ScaCas9 single guide RNA targeting the WXY site.

[0141] **SEQ ID NO: 34** is a Tad1 PRT sequence from Clostridium botulinum prophage.

[0142] **SEQ ID NO: 35** is a Tad1 PRT sequence from Clostridioides mangenotii prophage.

[0143] **SEQ ID NO: 36** is a Zea mays U6 terminator DNA sequence.

[0144] **SEQ ID NO: 37 – SEQ ID NO: 84** are DNA sequences of the genomic target sites of FIG. 8.

[0145] **SEQ ID NO: 85** is an artificial DNA sequence for a Zea mays U6 promoter consensus sequence.

[0146] **SEQ ID NO: 86** is a DNA sequence for truncated Zea mays U6 promoter 6.

[0147] **SEQ ID NO: 87** is a DNA sequence for truncated Zea mays U6 promoter 7.

[0148] **SEQ ID NO: 88** is a DNA sequence for truncated Zea mays U6 promoter 8.

[0149] **SEQ ID NO: 89** is a DNA sequence for truncated Zea mays U6 promoter 9.

[0150] **SEQ ID NO: 90** is a DNA sequence for truncated Zea mays U6 promoter 10.

[0151] **SEQ ID NO: 91** is a DNA sequence for truncated Zea mays U6 promoter 11.

[0152] **SEQ ID NO: 92** is an artificial DNA sequence for Zea mays U6 promoter motif 1.

[0153] **SEQ ID NO: 93** is an artificial DNA sequence for Zea mays U6 promoter motif 2.

[0154] **SEQ ID NO: 94** is an artificial DNA sequence for Zea mays U6 promoter motif 3.

[0155] **SEQ ID NO: 95** is an artificial DNA sequence for a Glycine max U6 promoter consensus sequence.

[0156] **SEQ ID NO: 96** is a DNA sequence for truncated Glycine max U6 promoter 1.

[0157] **SEQ ID NO: 97** is a DNA sequence for truncated Glycine max U6 promoter 2.

[0158] **SEQ ID NO: 98** is a DNA sequence for truncated Glycine max U6 promoter 3.

[0159] **SEQ ID NO: 99** is a DNA sequence for truncated Glycine max U6 promoter 4.

[0160] **SEQ ID NO: 100** is a DNA sequence for truncated Glycine max U6 promoter 5.

[0161] **SEQ ID NO: 101** is a DNA sequence for truncated Glycine max U6 promoter 6.

[0162] **SEQ ID NO: 102** is a DNA sequence for truncated Glycine max U6 promoter 7.

[0163] **SEQ ID NO: 103** is a DNA sequence for truncated Glycine max U6 promoter 8.

[0164] **SEQ ID NO: 104** is a DNA sequence for truncated Glycine max U6 promoter 9.

[0165] **SEQ ID NO: 105** is a DNA sequence for truncated Glycine max U6 promoter 10.

[0166] **SEQ ID NO: 106** is a DNA sequence for truncated Glycine max U6 promoter 11.

[0167] **SEQ ID NO: 107** is a DNA sequence for truncated Glycine max U6 promoter 12.

[0168] **SEQ ID NO: 108** is a DNA sequence for truncated Glycine max U6 promoter 13.

[0169] **SEQ ID NO: 109** is a DNA sequence for truncated Glycine max U6 promoter 14.

[0170] **SEQ ID NO: 110** is an artificial DNA sequence for Glycine max U6 promoter motif 1.

[0171] **SEQ ID NO: 111** is an artificial DNA sequence for Glycine max U6 promoter motif 2.

[0172] **SEQ ID NO: 112** is an artificial DNA sequence for Glycine max U6 promoter motif 3.

[0173] **SEQ ID NO: 113** is a DNA sequence for Medicago truncatula promoter 1.

[0174] **SEQ ID NO: 114** is a DNA sequence for Medicago truncatula promoter 2.

[0175] **SEQ ID NO: 115** is a DNA sequence for Medicago truncatula promoter 3.

[0176] **SEQ ID NO: 116** is a DNA sequence for Medicago truncatula promoter 4.

[0177] **SEQ ID NO: 117** is a DNA sequence for Medicago truncatula promoter 5.

[0178] **SEQ ID NO: 118** is a DNA sequence for Medicago truncatula promoter 6.

[0179] **SEQ ID NO: 119** is a DNA sequence for Medicago truncatula promoter 7.

[0180] **SEQ ID NO: 120** is a DNA sequence for Medicago truncatula promoter 8.

[0181] **SEQ ID NO: 121** is a DNA sequence for Medicago truncatula promoter 9.

## DETAILED DESCRIPTION

[0182] Terms used in the claims and specification are defined as set forth below unless otherwise specified. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[0183] The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "d" means day(s), "microliters" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "uM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "umole" mean micromole(s), "g" means gram(s), "micrograms" or "ug" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s) and "kb" means kilobase(s).

[0184] 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 or modification when compared to a non-altered target sequence. Such alterations or modifications 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).

[0185] As used herein, the term "before", in reference to a sequence position, refers to an occurrence of one sequence upstream, or 5', to another sequence.

[0186] The compositions and methods herein can provide for an improved "agronomic trait" or "trait of agronomic importance" or "trait of agronomic interest" to a plant, which can include, but

not be limited to, the following: disease resistance, drought tolerance, heat tolerance, cold tolerance, salinity tolerance, metal tolerance, herbicide tolerance, improved water use efficiency, improved nitrogen utilization, improved nitrogen fixation, pest resistance, herbivore resistance, pathogen resistance, yield improvement, health enhancement, vigor improvement, growth improvement, photosynthetic capability improvement, nutrition enhancement, altered protein content, altered oil content, increased biomass, increased shoot length, increased root length, improved root architecture, modulation of a metabolite, modulation of the proteome, increased seed weight, altered seed carbohydrate composition, altered seed oil composition, altered seed protein composition, altered seed nutrient composition, as compared to an isoline plant not comprising a modification derived from the methods or compositions herein.

[0187] "Agronomic trait potential" is intended to mean a capability of a plant element for exhibiting a phenotype, preferably an improved agronomic trait, at some point during its life cycle, or conveying said phenotype to another plant element with which it is associated in the same plant.

[0188] 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.

[0189] A "Cas endonuclease" may comprise domains that enable it to function as a double-strand-break-inducing agent. A Cas endonuclease may also comprise one or more modifications or mutations that abolish or reduce its ability to cleave a double-strand polynucleotide (dCas). In some aspects, the Cas endonuclease molecule may retain the ability to nick a single-strand polynucleotide (for example, a D10A mutation in a Cas9 endonuclease molecule) (nCas9). A "functional fragment", "fragment that is functionally equivalent" and "functionally equivalent fragment" of a Cas endonuclease are used interchangeably herein, and refer to a portion or subsequence of the Cas endonuclease of the present disclosure in which the ability to recognize, bind to, and optionally unwind, nick or cleave (introduce a single or double-strand break in) the target site is retained. The terms "functional variant", "variant that is functionally equivalent" and "functionally equivalent variant" of a Cas endonuclease or Cas effector polypeptide are used interchangeably herein, and refer to a variant of the Cas effector polypeptide disclosed herein in which the ability to recognize, bind to, and optionally unwind, nick or cleave all or part of a target sequence is retained.

[0190] A Cas endonuclease may also include a multifunctional Cas endonuclease. The term "multifunctional Cas endonuclease" and "multifunctional Cas endonuclease polypeptide" are used interchangeably herein and includes reference to a single polypeptide that has Cas endonuclease functionality (comprising at least one protein domain that can act as a Cas endonuclease) and at least one other functionality, such as but not limited to, the functionality to form a cascade (comprises at least a second protein domain that can form a cascade with other proteins). In one aspect, the multifunctional Cas endonuclease comprises at least one additional protein domain relative (either internally, upstream (5'), downstream (3'), or both internally 5' and 3', or any combination thereof) to those domains typical of a Cas endonuclease.

[0191] A "centimorgan" (cM) or "map unit" is the distance between two polynucleotide sequences, 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.

[0192] "Coding sequence" refers to a polynucleotide sequence that codes for a specific amino acid sequence.

[0193] A "codon-modified gene", "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 a host cell.

[0194] A "complex trait locus" includes a genomic locus that has multiple transgenes genetically linked to each other.

[0195] "CRISPR" (Clustered Regularly Interspaced Short Palindromic Repeats) loci refers to certain genetic loci encoding components of DNA cleavage systems, for example, used by bacterial and archaeal cells to destroy foreign DNA (Horvath and Barrangou, 2010, Science 327:167-170; WO2007025097, published 01 March 2007). A CRISPR locus can include of a CRISPR array, comprising short direct repeats (CRISPR repeats) separated by short variable DNA sequences (called spacers), which can be flanked by diverse Cas (CRISPR-associated) genes.

[0196] The term "Cas polypeptide" refers to a polypeptide encoded by a Cas (CRISPR-associated) gene. A Cas polypeptide includes but is not limited to: Cas9, Cas12f (Cas-alpha, Cas14), Cas12l (Cas-beta), Cas12a (Cpf1), Cas12b (a C2c1 protein), Cas13 (a C2c2 protein), Cas12c (a C2c3 protein), Cas12d, Cas12e, Cas12g, Cas12h, Cas12i, Cas12j, Cas12k, Cas3, Cas3-HD, Cas 5, Cas6,

Cas7, Cas8, Cas10, or combinations or complexes of these. A Cas polypeptide may be a "Cas endonuclease" that when in complex with a suitable polynucleotide component, is capable of recognizing, binding to, and optionally nicking or cleaving all or part of a specific polynucleotide target sequence. A Cas endonuclease described herein comprises one or more nuclease domains. The endonucleases of the disclosure may include those having one or more RuvC nuclease domains. A Cas polypeptide is further defined as a functional fragment or functional variant of a native Cas polypeptide, or a protein that shares at least 50%, between 50% and 55%, at least 55%, between 55% and 60%, at least 60%, between 60% and 65%, at least 65%, between 65% and 70%, at least 70%, between 70% and 75%, at least 75%, between 75% and 80%, at least 80%, between 80% and 85%, at least 85%, between 85% and 90%, at least 90%, between 90% and 95%, at least 95%, between 95% and 96%, at least 96%, between 96% and 97%, at least 97%, between 97% and 98%, at least 98%, between 98% and 99%, at least 99%, between 99% and 100%, or 100% sequence identity with at least 50, between 50 and 100, at least 100, between 100 and 150, at least 150, between 150 and 200, at least 200, between 200 and 250, at least 250, between 250 and 300, at least 300, between 300 and 350, at least 350, between 350 and 400, at least 400, between 400 and 450, at least 500, or greater than 500 contiguous amino acids of a native Cas polypeptide, and retains at least partial activity.

[0197] As used herein, "crossed" or "cross" or "crossing" refers to 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 (or microspores and megaspores) are from the same plant or genetically identical plants).

[0198] A "deaminase" is an enzyme that catalyzes a deamination reaction. For example, deamination of adenine with an adenine deaminase results in the formation of inosine. Inosine selectively base pairs with cytosine instead of thymine. This results in a post-replicative transition mutation, such that the original A – T base pair transforms into a G – C base pair. In another example, cytosine deamination results in the formation of uracil, which can be repaired by cellular repair mechanisms back to a C – T base pair or to a T – A, G – C, or A – T base pair. This heterogeneity in repair can be suppressed by the introduction of a uracil glycosylase inhibitor, such that DNA repair or replication transforms the original C – T base pair into a T – A base pair (Burnett *et al.* (2022) *Frontiers in Genome Editing*. 4, 923718). In the case of both adenine and

cytosine deaminases, the introduction of a nick promotes the respective base pair change (Burnett *et al.*, 2022).

[0199] The terms "decreased", "fewer", "reduced", "slower" and "increased", "faster", "enhanced", "greater" as used herein refer to a decrease or increase in a characteristic of a modified plant element or resulting plant compared to an unmodified plant element or resulting plant. For example, a decrease in a characteristic can be at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, between 5% and 10%, at least 10%, between 10% and 20%, at least 15%, at least 20%, between 20% and 30%, at least 25%, at least 30%, between 30% and 40%, at least 35%, at least 40%, between 40% and 50%, at least 45%, at least 50%, between 50% and 60%, at least about 60%, between 60% and 70%, between 70% and 80%, at least 75%, at least about 80%, between 80% and 90%, at least about 90%, between 90% and 100%, at least 100%, between 100% and 200%, at least 200%, at least about 300%, at least about 400%) or more lower than the untreated control and an increase may be at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, between 5% and 10%, at least 10%, between 10% and 20%, at least 15%, at least 20%, between 20% and 30%, at least 25%, at least 30%, between 30% and 40%, at least 35%, at least 40%, between 40% and 50%, at least 45%, at least 50%, between 50% and 60%, at least about 60%, between 60% and 70%, between 70% and 80%, at least 75%, at least about 80%, between 80% and 90%, at least about 90%, between 90% and 100%, at least 100%, between 100% and 200%, at least 200%, at least about 300%, at least about 400% or more higher than an untreated or unmodified control.

[0200] The terms "dicotyledonous" and "dicot" refer to the subclass of angiosperm plants also knows as "dicotyledoneae", whose seeds typically comprise two embryonic leaves, or cotyledons. The term includes references to whole plants, plant elements, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of the same.

[0201] As used herein, "domain" refers to a contiguous stretch of nucleotides (that can be RNA, DNA, and/or an RNA-DNA-combination sequence) or amino acids. The terms "conserved domain" or "motif" refer to a set of polynucleotides or 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.

[0202] As used herein, "donor DNA" is a DNA construct that comprises a polynucleotide of interest to be inserted into a target site.

[0203] The term "endogenous" refers to a sequence or other molecule that naturally occurs in a cell or organism. In one aspect, an endogenous polynucleotide is normally found in the genome of a cell (i.e., is not heterologous).

[0204] "Expression" as used herein refers to the production of a functional end-product (e.g., an mRNA, guide RNA, or a protein) in either precursor or mature form.

[0205] The term "fragment" refers to a contiguous set of nucleotides or amino acids. In some aspects, a fragment is 2, 3, 4, 5, 6, 7 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 contiguous nucleotides. In some aspects, a fragment is 2, 3, 4, 5, 6, 7 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or greater than 20 contiguous amino acids. A fragment may or may not exhibit the function of a sequence sharing some percent identity over the length of said fragment.

[0206] A "fragment that is functionally equivalent" and "functionally equivalent fragment" are used interchangeably herein. These terms refer to a portion or sub-sequence of an isolated nucleic acid fragment or polypeptide that displays the same activity or function as the longer sequence from which it is derived. For example, the fragment retains the ability to alter gene expression or produce a certain phenotype whether or not the fragment encodes an active protein. For example, the fragment can be used in the design of genes to produce the desired phenotype in a modified plant. Genes can be designed for use in suppression by linking a nucleic acid fragment, whether or not it encodes an active enzyme, in the sense or antisense orientation relative to a plant promoter sequence.

[0207] As used herein, "gene" refers to 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 its natural endogenous location with its own regulatory sequences.

[0208] As used herein "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. The term "genome" as

it applies to a prokaryotic and eukaryotic cell or organism 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.

[0209] As used herein, a "genomic region" is a segment of a chromosome in the genome of a cell that is present on either side of a target site or, alternatively, also comprises a portion of a target site. A 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.

[0210] As used herein, "guide polynucleotide" refers to a polynucleotide sequence that can form a complex with a Cas endonuclease, including the Cas endonuclease described herein, and enables the Cas endonuclease to recognize, optionally bind to, and optionally cleave a DNA target site. The guide polynucleotide sequence can be an RNA sequence, a DNA sequence, or a combination thereof (a RNA-DNA combination sequence). The guide polynucleotide can contain modified or substitute bases. The terms "functional fragment", "fragment that is functionally equivalent" and "functionally equivalent fragment" of a guide RNA, crRNA or tracrRNA are used interchangeably herein, and refer to a portion or subsequence of the guide RNA, crRNA or tracrRNA, respectively, of the present disclosure in which the ability to function as a guide RNA, crRNA or tracrRNA, respectively, is retained. The terms "functional variant", "variant that is functionally equivalent" and "functionally equivalent variant" of a guide RNA, crRNA or tracrRNA (respectively) are used interchangeably herein, and refer to a variant of the guide RNA, crRNA or tracrRNA, respectively, of the present disclosure in which the ability to function as a guide RNA, crRNA or tracrRNA, respectively, is retained. The terms "single guide RNA" and "sgRNA" are used interchangeably herein and relate to a synthetic fusion of two RNA molecules, a crRNA (CRISPR RNA) comprising a variable targeting domain (linked to a tracr mate sequence that hybridizes to a tracrRNA), fused to a tracrRNA (trans-activating CRISPR RNA). The single guide RNA can comprise a crRNA or crRNA fragment and a tracrRNA or tracrRNA fragment of the type II CRISPR/Cas system that can form a complex with a type II Cas endonuclease, wherein said guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling

the Cas endonuclease to recognize, optionally bind to, and optionally nick or cleave (introduce a single or double-strand break) the DNA target site.

[0211] As used herein, the terms "guide polynucleotide/Cas endonuclease complex", "guide polynucleotide/Cas endonuclease system", "guide polynucleotide/Cas complex", "guide polynucleotide/Cas system", "guided Cas system", and "polynucleotide-guided endonuclease" ("PGEN") are used interchangeably herein and refer to at least one guide polynucleotide and at least one Cas endonuclease that are capable of forming a complex, wherein the guide polynucleotide/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double-strand break) the DNA target site. A guide polynucleotide/Cas endonuclease complex herein can comprise Cas polypeptide(s) and suitable polynucleotide component(s) of any of the known CRISPR systems (Horvath and Barrangou, 2010, Science 327:167-170; Makarova et al. 2015, Nature Reviews Microbiology Vol. 13:1-15; Zetsche et al., 2015, Cell 163, 1-13; Shmakov et al., 2015, Molecular Cell 60, 1-13).

[0212] The terms "guide RNA/Cas endonuclease complex", "guide RNA/Cas endonuclease system", "guide RNA/Cas complex", "guide RNA/Cas system", "gRNA/Cas complex", "gRNA/Cas system", and "RNA-guided endonuclease" ("RGEN") are used interchangeably herein and refer to at least one RNA component and at least one Cas endonuclease that are capable of forming a complex , wherein said guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double-strand break) the DNA target site.

[0213] The term "heterologous" refers to the difference between the original environment, location, or composition of a particular polynucleotide or polypeptide sequence and its current environment, location, or composition. Non-limiting examples include differences in taxonomic derivation (e.g., a polynucleotide sequence obtained from Zea mays would be heterologous if inserted into the genome of an Oryza sativa plant, or of a different variety or cultivar of Zea mays; or a polynucleotide obtained from a bacterium was introduced into a cell of a plant), or sequence (e.g., a polynucleotide sequence obtained from Zea mays, isolated, modified, and re-introduced into a maize plant). As used herein, "heterologous" in reference to a sequence can refer to a sequence that originates from a different species, variety, foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by

deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. Alternatively, one or more regulatory region(s) and/or a polynucleotide provided herein may be entirely synthetic. In some aspects, a discrete component of a poly-gRNA molecule is heterologous to at least one other component, i.e., do not occur together in nature.

[0214] As used herein, "homology" is meant describe DNA sequences that are similar. For example, a "region of homology to a genomic region" that is found on a donor DNA is a region of DNA that has a similar sequence to a given "genomic region" in the genome of a cell or organism. A region of homology can be of any length that is sufficient to promote homologous recombination at a cleaved target site. For example, a 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, 5-1100, 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 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 structural similarity such that they are capable of acting as substrates for homologous recombination. 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.

[0215] As used herein, "homologous recombination" (HR) includes the exchange of DNA fragments between two DNA molecules at the sites of homology. The frequency of homologous recombination is influenced by a number of factors. Different organisms vary with respect to the amount of homologous recombination and the relative proportion of homologous to non-homologous recombination. Generally, the length of a region of homology affects the frequency of homologous recombination events: the longer the region of homology, the greater the frequency. The length of a homology region needed to observe homologous recombination is also species-

variable. In many cases, at least 5 kb of homology has been utilized, but homologous recombination has been observed with as little as 25-50 bp of homology.

[0216] As used herein, "host" refers to an organism or cell into which a heterologous component (polynucleotide, polypeptide, other molecule, cell) has been introduced. As used herein, a "host cell" refers to an *in vivo* or *in vitro* eukaryotic cell, prokaryotic cell (e.g., bacterial or archaeal cell), or cell from a multicellular organism (e.g., a cell line) cultured as a unicellular entity, into which a heterologous polynucleotide or polypeptide has been introduced. In some embodiments, the cell is selected from the group consisting of: an archaeal cell, a bacterial cell, a eukaryotic cell, a eukaryotic single-cell organism, a somatic cell, a germ cell, a stem cell, a plant cell, an algal cell, an animal cell, in invertebrate cell, a vertebrate cell, a fish cell, a frog cell, a bird cell, an insect cell, a mammalian cell, a pig cell, a cow cell, a goat cell, a sheep cell, a rodent cell, a rat cell, a mouse cell, a non-human primate cell, and a human cell. In some cases, the cell is *in vitro*. In some cases, the cell is *in vivo*.

[0217] As used herein, "introducing" and "providing" are intended to mean presenting a subject molecule to a target, such as a cell or organism, a polynucleotide or polypeptide or polynucleotide-protein complex, in such a manner that the subject gains access to the target, such as the interior of a cell of the organism or to the cell itself, or in the case of a target polynucleotide, presented to the polynucleotide in such a way that the subject has capability of physical or chemical contact with the polynucleotide.

[0218] 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, a modified (mutated or edited) native allele, or a selected allele of a marker or QTL.

[0219] An "isolated" or "purified" nucleic acid molecule, polynucleotide, polypeptide, or protein, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide or protein as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide, polypeptide, or protein is

substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Generally, an "isolated" polynucleotide is free of sequences (e.g., protein encoding sequences) that naturally flank the polynucleotide (i.e., sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. For example, in various embodiments, the isolated polynucleotide can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequence that naturally flank the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. Isolated polynucleotides may be purified from a cell in which they naturally occur. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

[0220] The term "isoline" is a comparative term, and references organisms that are genetically identical, but differ in treatment. In one example, two genetically identical maize plant embryos may be separated into two different groups, one receiving a treatment (such as the introduction of a CRISPR-Cas effector endonuclease) and one control that does not receive such treatment. Any phenotypic differences between the two groups may thus be attributed solely to the treatment and not to any inherency of the plant's endogenous genetic makeup.

[0221] The terms "knock-out", "gene knock-out", and "genetic knock-out" are used interchangeably herein. A knock-out refers to a DNA sequence of a cell that has been rendered partially or completely inoperative by targeting with a genome editing system; for example, a DNA sequence prior to knock-out could have encoded an amino acid sequence, or could have had a regulatory function (e.g., promoter).

[0222] The terms "knock-in", "gene knock-in, "gene insertion", and "genetic knock-in" are used interchangeably herein. A knock-in represents the replacement or insertion of a DNA sequence at a specific DNA sequence in cell by targeting with a genome editing system (for example by homologous recombination (HR), wherein a suitable donor DNA polynucleotide is also used). Examples of knock-ins are a specific insertion of a heterologous amino acid coding sequence in a coding region of a gene, or a specific insertion of a transcriptional regulatory element in a genetic locus.

[0223] A "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).

[0224] 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).

[0225] The terms "monocotyledonous" and "monocot" refer to the subclass of angiosperm plants also known as "monocotyledoneae", whose seeds typically comprise only one embryonic leaf, or cotyledon. The term includes references to whole plants, plant elements, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of the same.

[0226] 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 some aspects, 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.

[0227] As used herein, "nucleic acid" generally refers to 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 and/or RNA-DNA that is single- or double-stranded, optionally comprising 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.

[0228] An "optimized" polynucleotide is a sequence that has been optimized for improved expression in a particular heterologous host cell.

[0229] An "optimized nucleotide sequence" is a nucleotide sequence that has been optimized for expression in a particular organism. A plant-optimized nucleotide sequence includes a codon-optimized gene. A plant-optimized nucleotide sequence can be synthesized by modifying a nucleotide sequence encoding a protein such as, for example, a Cas endonuclease as disclosed

herein, using one or more plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) Plant Physiol. 92:1-11 for a discussion of host-preferred codon usage.

[0230] As used herein, "open reading frame" is abbreviated ORF.

[0231] 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.

[0232] The terms "plasmid", "vector", and "cassette" refer to a linear or circular 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 polynucleotide of interest into a cell. "Transformation cassette" refers to a specific vector comprising a gene and having elements in addition to the gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a specific vector comprising a gene and having elements in addition to the gene that allow for expression of that gene in a host. In some aspects, a "Donor DNA cassette" comprises a heterologous polynucleotide to be inserted at the double-strand break site created by a double-strand-break inducing agent (e.g. a Cas endonuclease and guide RNA complex), that is operably linked to a noncoding expression regulatory element. In some aspects, the Donor DNA cassette further comprises polynucleotide sequences that are homologous to the target site, that flank the polynucleotide of interest operably linked to a noncoding expression regulatory element.

[0233] The term "plant" generically includes 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 cells comprise a plant cell wall, and as such are distinct, with different biochemical characteristics, from protoplasts that lack a cell wall.

[0234] A "plant element" or “plant part” is intended to reference either a whole plant or a plant component, which may comprise differentiated and/or undifferentiated tissues, for example but not limited to plant tissues, parts, and cell types. In one embodiment, a plant element is one of the following: whole plant, seedling, meristematic tissue, ground tissue, vascular tissue, dermal tissue, seed, leaf, root, shoot, stem, flower, fruit, stolon, bulb, tuber, corm, keiki, shoot, bud, tumor tissue, and various forms of cells and culture (e.g., single cells, protoplasts, embryos, callus tissue), plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like, as well as the parts themselves. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides. The term "plant organ" refers to plant tissue or a group of tissues that constitute a morphologically and functionally distinct part of a plant. As used herein, a "plant element" is synonymous to a "portion" or “part” of a plant, and refers to any part of the plant, and can include distinct tissues and/or organs, and may be used interchangeably with the term "tissue" throughout. Similarly, a "plant reproductive element" is intended to generically reference any part of a plant that is able to initiate other plants via either sexual or asexual reproduction of that plant, for example but not limited to: seed, seedling, root, shoot, cutting, scion, graft, stolon, bulb, tuber, corm, keiki, or bud. The plant element may be in plant or in a plant organ, tissue culture, or cell culture.

[0235] The term “polynucleotide modification template” includes a polynucleotide that comprises at least one nucleotide modification when compared to a 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.

[0236] As used herein, a “polynucleotide of interest” encodes a protein or polypeptide that is “of interest” for a particular purpose, e.g. a selectable marker. In some aspects, a trait or polynucleotide

"of interest" is one that improves a desirable phenotype of a plant, particularly a crop plant, i.e. a trait of agronomic interest. Polynucleotides of interest: include, but are not limited to, polynucleotides encoding important traits for agronomics, herbicide-resistance, insecticidal resistance, disease resistance, nematode resistance, herbicide resistance, microbial resistance, fungal resistance, viral resistance, fertility or sterility, grain characteristics, commercial products, phenotypic marker, or any other trait of agronomic or commercial importance. A polynucleotide of interest may additionally be utilized in either the sense or anti-sense orientation. Further, more than one polynucleotide of interest may be utilized together, or "stacked", to provide additional benefit. In some aspects, a "polynucleotide of interest" may encode a gene expression regulatory element, for example a promoter, intron, terminator, 5'UTR, 3'UTR, or other noncoding sequence. In some aspects, a "polynucleotide of interest" may comprise a DNA sequences that encodes for an RNA molecule, for example a functional RNA, siRNA, miRNA, or a guide RNA that is capable of interacting with a Cas endonuclease to bind to a target polynucleotide sequence.

[0237] A "population" of plants refers to a plurality of individual plants that share temporal and spatial location, and may further share one or more characteristic(s), such as a common genotype.

[0238] A "precursor" protein refers to the primary product of translation of mRNA (i.e., with pre- and propeptides still present). Pre- and pro-peptides may be but are not limited to intracellular localization signals.

[0239] "Progeny" comprises any subsequent generation of a plant.

[0240] A "promoter" is a region of DNA involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. 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.

[0241] Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". The term "inducible promoter" refers to a promoter that selectively expresses a coding sequence or functional RNA in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters induced or regulated by light, heat, stress, flooding or drought, salt stress, osmotic stress, phytohormones, wounding, or chemicals such as ethanol, abscisic acid (ABA), jasmonate, salicylic acid, or safeners.

[0242] A "protospacer adjacent motif" (PAM) herein refers to a short nucleotide sequence adjacent to a target sequence (protospacer) that is recognized (targeted) by a guide polynucleotide/Cas endonuclease system described herein. The Cas endonuclease may not successfully recognize a target DNA sequence if the target DNA sequence is not followed by a PAM sequence. The sequence and length of a PAM herein can differ depending on the Cas polypeptide or Cas polypeptide complex used. The PAM sequence can be of any length but is typically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides long.

[0243] 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.

[0244] The terms "recombinant DNA molecule", "recombinant DNA construct", "expression construct", "construct", and "recombinant construct" are used interchangeably herein. A recombinant DNA construct comprises an artificial combination of nucleic acid sequences, e.g., regulatory and coding sequences that are not all found together in nature. For example, a recombinant DNA 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 introduce the vector into the host cells as is well known to those skilled in the art.

[0245] "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 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.

[0246] "3' non-coding sequences", "transcription terminator", and "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.

[0247] "Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window. As used herein, "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 percentage from 50% to 100%.

[0248] 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 sequences 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 sequences that do not substantially alter the functional properties of the resulting nucleic acid relative to the initial, unmodified nucleic acid. These modifications

include deletion, substitution, and/or insertion of one or more nucleotides in the nucleic acid fragment. Substantially similar nucleic acid sequences encompassed can 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.

[0249] As used herein, a "targeted mutation" is a mutation in a gene (referred to as the target gene), including a native gene, made by altering a target sequence within the target gene using any method known to one skilled in the art, including a method involving a guided Cas endonuclease system as disclosed herein.

[0250] The terms "target site", "target sequence", "target site sequence, "target DNA", "target locus", "genomic target site", "genomic target sequence", "genomic target locus", "target polynucleotide", and "protospacer", are used interchangeably herein and refer to a polynucleotide sequence such as, but not limited to, a nucleotide sequence on a chromosome, episome, a locus, or any other DNA molecule in the genome (including chromosomal, chloroplastic, mitochondrial DNA, plasmid DNA) of a cell, at which a guide polynucleotide/Cas endonuclease complex can recognize, bind to, and optionally nick or cleave. The target site can be an endogenous site in the genome of a cell, or alternatively, the target site can be heterologous to the cell and thereby not be naturally occurring in the genome of the cell, 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 and is at the endogenous or native position of that target sequence in the genome of the cell. 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. 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. Methods for

"modifying a target site" and "altering a target site" are used interchangeably herein and refer to methods for producing an altered target site.

[0251] The present disclosure relates to methods and compositions for delivering multiple guide polynucleotides to a cell. More specifically, described herein are methods and compositions comprising exogenous treatments and/or expression of heterologous polypeptides to facilitate or enhance co-delivery of T-DNAs expressing multiple guide polynucleotides to a plant cell.

[0252] In a first aspect, the disclosure provides methods for co-delivery of multiple *Agrobacterium* T-DNAs comprising transforming a plant cell with a first T-DNA expressing a first set of guide polynucleotides (i.e., two or more) targeting a first set of nucleotide sequences in the plant cell and a second T-DNA expressing a second set of guide polynucleotides (i.e., two or more) targeting a second set of nucleotide sequences in the plant cell, wherein the optical density (OD) of the first and the second T-DNAs ranges from about 0.2 to about 3.0, and more preferably from about 0.8 to about 2.0 (e.g., 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0) and the concentration, based on the OD, of the first and the second T-DNAs is the same. Each guide polynucleotide within a set of guide polynucleotides has a unique genomic target. For example, a first *Agrobacterium* T-DNA expressing a first set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target A and genomic target B) and a second T-DNA expressing a second set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target C and genomic target D) that differ from the genomic targets of the first set of guide polynucleotides.

[0253] In a second aspect, the disclosure provides methods for editing a plant genome, the methods comprising providing a plant cell with a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide, a first T-DNA expressing a first set of guide polynucleotides (i.e., two or more) targeting a first set of nucleotide sequences in the plant cell, and a second T-DNA expressing a second set of guide polynucleotides (i.e., two or more) targeting a second set of nucleotide sequences in the plant cell, wherein the OD of the first and the second T-DNAs ranges from about 0.2 to about 3.0, and more preferably from about 0.8 to about 2.0 (e.g., 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0) and the concentration, based on the OD, of the first and the second T-DNAs is the same; introducing site-specific modifications in the first set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the first set of guide polynucleotides; and introducing site-specific modifications in the second

set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the second set of guide polynucleotides. Each guide polynucleotide within a set of guide polynucleotides has a unique genomic target. For example, a first T-DNA expressing a first set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target A and genomic target B) and a second T-DNA expressing a second set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target C and genomic target D) that differ from the genomic targets of the first set of guide polynucleotides.

[0254] In a third aspect, the disclosure provides compositions for delivering guide polynucleotides, the compositions comprising a first T-DNA expressing multiple (i.e., two or more) guide polynucleotides targeting a first set of nucleotide sequences in the plant cell, a second T-DNA expressing multiple (i.e., two or more) guide polynucleotides targeting a second set of nucleotide sequences in the plant cell, and optionally an inhibitor of cyclic adenine diphosphate ribose, or a variant or isomer thereof. In some aspects, the composition further comprises a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide. Each guide polynucleotide within a set of guide polynucleotides has a unique genomic target. For example, a first T-DNA expressing a first set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target A and genomic target B) and a second T-DNA expressing a second set of guide polynucleotides can comprise two guide polynucleotides targeting two different genomic targets (genomic target C and genomic target D) that differ from the genomic targets of the first set of guide polynucleotides.

[0255] Suitable *Agrobacterium* strains of the present disclosure include, but are not limited to, EHA101, EHA105, GV3101, MP90, LBA288, LBA1100, NT1, A136, A281, A348, AGL-0, AGL-1, C58-Z707, C58C1, LBA4404, and KYRT1. In some aspects of the method, the first and second *Agrobacterium* T-DNAs are LBA4404.

[0256] In some aspects of the methods disclosed herein (i.e., methods for co-delivery of multiple T-DNAs and methods for editing a plant genome), the method further comprises regenerating a plantlet or plant from the transformed plant cell.

[0257] In some aspects of the methods disclosed herein, the method further comprises pre-treating the plant cell prior to transformation by incubating the plant cell at a temperature above about 28°C for about 2 minutes to about 10 minutes. In some aspects, the pre-treatment incubation temperature

ranges from about 28°C to about 50°C, alternatively about 35°C to about 50°C, alternatively about 40°C to about 50°C, alternatively about 45°C to about 50°C, alternatively about 45°C. In some aspects, the pre-treatment incubation duration is about 2 minutes, alternatively about 3 minutes, alternatively about 4 minutes, alternatively about 5 minutes, alternatively about 6 minutes, alternatively about 7 minutes, alternatively about 8 minutes, alternatively about 9 minutes, alternatively about 10 minutes. In some aspects, pre-treating the plant cell comprises incubating the plant cell at about 45°C for about 5 minutes.

[0258] In some aspects, the methods and compositions described herein can result in stable or transient T-DNA delivery. In some aspects, the methods and compositions described herein result in stable T-DNA delivery.

[0259] In some aspects of the methods disclosed herein, the method further comprises inhibiting, reducing, or attenuating an immune response in the plant cell to promote or facilitate increased bacteria-mediated infection and delivery of multiple T-DNAs. In some aspects, inhibiting immune signaling in the plant cell makes the plant cell more receptive to an increased *Agrobacterium* concentration (e.g., OD of 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0) or an increased number (i.e., two or more) of T-DNAs.

[0260] In some aspects of the methods disclosed herein, inhibiting or attenuating a plant cell's immune response comprises blocking, inhibiting, or disrupting, through decreased function or expression, of cyclic adenine diphosphate ribose (cADPR), or a variant or isomer thereof, in the plant cell.

[0261] In some aspects of the methods disclosed herein, inhibiting or attenuating a plant cell's immune response comprises providing to the plant cell a bacteriophage-derived, heterologous Thoeris anti-defense 1 (Tad1) polypeptide. In some aspects, the method comprises providing a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide to the plant cell, which can be provided simultaneously with the first and second *Agrobacterium* T-DNAs. In some aspects, the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide can be provided to the plant cell prior to transformation with the first and second *Agrobacterium* T-DNAs.

[0262] In some aspects of the methods described herein, a polynucleotide sequence encoding a Tad1 polypeptide can be codon-optimized and incorporated into the first and/or the second T-DNA or incorporated into an associated *Agrobacterium* virulence plasmid.

[0263] In some aspects of the compositions disclosed herein (i.e., compositions for delivering guide polynucleotides), the inhibitor of cyclic adenine diphosphate ribose, or a variant or isomer thereof, is a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide.

[0264] The methods and compositions disclosed can be used to co-deliver more than two T-DNAs. In some aspects of the methods disclosed herein, the method further comprises transforming a plant cell with a third T-DNA expressing a third set of guide polynucleotides (i.e., two or more) targeting a third set of nucleotide sequences in the plant cell. In some aspects of the methods disclosed herein, the method further comprises transforming a plant cell with a fourth T-DNA expressing a fourth set of guide polynucleotides (i.e., two or more) targeting a fourth set of nucleotide sequences in the plant cell. In some aspects of the methods disclosed herein, the method further comprises transforming a plant cell with a fifth T-DNA expressing a fifth set of guide polynucleotides (i.e., two or more) targeting a fifth set of nucleotide sequences in the plant cell.

[0265] In some aspects of the methods disclosed herein, the method further comprises providing the plant cell with a third T-DNA, the third T-DNA expressing a third set of guide polynucleotides (i.e., two or more) targeting a third set of nucleotide sequences in the plant cell, and introducing site-specific modifications in the third set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the third set of guide polynucleotides. In some aspects of the methods disclosed herein, the method further comprises providing the plant cell with a fourth T-DNA, the fourth T-DNA expressing a fourth set of guide polynucleotides (i.e., two or more) targeting a fourth set of nucleotide sequences in the plant cell, and introducing site-specific modifications in the fourth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fourth set of guide polynucleotides. In some aspects of the methods disclosed herein, the method further comprises providing the plant cell with a fifth T-DNA, the fifth T-DNA expressing a fifth set of guide polynucleotides (i.e., two or more) targeting a fifth set of nucleotide sequences in the plant cell, and introducing site-specific modifications in the fifth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fifth set of guide polynucleotides.

[0266] In some aspects of the compositions disclosed herein, the composition further comprises a third T-DNA expressing a third set of guide polynucleotides (i.e., two or more) targeting a third set of nucleotide sequences in the plant cell. In some aspects of the compositions disclosed herein, the composition further comprises a fourth T-DNA expressing a fourth set of guide

polynucleotides (i.e., two or more) targeting a fourth set of nucleotide sequences in the plant cell. In some aspects of the compositions disclosed herein, the composition further comprises a fifth T-DNA expressing a fifth set of guide polynucleotides (i.e., two or more) targeting a fifth set of nucleotide sequences in the plant cell.

[0267] In some aspects, the first, second, third, fourth, and/or fifth set of guide polynucleotides expressed from the respective T-DNA are for multiplexed genome editing of the unique first, second, third, fourth, and/or fifth set of target nucleotide sequences in the plant cell.

[0268] In some aspects of the methods and compositions described herein, the first, second, third, fourth, and/or fifth set of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

[0269] ***Promoters***

[0270] In the methods and compositions described herein, each guide polynucleotide can be expressed by a separate promoter, rather than utilizing mono-cistronic expression. For example, each guide polynucleotide can be operably linked to a U6 promoter comprising: (a) a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94; or (b) a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112; or (c) a polynucleotide sequence having at least 85%, alternatively at least 90%, alternatively at least 95%, alternatively 100% sequence identity to SEQ ID Nos: 3-7 or 86-91; or (d) a polynucleotide sequence having at least 85%, alternatively at least 90%, alternatively at least 95%, alternatively 100% sequence identity to SEQ ID Nos: 96-109; or (e) a polynucleotide sequence having at least 85%, alternatively at least 90%, alternatively at least 95%, alternatively 100% sequence identity to SEQ ID Nos: 113-121.

[0271] When the methods and compositions described herein are used to deliver or express multiple guide polynucleotides, the above U6 promoters can be used in a repeating manner or pattern to express the desired number of guide polynucleotides. For example, a first guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 4, a second guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 5, a third guide

polynucleotide operably linked to a U6 promoter having SEQ ID NO: 6, a fourth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 7, a fifth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 86, a sixth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 4, a seventh guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 5, an eighth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 6, a ninth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 7, and a tenth guide polynucleotide operably linked to a U6 promoter having SEQ ID NO: 86.

[0272] ***Cas Endonucleases***

[0273] Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain. Examples of endonucleases include restriction endonucleases, meganucleases, TAL effector nucleases (TALENs), zinc finger nucleases, and Cas (CRISPR-associated) effector endonucleases.

[0274] 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).

[0275] Cas endonucleases, either as single effector polypeptides or in an effector complex with other components, unwind the DNA duplex at a target sequence and, optionally, cleave at least one DNA strand, as mediated by recognition of the target DNA sequence by a guide polynucleotide (such as, but not limited to, a CRISPR RNA (crRNA) or guide RNA) that is complexed with a Cas endonuclease. Such recognition and cutting of a target DNA sequence by a Cas endonuclease typically occurs if the correct protospacer-adjacent motif (PAM) is located at or adjacent to the 3' end of the target DNA sequence. Alternatively, a Cas endonuclease can lack DNA cleavage or nicking activity, but can still specifically bind to a target DNA sequence when complexed with a suitable guide polynucleotide. (See also U.S. Patent Application US20150082478 published 19 March 2015 and US20150059010 published 26 February 2015).

[0276] Cas endonucleases that have been described include, but are not limited to, for example: Cas9, Cas12f (Cas-alpha, Cas14), Cas12l (Cas-beta), Cas12a (Cpf1), Cas12b (a C2c1 protein),

Cas13 (a C2c2 protein), Cas12c (a C2c3 protein), Cas12d, Cas12e, Cas12g, Cas12h, Cas12i, Cas12j, Cas12k, Cas3, Cas3-HD, Cas 5, Cas6, Cas7, Cas8, Cas10, or combinations or complexes of these. In some aspects, the methods and compositions described herein can utilize transposon-associated TnpB, a programmable RNA-guided DNA endonuclease.

[0277] Cas endonucleases and effector polypeptides can be used for targeted genome editing (via simplex and multiplex double-strand breaks and nicks) and targeted genome regulation (via tethering of epigenetic effector domains to either the Cas polypeptide or sgRNA. A Cas endonuclease can also be engineered to function as an RNA-guided recombinase, and via RNA tethers could serve as a scaffold for the assembly of multiprotein and nucleic acid complexes (Mali *et al.*, 2013, *Nature Methods Vol.* 10: 957-963).

[0278] A Cas endonuclease, effector polypeptide, or functional fragment thereof, for use in the disclosed methods, can be isolated from a native source, or from a recombinant source where the genetically modified host cell is modified to express the nucleic acid sequence encoding the protein. Alternatively, the Cas endonuclease protein can be produced using cell free protein expression systems, or be synthetically produced. Cas endonucleases may be isolated and introduced into a heterologous cell, or may be modified from its native form to exhibit a different type or magnitude of activity than what it would exhibit in its native source. Such modifications include but are not limited to: fragments, variants, substitutions, deletions, and insertions.

[0279] Fragments and variants of Cas endonucleases can be obtained via methods such as site-directed mutagenesis and synthetic construction. Methods for measuring endonuclease activity are well known in the art such as, but not limiting to, WO2013166113 published 07 November 2013, WO2016186953 published 24 November 2016, and WO2016186946 published 24 November 2016.

[0280] The Cas endonuclease can comprise a modified form of the Cas polypeptide. The modified form of the Cas polypeptide can include an amino acid change (*e.g.*, deletion, insertion, or substitution) that reduces the naturally-occurring nuclease activity of the Cas polypeptide. For example, in some instances, the modified form of the Cas polypeptide has less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 5%, or less than 1% of the nuclease activity of the corresponding wild-type Cas polypeptide (US20140068797 published 06 March 2014). In some cases, the modified form of the Cas polypeptide has no substantial nuclease activity and is referred to as catalytically "inactivated Cas" or "deactivated Cas (dCas)." An inactivated

Cas/deactivated Cas includes a deactivated Cas endonuclease (dCas). A catalytically inactive Cas endonuclease can be fused to a heterologous sequence to induce or modify activity.

[0281] A Cas endonuclease can be part of a fusion protein comprising one or more heterologous protein domains (*e.g.*, 1, 2, 3, or more domains in addition to the Cas polypeptide). Suitable fusion partners include, but are not limited to, a polypeptide that provides an activity that indirectly increases transcription by acting directly on the target DNA or on a polypeptide (*e.g.*, a histone or other DNA-binding protein) associated with the target DNA. Additional suitable fusion partners include, but are not limited to, a polypeptide that provides for methyltransferase activity, demethylase activity, acetyltransferase activity, deacetylase activity, kinase activity, phosphatase activity, ubiquitin ligase activity, deubiquitinating activity, adenylation activity, deadenylation activity, SUMOylating activity, deSUMOylating activity, ribosylation activity, deribosylation activity, myristoylation activity, or demyristoylation activity. Further suitable fusion partners include, but are not limited to, a polypeptide that directly provides for increased transcription of the target nucleic acid (*e.g.*, a transcription activator or a fragment thereof, a protein or fragment thereof that recruits a transcription activator, a small molecule/drug-responsive transcription regulator, etc.). A catalytically inactive Cas can also be fused to a FokI nuclease to generate double-strand breaks (Guilinger *et al. Nature Biotechnology*, volume 32, number 6, June 2014). In some aspects, the Cas endonuclease is a fusion protein further comprising a nuclease domain, a transcriptional activator domain, a transcriptional repressor domain, an epigenetic modification domain, a cleavage domain, a nuclear localization signal, a cell-penetrating domain, a translocation domain, a marker, or a transgene that is heterologous to the target polynucleotide sequence or to the cell from which said target polynucleotide sequence is obtained or derived. In some aspects, the nuclease fusion protein comprises Clo51 or Fok1.

[0282] The Cas endonucleases described herein can be expressed and purified by methods known in the art, for example as described in WO/2016/186953.

[0283] A Cas endonuclease can comprise a heterologous nuclear localization sequence (NLS). A heterologous NLS amino acid sequence herein may be of sufficient strength to drive accumulation of a Cas polypeptide in a detectable amount in the nucleus of a yeast cell herein, for example.

[0284] ***Cas9 Endonuclease***

[0285] In some aspects of the methods disclosed herein, a genome editing system comprises a Cas9 endonuclease and one or more guide polynucleotides that introduce one or more site-specific

modifications in a target polynucleotide sequence. In some aspects, a genome editing system comprises a Cas9 endonuclease, one or more guide polynucleotides, and a donor DNA. Some exemplary Cas9 endonucleases are described, for example, in WO2019165168.

[0286] Cas9 (formerly referred to as Cas5, Csn1, or Csx12) is a Cas endonuclease that forms a complex with a crNucleotide and a tracrNucleotide, or with a single guide polynucleotide, for specifically recognizing and cleaving all or part of a DNA target sequence. The canonical Cas9 recognizes a 3' GC-rich PAM sequence on the target dsDNA, typically comprising an NGG motif. The Cas endonucleases described herein may recognize additional PAM sequences and used to modify target sites with different recognition sequence specificity.

[0287] A Cas9 polypeptide comprises a RuvC nuclease with an HNH (H-N-H) nuclease adjacent to the RuvC-II domain. The RuvC nuclease and HNH nuclease each can cleave a single DNA strand at a target sequence (the concerted action of both domains leads to DNA double-strand cleavage, whereas activity of one domain leads to a nick). In general, the RuvC domain comprises subdomains I, II and III, where domain I is located near the N-terminus of Cas9 and subdomains II and III are located in the middle of the protein, flanking the HNH domain (Hsu *et al.*, 2013, *Cell* 157:1262-1278). Cas9 endonucleases are typically derived from a type II CRISPR system, which includes a DNA cleavage system utilizing a Cas9 endonuclease in complex with at least one polynucleotide component. For example, a Cas9 can be in complex with a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA). In another example, a Cas9 can be in complex with a single guide RNA (Makarova *et al.* 2015, *Nature Reviews Microbiology* Vol. 13:1-15).

[0288] 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. In some aspects, a guide polynucleotide comprises a synthetic fusion of two RNA molecules, a crRNA (CRISPR RNA) comprising a variable targeting domain, and a tracrRNA. In some aspects, a guide polynucleotide comprises a variable targeting domain of 12 to 30 nucleotides and an RNA fragment that interacts with a Cas9 endonuclease.

[0289] ***Cas-alpha Endonuclease***

[0290] In some aspects of the methods disclosed herein, a genome editing system comprises a Cas-alpha (e.g., Cas12f) endonuclease and one or more guide polynucleotides that introduce one

or more site-specific modifications in a target polynucleotide sequence. In some aspects, a genome editing system comprises a Cas-alpha endonuclease, one or more guide polynucleotides, and a donor DNA. Some exemplary Cas-alpha endonucleases are described, for example, in US10934536 and WO2022082179.

[0291] A Cas-alpha endonuclease is a functional RNA-guided, PAM-dependent dsDNA cleavage protein of fewer than 800 amino acids, comprising: a C-terminal RuvC catalytic domain split into three subdomains and further comprising bridge-helix and one or more Zinc finger motif(s); and an N-terminal Rec subunit with a helical bundle, WED wedge-like (or "Oligonucleotide Binding Domain", OBD) domain, and, optionally, a Zinc finger motif.

[0292] Cas-alpha endonucleases comprise one or more Zinc Finger (ZFN) coordination motif(s) that may form a Zinc binding domain. Zinc Finger-like motifs can aid in target and non-target strand separation and loading of the guide polynucleotide into the DNA target. Cas-alpha endonucleases comprising one or more Zinc Finger motifs can provide additional stability to a ribonucleoprotein complex on a target polynucleotide. Cas-alpha endonucleases comprise C4 or C3H zinc binding domains.

[0293] A Cas-alpha endonuclease can function as a double-strand-break-inducing agent, a single-strand-break inducing agent, or as a nickase. In some aspects, a catalytically inactive Cas-alpha endonuclease can be used to target or recruit to a target DNA sequence but not induce cleavage. In some aspects, a catalytically inactive Cas-alpha protein can be combined with a base editing molecule, such as a cytidine deaminase or an adenine deaminase.

[0294] ***Protospacer Adjacent Motif***

[0295] A "protospacer adjacent motif" (PAM) herein refers to a short nucleotide sequence adjacent to a target sequence (protospacer) that can be recognized (targeted) by a guide polynucleotide/Cas endonuclease system. In some aspects, the Cas endonuclease may not successfully recognize a target DNA sequence if the target DNA sequence is not adjacent to, or near, a PAM sequence. In some aspects, the PAM precedes the target sequence (*e.g.* Cas12a). In some aspects, the PAM follows the target sequence (*e.g. S. pyogenes* Cas9).. The sequence and length of a PAM herein can differ depending on the Cas polypeptide or Cas polypeptide complex used. The PAM sequence can be of any length but is typically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides long.

[0296] A "randomized PAM" and "randomized protospacer adjacent motif" are used interchangeably herein, and refer to a random DNA sequence adjacent to a target sequence (protospacer) that is recognized (targeted) by a guide polynucleotide/Cas endonuclease system. The randomized PAM sequence can be of any length but is typically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides long. A randomized nucleotide includes anyone of the nucleotides A, C, G or T.

[0297] Many Cas endonucleases have been described to date that can recognize specific PAM sequences (WO2016186953 published 24 November 2016, WO2016186946 published 24 November 2016, and Zetsche B et al. 2015. Cell 163, 1013) and cleave the target DNA at a specific position. It is understood that based on the methods and embodiments described herein utilizing a novel guided Cas system one skilled in the art can now tailor these methods such that they can utilize any guided endonuclease system.

[0298] ***Guide Polynucleotide/Cas Endonuclease Complexes***

[0299] The guide polynucleotide enables target recognition, binding, and optionally cleavage by the Cas endonuclease, and 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. A guide polynucleotide that solely comprises ribonucleic acids is also referred to as a "guide RNA" or "gRNA" (US20150082478 published 19 March 2015 and US20150059010 published 26 February 2015). A guide polynucleotide may be engineered or synthetic.

[0300] A guide polynucleotide includes a chimeric non-naturally occurring guide polynucleotide comprising regions that are not found together in nature (*i.e.*, they are heterologous with respect to each other). For example, a chimeric non-naturally occurring guide RNA comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA, linked to a second nucleotide sequence that

can recognize the Cas endonuclease, such that the first and second nucleotide sequence are not found linked together in nature.

[0301] A guide polynucleotide can be a double molecule (also referred to as duplex guide polynucleotide) comprising a crNucleotide sequence and a tracrNucleotide sequence. The crNucleotide includes a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA and a second nucleotide sequence (also referred to as a tracr mate sequence) that is part of a Cas endonuclease recognition (CER) domain. The tracr mate sequence can hybridized to a tracrNucleotide along a region of complementarity and together form the Cas endonuclease recognition domain or CER domain. The CER domain is capable of interacting with a Cas endonuclease polypeptide. The crNucleotide and the tracrNucleotide of the duplex guide polynucleotide can be RNA, DNA, and/or RNA-DNA combination sequences.

[0302] In some embodiments, the crNucleotide molecule of the duplex guide polynucleotide 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 crNucleotide can comprise a fragment of the crRNA naturally occurring in Bacteria and Archaea. The size of the fragment of the crRNA naturally occurring in Bacteria and Archaea that can be present in a crNucleotide disclosed herein can range from, but is not limited to, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides.

[0303] The tracrRNA (trans-activating CRISPR RNA) comprises, in the 5'-to-3' direction, (i) an "anti-repeat" sequence that anneals with the repeat region of CRISPR type II crRNA and (ii) a stem loop-comprising portion (Deltcheva *et al.*, *Nature* 471:602-607). The duplex guide polynucleotide can form a complex with a Cas endonuclease, wherein said guide polynucleotide/Cas endonuclease complex (also referred to as a guide polynucleotide/Cas endonuclease system) can direct the Cas endonuclease to a genomic target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double-strand break) into the target site. (US20150082478 published 19 March 2015 and US20150059010 published 26 February 2015). In some embodiments, the 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.

[0304] In some aspects, the RNA of the RNA-Cas endonuclease complex is a duplexed RNA comprising a duplex crRNA-tracrRNA.

[0305] In some aspects, the guide polynucleotide is a guide polynucleotide capable of forming a PGEN as described herein, wherein said guide polynucleotide comprises a first nucleotide sequence domain that is complementary to a nucleotide sequence in a target DNA, and a second nucleotide sequence domain that interacts with said Cas endonuclease polypeptide.

[0306] In some aspects, the guide polynucleotide is a guide polynucleotide described herein, wherein the first nucleotide sequence and the second nucleotide sequence domain is selected from the group consisting of a DNA sequence, a RNA sequence, and a combination thereof.

[0307] In some aspects, the guide polynucleotide is a guide polynucleotide described herein, wherein the first nucleotide sequence and the second nucleotide sequence domain is selected from the group consisting of RNA backbone modifications that enhance stability, DNA backbone modifications that enhance stability, and a combination thereof (see Kanasty *et al.*, 2013, Common RNA-backbone modifications, *Nature Materials* 12:976-977; US20150082478 published 19 March 2015 and US20150059010 published 26 February 2015)

[0308] The guide RNA includes a dual molecule comprising a chimeric non-naturally occurring crRNA linked to at least one tracrRNA. A chimeric non-naturally occurring crRNA includes a crRNA that comprises regions that are not found together in nature (*i.e.*, they are heterologous with each other. For example, a crRNA comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA, linked to a second nucleotide sequence (also referred to as a tracr mate sequence) such that the first and second sequence are not found linked together in nature.

[0309] The guide polynucleotide can also be a single molecule (also referred to as single guide polynucleotide) comprising a crNucleotide sequence linked to a tracrNucleotide sequence. The single guide polynucleotide comprises a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA and a Cas endonuclease recognition domain (CER domain), that interacts with a Cas endonuclease polypeptide.

[0310] 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. The single guide polynucleotide being comprised of sequences from the crNucleotide and the 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). The single guide polynucleotide can form a complex with a Cas endonuclease, wherein said guide polynucleotide/Cas endonuclease complex (also referred to as a guide polynucleotide/Cas endonuclease system) can direct the Cas endonuclease to a genomic target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double-strand break) the target site. (US20150082478 published 19 March 2015 and US20150059010 published 26 February 2015).

[0311] A chimeric non-naturally occurring single guide RNA (sgRNA) includes a sgRNA that comprises regions that are not found together in nature (*i.e.*, they are heterologous with each other. For example, a sgRNA comprising a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA linked to a second nucleotide sequence (also referred to as a tracr mate sequence) that are not found linked together in nature.

[0312] 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 some aspects, the nucleotide sequence linking the crNucleotide and the tracrNucleotide of a single guide polynucleotide (also referred to as "loop") 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, 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.

[0313] The guide polynucleotide can be produced by any method known in the art, including chemically synthesizing guide polynucleotides (such as but not limiting to Hendel *et al.* 2015,

*Nature Biotechnology* 33, 985–989), *in vitro* generated guide polynucleotides, and/or self-splicing guide RNAs (such as but not limited to Xie *et al.* 2015, *PNAS* 112:3570-3575).

[0314] A method of expressing RNA components such as gRNA in eukaryotic cells for performing Cas9-mediated DNA targeting has been to use RNA polymerase III (Pol III) promoters, which allow for transcription of RNA with precisely defined, unmodified, 5'- and 3'-ends (DiCarlo et al., Nucleic Acids Res. 41: 4336-4343; Ma et al., Mol. Ther. Nucleic Acids 3:e161). This strategy has been successfully applied in cells of several different species including maize and soybean (US 20150082478, published on March 19, 2015). Methods for expressing RNA components that do not have a 5' cap have been described (WO 2016/025131, published on February 18, 2016).

[0315] The terms "single guide RNA" and "sgRNA" are used interchangeably herein and relate to a synthetic fusion of two RNA molecules, a crRNA (CRISPR RNA) comprising a variable targeting domain (linked to a tracr mate sequence that hybridizes to a tracrRNA), fused to a tracrRNA (trans-activating CRISPR RNA). The single guide RNA can comprise a crRNA or crRNA fragment and a tracrRNA or tracrRNA fragment of the type II CRISPR/Cas9 system that can form a complex with a type II Cas9 endonuclease, wherein said guide RNA/Cas9 endonuclease complex can direct the Cas9 endonuclease to a DNA target site, enabling the Cas9 endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) the DNA target site.

[0316] Single guide RNAs targeting a target site in the genome of an organism can be designed by changing the Variable Targeting Domain (VT) of any of the guide polynucleotides described herein, with any random nucleotide that can hybridize to any desired target sequence.

[0317] In some embodiments, a subject nucleic acid (*e.g.*, a guide polynucleotide, a nucleic acid comprising a nucleotide sequence encoding a guide polynucleotide; a nucleic acid encoding Cas9 endonuclease of the present disclosure; a crRNA or a nucleotide encoding a crRNA, a tracrRNA or a nucleotide encoding a tracrRNA, a nucleotide encoding a VT domain, a nucleotide encoding a CER domain, etc.) comprises a modification or sequence that provides for an additional desirable feature (*e.g.*, modified or regulated stability; subcellular targeting; tracking, *e.g.*, a fluorescent label; a binding site for a protein or protein complex; etc.). 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.

[0318] Functional variants of a guide polynucleotide of the present disclosure can comprise a modified guide polynucleotide wherein the modification comprises adding, removing, or otherwise altering loops and/or hairpins in the single guide RNA.

[0319] Functional variants of a guide polynucleotide of the present disclosure can comprise a modified guide polynucleotide wherein the modification comprises one or more modified nucleotides in the nucleotide sequence, wherein the one or more modified nucleotides comprises at least one non-naturally-occurring nucleotide, nucleotide mimetic (as described in US application US2014/0068797, published March 6, 2014), or analog thereof, or wherein the one or more modified nucleotides are selected from the group consisting of 2'-0-methylanalogs, 2'-fluoro analogs 2-aminopurine, 5-bromo-uridine, pseudouridine, and 7 -methylguanosine.

[0320] In some aspects, the functional variant of the guide RNA can form a guide RNA/Cas9 endonuclease complex that can recognize, bind to, and optionally nick or cleave a target sequence.

[0321] A guide polynucleotide/Cas endonuclease complex described herein is capable of recognizing, binding to, and optionally nicking, unwinding, or cleaving all or part of a target sequence.

[0322] A guide polynucleotide/Cas endonuclease complex that can cleave both strands of a DNA target sequence typically comprises a Cas polypeptide that has all of its endonuclease domains in a functional state (*e.g.*, wild type endonuclease domains or variants thereof retaining some or all activity in each endonuclease domain). Thus, a wild type Cas polypeptide (*e.g.*, a Cas polypeptide disclosed herein), or a variant thereof retaining some or all activity in each endonuclease domain

of the Cas polypeptide, is a suitable example of a Cas endonuclease that can cleave both strands of a DNA target sequence.

[0323] A guide polynucleotide/Cas endonuclease complex that can cleave one strand of a DNA target sequence can be characterized herein as having nickase activity (*e.g.*, partial cleaving capability). A Cas nickase typically comprises one functional endonuclease domain that allows the Cas to cleave only one strand (*i.e.*, make a nick) of a DNA target sequence. For example, a Cas nickase may comprise (i) a mutant, dysfunctional RuvC domain and (ii) a functional HNH domain (*e.g.*, wild type HNH domain). As another example, a Cas nickase may comprise (i) a functional RuvC domain (*e.g.*, wild type RuvC domain) and (ii) a mutant, dysfunctional HNH domain. Non-limiting examples of Cas nickases suitable for use herein are disclosed in US20140189896 published on 03 July 2014. A pair of Cas nickases can be used to increase the specificity of DNA targeting. In general, this can be done by providing two Cas nickases that, by virtue of being associated with RNA components with different guide sequences, target and nick nearby DNA sequences on opposite strands in the region for desired targeting. Such nearby cleavage of each DNA strand creates a double-strand break (*i.e.*, a DSB with single-stranded overhangs), which is then recognized as a substrate for non-homologous-end-joining, NHEJ (prone to imperfect repair leading to mutations) or homologous recombination, HR. Each nick in these embodiments can be at least 5, between 5 and 10, at least 10, between 10 and 15, at least15, between 15 and 20, at least 20, between 20 and 30, at least 30, between 30 and 40, at least 40, between 40 and 50, at least 50, between 50 and 60, at least 60, between 60 and 70, at least 70, between 70 and 80, at least 80, between 80 and 90, at least 90, between 90 and 100, or 100 or greater (or any number between 5 and 100) bases apart from each other, for example. One or two Cas nickase proteins herein can be used in a Cas nickase pair. For example, a Cas nickase with a mutant RuvC domain, but functioning HNH domain (*i.e.*, Cas HNH+/RuvC-), can be used (*e.g.*, *Streptococcus pyogenes* Cas HNH+/RuvC-). Each Cas nickase (*e.g.*, Cas HNH+/RuvC-) can be directed to specific DNA sites nearby each other (up to 100 base pairs apart) by using suitable RNA components herein with guide RNA sequences targeting each nickase to each specific DNA site.

[0324] A guide polynucleotide/Cas endonuclease complex in certain embodiments can bind to a DNA target site sequence, but does not cleave any strand at the target site sequence. Such a complex may comprise a Cas polypeptide in which all of its nuclease domains are mutant, dysfunctional. For example, a Cas polypeptide that can bind to a DNA target site sequence, but

does not cleave any strand at the target site sequence, may comprise both a mutant, dysfunctional RuvC domain and a mutant, dysfunctional HNH domain. A Cas polypeptide herein that binds, but does not cleave, a target DNA sequence can be used to modulate gene expression, for example, in which case the Cas polypeptide could be fused with a transcription factor (or portion thereof) (*e.g.*, a repressor or activator, such as any of those disclosed herein).

[0325] In some aspects of the disclosure, the guide polynucleotide/Cas endonuclease complex is a guide polynucleotide/Cas endonuclease complex (PGEN) comprising at least one guide polynucleotide and at least one Cas endonuclease polypeptide. In some aspects, the Cas endonuclease polypeptide comprises at least one protein subunit of another Cas polypeptide, or a functional fragment thereof, wherein said guide polynucleotide is a chimeric non-naturally occurring guide polynucleotide, wherein said guide polynucleotide/Cas endonuclease complex is capable of recognizing, binding to, and optionally nicking, unwinding, or cleaving all or part of a target sequence.

[0326] In some aspects, the PGEN is a ribonucleoprotein complex (RNP), wherein the Cas endonuclease is provided as a protein and the guide polynucleotide is provided as a ribonucleotide.

[0327] In some aspects of the disclosure, the guide polynucleotide/Cas effector complex is a guide polynucleotide/Cas endonuclease complex (PGEN) comprising at least one guide polynucleotide and a Cas endonuclease, wherein said guide polynucleotide/Cas endonuclease complex is capable of recognizing, binding to, and optionally nicking, unwinding, or cleaving all or part of a target sequence.

[0328] The PGEN can be a guide polynucleotide/Cas endonuclease complex, wherein said Cas endonuclease further comprises one copy or multiple copies of at least one protein subunit, or a functional fragment thereof, of an additional Cas polypeptide.

[0329] In some aspects, the guide polynucleotide/Cas endonuclease complex (PGEN) described herein is a PGEN, wherein said Cas endonuclease is covalently or non-covalently linked to at least one Cas polypeptide subunit, or functional fragment thereof. The PGEN can be a guide polynucleotide/Cas endonuclease complex, wherein said Cas endonuclease polypeptide is covalently or non-covalently linked, or assembled to one copy or multiple copies of at least one protein subunit, or a functional fragment thereof, of a Cas polypeptide selected from the group consisting of a Cas1 protein subunit, a Cas2 protein subunit, a Cas4 protein subunit, and any combination thereof, in some aspects effectively forming a cleavage ready Cascade. The PGEN

can be a guide polynucleotide/Cas endonuclease complex, wherein said Cas endonuclease is covalently or non-covalently linked or assembled to at least two different protein subunits of a Cas polypeptide selected from the group consisting of a Cas1, a Cas2, and Cas4. The PGEN can be a guide polynucleotide/Cas endonuclease complex, wherein said Cas endonuclease is covalently or non-covalently linked to at least three different protein subunits, or functional fragments thereof, of a Cas polypeptide selected from the group consisting of a Cas1, a Cas2, and Cas4, and any combination thereof.

[0330] Any component of the guide polynucleotide/Cas endonuclease complex, the guide polynucleotide/Cas endonuclease complex itself, as well as the polynucleotide modification template(s) and/or donor DNA(s), can be introduced into a heterologous cell or organism by any method known in the art.

[0331] Some uses for guide RNA/Cas9 endonuclease systems include but are not limited to modifying or replacing nucleotide sequences of interest (such as a regulatory elements), insertion of polynucleotides of interest, gene knock-out, gene-knock in, modification of splicing sites and/or introducing alternate splicing sites, modifications of nucleotide sequences encoding a protein of interest, amino acid and/or protein fusions, and gene silencing by expressing an inverted repeat into a gene of interest.

[0332] Methods and compositions are provided herein for the chemical modification or alteration of one or more nucleobases of a target polynucleotide, to change the base(s) from one type to another, for example from a Cytosine to a Thymine or an Adenine to a Guanine, using an RNA-guided Cas endonuclease that has been modified to lack double- or single-strand cleaving activity.

[0333] ***NHEJ and HDR***

[0334] In some aspects of the methods and compositions described herein, a genome editing system comprises a Cas endonuclease, one or more guide polynucleotides, and optionally donor DNA, and editing a target polynucleotide sequence comprises nonhomologous end-joining (NHEJ) or homologous recombination (HR) following a Cas endonuclease-mediated double-strand break. Once a double-strand break is induced in the DNA, the cell's DNA repair mechanism is activated to repair the break. The most common repair mechanism to bring the broken ends together is the nonhomologous end-joining 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 14:1121-

31; Pacher et al., (2007) Genetics 175:21-9). 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 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 152:1173-81).

[0335] 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. Once a double-strand break is introduced in the target site by the 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 target 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 plant genome, thereby altering the original target site and producing an altered genomic target site.

[0336] In some aspects of the methods and compositions described herein, the Cas polypeptide has endonuclease activity. In some aspects, the Cas polypeptide is Cas12f or Cas9. In some aspects of compositions for delivering guide polynucleotides, the composition further comprises a donor DNA. In some aspects of methods for editing a plant genome, the method further comprises providing the plant cell with a donor DNA.

[0337] ***Base Editing***

[0338] In some aspects of the methods and compositions described herein, a genome editing system comprises a base editing agent and a plurality of guide polynucleotides and editing a target polynucleotide sequence comprises introducing a plurality of nucleobase edits in the target polynucleotide sequence resulting in a variant nucleotide sequence.

[0339] One or more nucleobases of a target polynucleotide can be chemically altered, in some cases to change the base from one type to another, for example from a Cytosine to a Thymine, or an Adenine to a Guanine. In some aspects, a plurality of bases, for example, 2 or more, 5 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 60 or more, 70 or more, 80 or more

90 or more, 100 or more, or even greater than 100, 200 or more, up to thousands of bases may be modified or altered, to produce a plant with a plurality of modified bases.

[0340] Any base editing complex, such as a base editing agent associated with an RNA-guided protein, may be used to target and bind to a desired locus in the genome of an organism and chemically modify one or more components of a target polynucleotide.

[0341] Site-specific base conversions can be achieved to engineer one or more nucleotide changes to create one or more edits into the genome. These include for example, a site-specific base edit mediated by an C•G to T•A or an A•T to G•C base editing deaminase enzymes (Gaudelli et al., Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage." Nature (2017); Nishida et al. "Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems." Science 353 (6305) (2016); Komor et al. "Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage." Nature 533 (7603) (2016):420-4. A catalytically "dead" or inactive Cas9 (dCas9), for example a catalytically inactive "dead" version of a Cas endonuclease disclosed herein, fused to a cytidine deaminase or an adenine deaminase protein becomes a specific base editor that can alter DNA bases without inducing a DNA break. Base editors convert C->T (or G->A on the opposite strand) or an adenine base editor that would convert adenine to inosine, resulting in an A->G change within an editing window specified by the gRNA. Any molecule that effects a change in a nucleobase is a "base editing agent".

[0342] For many traits of interest, the creation of single double-strand breaks and the subsequent repair via HDR or NHEJ is not ideal for quantitative traits. An observed phenotype includes both genotype effects and environmental effects. The genotype effects further comprise additive effects, dominance effects, and epistatic effects. The probability of no effect per any single edit can be greater than zero, and any single phenotypic effect can be small, depending on the method used and site selected. Double-stranded break repair can additionally be "noisy" and have low repeatability.

[0343] One approach to ameliorate the probability of no effect per edit or small phenotypic effect outcome is to multiplex genome modification, such that a plurality of target sites are modified. Methods to modify a genomic sequence that do not introduce double-strand breaks would allow for single base substitutions. Combining these approaches, multiplexed base editing is beneficial for creating large numbers of genotype edits that can produce observable phenotype modifications.

In some cases, dozens or hundreds or thousands of sites can be edited within one or a few generations of an organism.

[0344] A multiplexed approach to base editing in an organism, has the potential to create a plurality of significant phenotypic variations in one or a few generations, with a positive directional bias to the effects. In some aspects, the organism is a plant. A plant or a population of plants with a plurality of edits can be cross-bred to produce progeny plants, some of which will comprise multiple pluralities of edits from the parental lines. In this way, accelerated breeding of desired traits can be accomplished in parallel in one or a few generations, replacing time-consuming traditional sequential crossing and breeding across multiple generations.

[0345] A base editing deaminase, such as a cytidine deaminase or an adenine deaminase, may be fused to an RNA-guided endonuclease that can be deactivated ("dCas", such as a deactivated Cas9) or partially active ("nCas", such as a Cas9 nickase) so that it does not cleave a target site to which it is guided. The dCas forms a functional complex with a guide polynucleotide that shares homology with a polynucleotide sequence at the target site, and is further complexed with the deaminase molecule. The guided Cas endonuclease recognizes and binds to a double-stranded target sequence, opening the double-strand to expose individual bases. In the case of a cytidine deaminase, the deaminase deaminates the cytosine base and creates a uracil. Uracil glycosylase inhibitor (UGI) is provided to prevent the conversion of U back to C. DNA replication or repair mechanisms then convert the Uracil to a thymine (U to T), and subsequent repair of the opposing base (formerly G in the original G-C pair) to an Adenine, creating a T-A pair. For example, see Komor *et al.* Nature Volume 533, Pages 420-424, 19 May 2016.

[0346] In some aspects of the methods and compositions described herein, the Cas polypeptide comprises a deactivated Cas endonuclease (dCas) operably associated with a deaminase such as a cytosine deaminase or an adenine deaminase. In some aspects, the dCas polypeptide is dCas12f or dCas9.

[0347] ***Prime Editing***

[0348] In some aspects of the methods and compositions described herein, a genome editing system comprises a prime editing agent and a guide polynucleotide and editing a target nucleotide

sequence comprises introducing one or more insertions, deletions, or nucleobase swaps in a target nucleotide sequence without generating a double-stranded DNA break.

[0349] In some aspects, the prime editing agent is a Cas polypeptide fused to a reverse transcriptase, wherein the Cas polypeptide is modified to nick DNA rather than generating double-strand break. This Cas-polypeptide-reverse transcriptase fusion can also be referred to as a "prime editor" or "PE". In some aspects, the guide polynucleotide comprises a prime editing guide polynucleotide (pegRNA), and is larger than standard sgRNAs commonly used for CRISPR gene editing (e.g., >100 nucleobases). The pegRNA comprises a primer binding sequence (PBS) and a template containing the desired or target RNA sequence at its 3' end.

[0350] During prime editing, the PE:pegRNA complex binds to a target DNA sequence and the modified Cas polypeptide nicks one target DNA strand resulting in a flap. The PBS on the pegRNA binds to the DNA flap and the target RNA sequence is reverse transcribed using the reverse transcriptase. The edited strand is incorporated into the target DNA at the end of the nicked flap, and the target DNA sequence is repaired with the new reverse transcribed DNA.

[0351] In some aspects of the methods and compositions described herein, the Cas polypeptide comprises a nickase Cas endonuclease (nCas) operably associated with a reverse transcriptase or co-expressed with a reverse transcriptase. In some aspects, the nCas polypeptide is nCas12f or nCas9.

[0352] ***Evaluation and Selection of Target Sites***

[0353] The length of the DNA sequence at 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 than 30 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.

[0354] Any cell genome can be evaluated for potential target sites for multiplexed base editing. In one non-limiting example, an elite inbred line of a particular plant is selected, wherein the elite inbred line displays one or more desirable phenotypes. However, the elite inbred line may further comprise some alleles that can be optimized. Candidate editing sites are selected via one of several methods that identifies such optimizable alleles, that may be neutral or deleterious in the original genome but positive, beneficial, or desirable after editing, such as with a deaminase base editor.

[0355] Any approach or combination of approaches may be used to find candidate sites in a genome for multiple site editing, for example but not limited to: evolutionary conservation, computational functional prediction, or candidate QTNs identified from experimentation or the literature. Because single edit effects are likely to be small, multiplexing of the edits is an important feature. In some cases, individual phenotypes affected may not be obvious, but fitness association would likely impact yield components of a plant.

[0356] In one approach, calculations of evolutionary conservation of individual nucleotides are performed and evaluated, such as Genomic Evolutionary Rate Profiling (GERP), which is a method for producing position-specific estimates of evolutionary constraint using maximum likelihood evolutionary rate estimation. Several "constrained elements" where multiple positions combine to give a signal that is indicative of a putative functional element are identified; this track shows the position-specific scores only, not the element predictions. Constraint intensity at each individual alignment position is quantified in terms of a "rejected substitutions" (RS) score, defined as the number of substitutions expected under neutrality minus the number of substitutions "observed" at the position.

[0357] Genomic sites for potential editing are scored independently. Positive scores represent a substitution deficit (*i.e.*, fewer substitutions than the average neutral site) and thus indicate that a site may be under evolutionary constraint. Negative scores indicate that a site is probably evolving neutrally; negative scores should not be interpreted as evidence of accelerated rates of evolution because of too many strong confounders, such as alignment uncertainty or rate variance. Positive scores scale with the level of constraint, such that the greater the score, the greater the level of evolutionary constraint inferred to be acting on that site.

[0358] Potential sites for selection for editing, including base editing, are made of alleles at evolutionarily conserved loci that display nucleotide positions that are different than the consensus across multiple genomes. In plants, incomplete dominance of deleterious alleles contributes to trait variation and heterosis in maize.

[0359] In another method, candidate edits can be selected based on attention-based predictor network algorithms. Sites with a mutation increasing expression levels of a gene are more conserved, while sites with a mutation decreasing expression levels of a gene are less conserved. For example, conserved sites with a particular allele that has predicted regulatory effects could be one target for base editing.

[0360] Identification of candidate alleles for editing can be, for example but not limited to, non-conserved bases in a particular cell line at a conserved (polymorphic) site, bases producing nonsense codons, and/or predicted rare nonsynonymous high impact substitutions.

[0361] ***Recombinant Constructs for Transformation of Cells***

[0362] The disclosed guide polynucleotides, Cas endonucleases, deaminases, and guide various molecular systems disclosed herein, and any one combination thereof, optionally further comprising one or more polynucleotide(s) or polypeptide(s) of interest, can be introduced into a cell. Cells include, but are not limited to, human, non-human, animal, bacterial, fungal, insect, yeast, non-conventional yeast, and plant cells as well as plants and seeds produced by the methods described herein.

[0363] 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*.

[0364] Vectors and constructs include circular plasmids, and linear polynucleotides, comprising a polynucleotide of interest and optionally other components including linkers, adapters, regulatory or analysis. In some examples a recognition site and/or target site can be comprised within an intron, coding sequence, 5' UTRs, 3' UTRs, and/or regulatory regions.

[0365] ***Components for Expression and Utilization of CRISPR-Cas Systems in Prokaryotic and Eukaryotic cells***

[0366] The invention further provides expression constructs for expressing in a prokaryotic or eukaryotic cell/organism a guide RNA/Cas system that is capable of recognizing, binding to, and optionally nicking, unwinding, or cleaving all or part of a target sequence.

[0367] In some aspects, the expression constructs of the disclosure comprise a promoter operably linked to a nucleotide sequence encoding a Cas gene (or optimized sequence, including a Cas endonuclease gene described herein) and a promoter operably linked to a guide RNA of the present disclosure. The promoter is capable of driving expression of an operably linked nucleotide sequence in a prokaryotic or eukaryotic cell/organism.

[0368] 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.

[0369] ***Expression Elements***

[0370] Any polynucleotide encoding a Cas endonuclease, guide RNA, or other CRISPR system component disclosed herein may be functionally linked to a heterologous expression element, to facilitate transcription or regulation in a host cell. Such expression elements include but are not limited to: promoter, leader, intron, and terminator. Expression elements may be "minimal" – meaning a shorter sequence derived from a native source, that still functions as an expression regulator or modifier. Alternatively, an expression element may be "optimized" – meaning that its polynucleotide sequence has been altered from its native state in order to function with a more desirable characteristic in a particular host cell. Alternatively, an expression element may be "synthetic" – meaning that it is designed in silico and synthesized for use in a host cell. Synthetic expression elements may be entirely synthetic, or partially synthetic (comprising a fragment of a naturally-occurring polynucleotide sequence).

[0371] A method of expressing RNA components such as gRNA in eukaryotic cells for performing Cas9-mediated DNA targeting has been to use RNA polymerase III (Pol III) promoters, which allow for transcription of RNA with precisely defined, unmodified, 5'- and 3'-ends (DiCarlo *et al.*, *Nucleic Acids Res.* 41: 4336-4343; Ma *et al.*, *Mol. Ther. Nucleic Acids* 3:e161). This strategy has been successfully applied in cells of several different species including maize and soybean (US20150082478 published 19 March 2015). Methods for expressing RNA components that do not have a 5' cap have been described (WO2016/025131 published 18 February 2016).

[0372] ***Optimization of Sequences for Expression in Plants***

[0373] 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.

[0374] ***Polynucleotides of Interest***

[0375] Polynucleotides of interest may be endogenous to the organism being edited, or maybe provided as heterologous molecules to the organism.

[0376] General categories of polynucleotides of interest include, for example, genes of interest 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 polynucleotides of interest include, but are not limited to, genes involved in crop yield, grain quality, crop nutrient content, starch and carbohydrate quality and quantity as well as those affecting kernel size, sucrose loading, protein quality and quantity, nitrogen fixation and/or utilization, fatty acid and oil composition, genes encoding proteins conferring resistance to abiotic stress (such as drought, nitrogen, temperature, salinity, toxic metals or trace elements, or those conferring resistance to molecules such as pesticides or herbicides), genes encoding proteins conferring resistance to biotic stress (such as attacks by fungi, viruses, bacteria, insects, or nematodes, and development of diseases associated with these organisms).

[0377] 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.

[0378] 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.

[0379] 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, also referred to as acetohydroxyacid synthase, AHAS), in particular the sulfonylurea (UK: sulphonylurea) 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 9,187,762. The bar gene encodes resistance to the herbicide basta, the nptII gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.

[0380] 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.

[0381] 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.

[0382] 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 comprises 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.

[0383] ***Introduction of CRISPR-Cas System Components into a Cell***

[0384] The methods and compositions described herein do not depend on a particular method for introducing a sequence into an organism or cell, only that the polynucleotide or polypeptide gains access to the interior of at least one cell of the organism. Introducing 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 (direct) provision of a nucleic acid, protein or polynucleotide-protein complex (PGEN, RGEN) to the cell.

[0385] Methods for introducing polynucleotides or polypeptides or a polynucleotide-protein complex into cells or organisms are known in the art including, but not limited to, microinjection, electroporation, stable transformation methods, transient transformation methods, ballistic particle acceleration (particle bombardment), whiskers mediated transformation, Agrobacterium-mediated transformation, direct gene transfer, viral-mediated introduction, transfection, transduction, cell-penetrating peptides, mesoporous silica nanoparticle (MSN)-mediated direct protein delivery, topical applications, sexual crossing , sexual breeding, and any combination thereof.

[0386] For example, the guide polynucleotide (guide RNA, crNucleotide + tracrNucleotide, guide DNA and/or guide RNA-DNA molecule) can be introduced into a cell directly (transiently) as a single stranded or double stranded polynucleotide molecule. The guide RNA (or crRNA + tracrRNA) can also be introduced into a cell indirectly by introducing a recombinant DNA molecule comprising a heterologous nucleic acid fragment encoding the guide RNA (or crRNA + tracrRNA), operably linked to a specific promoter that is capable of transcribing the guide RNA (crRNA+tracrRNA molecules) in said cell. The specific promoter can be, but is not limited to, a RNA polymerase III promoter, which allow for transcription of RNA with precisely defined, unmodified, 5'- and 3'-ends (Ma et al., 2014, Mol. Ther. Nucleic Acids 3:e161; DiCarlo et al.,

2013, Nucleic Acids Res. 41: 4336-4343; WO2015026887, published 26 February 2015). Any promoter capable of transcribing the guide RNA in a cell can be used and includes a heat shock /heat inducible promoter operably linked to a nucleotide sequence encoding the guide RNA.

[0387] The Cas endonuclease, such as the Cas endonuclease described herein, can be introduced into a cell by directly introducing the Cas polypeptide itself (referred to as direct delivery of Cas endonuclease), the mRNA encoding the Cas polypeptide, and/ or the guide polynucleotide/Cas endonuclease complex itself, using any method known in the art. The Cas endonuclease can also be introduced into a cell indirectly by introducing a recombinant DNA molecule that encodes the Cas endonuclease. The endonuclease can be introduced into a cell transiently or can be incorporated into the genome of the host cell using any method known in the art. Uptake of the endonuclease and/or the guided polynucleotide into the cell can be facilitated with a Cell Penetrating Peptide (CPP) as described in WO2016073433 published 12 May 2016. Any promoter capable of expressing the Cas endonuclease in a cell can be used and includes a heat shock /heat inducible promoter operably linked to a nucleotide sequence encoding the Cas endonuclease.

[0388] Direct delivery of a polynucleotide modification template into plant cells can be achieved through particle mediated delivery, and any other direct method of delivery, such as but not limiting to, polyethylene glycol (PEG)-mediated transfection to protoplasts, whiskers mediated transformation, electroporation, particle bombardment, cell-penetrating peptides, or mesoporous silica nanoparticle (MSN)-mediated direct protein delivery can be successfully used for delivering a polynucleotide modification template in eukaryotic cells, such as plant cells.

[0389] Direct delivery of any one of the guided Cas system components can be accompanied by direct delivery (co-delivery) of other mRNAs that can promote the enrichment and/or visualization of cells receiving the guide polynucleotide/Cas endonuclease complex components. For example, direct co-delivery of the guide polynucleotide/Cas endonuclease components (and/or guide polynucleotide/Cas endonuclease complex itself) together with mRNA encoding phenotypic markers (such as but not limiting to transcriptional activators such as CRC (Bruce et al. 2000 The Plant Cell 12:65-79) can enable the selection and enrichment of cells without the use of an exogenous selectable marker by restoring function to a non-functional gene product as described in WO2017070032 published 27 April 2017.

[0390] Introducing a guide RNA/Cas endonuclease complex described herein, into a cell includes introducing the individual components of said complex either separately or combined into the cell,

and either directly (direct delivery as RNA for the guide and protein for the Cas endonuclease and Cas polypeptide subunits, or functional fragments thereof) or via recombination constructs expressing the components (guide RNA, Cas endonuclease, Cas polypeptide subunits, or functional fragments thereof). Introducing a guide RNA/Cas endonuclease complex (RGEN) into a cell includes introducing the guide RNA/Cas endonuclease complex as a ribonucleotide-protein into the cell. The ribonucleotide-protein can be assembled prior to being introduced into the cell as described herein. The components comprising the guide RNA/Cas endonuclease ribonucleotide protein (at least one Cas endonuclease, at least one guide RNA, at least one Cas polypeptide subunits) can be assembled in vitro or assembled by any means known in the art prior to being introduced into a cell (targeted for genome modification as described herein).

[0391] Plant cells differ from human and animal cells in that plant cells comprise a plant cell wall which may act as a barrier to the direct delivery of the RGEN ribonucleoproteins and/or of the direct delivery of the RGEN components.

[0392] Direct delivery of the RGEN ribonucleoproteins into plant cells can be achieved through particle mediated delivery (particle bombardment. Based on the experiments described herein, a skilled artesian can now envision that any other direct method of delivery, such as but not limiting to, polyethylene glycol (PEG)-mediated transfection to protoplasts, electroporation, cell-penetrating peptides, or mesoporous silica nanoparticle (MSN)-mediated direct protein delivery, can be successfully used for delivering RGEN ribonucleoproteins into plant cells.

[0393] Direct delivery of the RGEN ribonucleoprotein, allows for genome editing at a target site in the genome of a cell which can be followed by rapid degradation of the complex, and only a transient presence of the complex in the cell. This transient presence of the RGEN complex may lead to reduced off-target effects. In contrast, delivery of RGEN components (guide RNA, Cas endonuclease) via plasmid DNA sequences can result in constant expression of RGENs from these plasmids which can intensify off target effects (Cradick, T. J. et al. (2013) Nucleic Acids Res 41:9584-9592; Fu, Y et al. (2014) Nat. Biotechnol. 31:822-826).

[0394] Direct delivery can be achieved by combining any one component of the guide RNA/Cas endonuclease complex (RGEN) (such as at least one guide RNA, at least one Cas polypeptide, and at least one Cas polypeptide), with a particle delivery matrix comprising a microparticle (such as but not limited to of a gold particle, tungsten particle, and silicon carbide whisker particle) (see also WO2017070032 published 27 April 2017).

[0395] In some aspects, the guide polynucleotide/Cas endonuclease complex is a complex wherein the guide RNA and Cas endonuclease protein forming the guide RNA /Cas endonuclease complex are introduced into the cell as RNA and protein, respectively.

[0396] In some aspects, the guide polynucleotide/Cas endonuclease complex is a complex wherein the guide RNA and Cas endonuclease protein and the at least one protein subunit of a Cas polypeptide forming the guide RNA/Cas endonuclease complex are introduced into the cell as RNA and proteins, respectively.

[0397] In some aspects, the guide polynucleotide/Cas endonuclease complex is a complex wherein the guide RNA and Cas endonuclease protein and the at least one protein subunit of a Cascade forming the guide RNA /Cas endonuclease complex (cleavage ready cascade) are preassembled in vitro and introduced into the cell as a ribonucleotide-protein complex.

[0398] Protocols for introducing polynucleotides, polypeptides or polynucleotide-protein complexes (PGEN, RGEN) into eukaryotic cells, such as plants or plant cells are known.

[0399] Alternatively, polynucleotides may be introduced into plant or plant cells by contacting cells or organisms 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 in vivo or 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.

[0400] The polynucleotide or recombinant DNA construct can be provided to or introduced into a prokaryotic and eukaryotic cell or organism using a variety of transient transformation methods. Such transient transformation methods include, but are not limited to, the introduction of the polynucleotide construct directly into the plant.

[0401] Methods for introducing more than one different transfer (T)-DNAs each encoding more than one guide RNA using Ochrobactrum-mediated transformation of plants are provided. Methods include, but are not limited to, using an Ochrobactrum strain to transfer a plurality of guide polynucleotides of interest to a plant cell. These methods include VirD2-dependent methods. Compositions include an Ochrobactrum strain, transfer DNAs, and constructs and/or plasmids. These compositions include Ochrobactrum strains having a plasmid comprising one or more

virulence gene(s), border region, and/or origin of replication. Plant cells, tissues, plants, and seeds comprising a polynucleotide of interest produced by the methods are also provided. The disclosure of US11236347B2, as it relates to the compositions and methods of transformation using a non-Agrobacterium strain, is incorporated herein by reference to its entirety. In an aspect, the Ochrobactrum is selected from the group consisting of Ochrobactrum haywardense H1, Ochrobactrum cytisi, Ochrobactrum daejeonense, Ochrobactrum lupine, Ochrobactrum oryzae, Ochrobactrum tritici, LBNL 124-A-10, HTG3-C-07 and Ochrobactrum pectoris.

[0402] Ochrobactrum is a bacterial genus in the Rhizobiales order, Brucellaceae family. Ochrobactrum strains are Gram-negative short rods, straight or slightly curved with one end flame-shaped. The cells are approximately 0.6 μm wide and 1.2 to 2 μm in length. Ochrobactrum are non-spore forming and are strictly aerobic and non-fermentative. The genomes of most Ochrobactrum species are complex with two independent circular chromosomes often associated to plasmids. The Ochrobactrum genus has been described by Holmes in 1988 (Holmes et al. (1988) Int J Syst Bacteriol 38:408) and Ochrobactrum anthropi was proposed as the type species of the genus. Further work led to the recognition of other species which include O. ciceri, O. cytisi, O. daejeonense, O. gallinifaecis, O. grignonense, O. guangzhouense, O. haematophilum, O. intermedium, O. lupini, O. oryzae, O. pecoris, O. pituitosum, O. pseudintermedium, O. pseudogrignonense, O. rhizosphaerae, O. thiophenivorans, and O. tritici.

[0403] In some examples of the present disclosure, an Ochrobactrum for transformation of cells is provided. In some examples, the Ochrobactrum is an Ochrobactrum grignonense. In some examples, the Ochrobactrum strain is Ochrobactrum haywardense H1 NRRL Deposit B-67078. Ochrobactrum haywardense H1 NRRL Deposit B-67078 may be referred to herein as Ochrobactrum haywardense H1 NRRL Deposit B-67078, Ochrobactrum haywardense H1, or EP1A09. In some examples, the Ochrobactrum vector comprises one or more virulence gene, a border region, and/or origin of replication. In some examples, the vector comprises a selectable marker(s) for plant and bacterial transformation. In some examples, one or more virulence genes is selected from the group consisting of virA, virB, virC, virD, virE, virF, virG, and variants thereof, and any combinations thereof.

[0404] Nucleic acids and proteins can be provided to a cell by any method including methods using molecules to facilitate the uptake of anyone or all components of a guided Cas system

(protein and/or nucleic acids), such as cell-penetrating peptides and nanocarriers. See also US20110035836 published 10 February 2011, and EP2821486A1 published 07 January 2015.

[0405] Other methods of introducing polynucleotides into a prokaryotic and eukaryotic cell or organism or plant part can be used, including plastid transformation methods, and the methods for introducing polynucleotides into tissues from seedlings or mature seeds.

[0406] Stable transformation is intended to mean that the nucleotide construct introduced into an organism integrates into a genome of the organism and is capable of being inherited by the progeny thereof. Transient transformation is intended to mean that a polynucleotide is introduced into the organism and does not integrate into a genome of the organism or a polypeptide is introduced into an organism. Transient transformation indicates that the introduced composition is only temporarily expressed or present in the organism.

[0407] A variety of methods are available to identify those cells having an altered genome at or near a 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.

[0408] The presently disclosed polynucleotides and polypeptides can be introduced into a cell. Cells include, but are not limited to, human, non-human, animal, mammalian, bacterial, protist, fungal, insect, yeast, non-conventional yeast, and plant cells, as well as plants and seeds produced by the methods described herein. In some aspects, the cell of the organism is a reproductive cell, a somatic cell, a meiotic cell, a mitotic cell, a stem cell, or a pluripotent stem cell.

[0409] ***Cells and Plants***

[0410] The presently disclosed polynucleotides and polypeptides can be introduced into a plant cell. Plant cells include, well as plants and seeds produced by the methods described herein. Any plant can be used with the compositions and methods described herein, including monocot and dicot plants, and plant elements.

[0411] The novel Cas endonucleases disclosed may be used to edit the genome of a plant cell in various ways. In some aspects, it may be desirable to delete one or more nucleotides. In another aspect, it may be desirable to insert one or more nucleotides. In some aspects, it may be desirable to replace one or more nucleotides. In another aspect, it may be desirable to modify one or more

nucleotides via a covalent or non-covalent interaction with another atom or molecule. In some aspects, the cell is diploid. In some aspects, the cell is haploid.

[0412] Genome modification via a Cas endonuclease may be used to effect a genotypic and/or phenotypic change on the target organism. Such a change is preferably related to an improved trait of interest or an agronomically-important characteristic, the correction of an endogenous defect, or the expression of some type of expression marker. In some aspects, the trait of interest or agronomically-important characteristic is related to the overall health, fitness, or fertility of the plant, the yield of a plant product, the ecological fitness of the plant, or the environmental stability of the plant. In some aspects, the trait of interest or agronomically-important characteristic is selected from the group consisting of: agronomics, herbicide resistance, insecticide resistance, disease resistance, nematode resistance, microbial resistance, fungal resistance, viral resistance, fertility or sterility, grain characteristics, commercial product production. In some aspects, the trait of interest or agronomically-important characteristic is selected from the group consisting of: disease resistance, drought tolerance, heat tolerance, cold tolerance, salinity tolerance, metal tolerance, herbicide tolerance, improved water use efficiency, improved nitrogen utilization, improved nitrogen fixation, pest resistance, herbivore resistance, pathogen resistance, yield improvement, health enhancement, vigor improvement, growth improvement, photosynthetic capability improvement, nutrition enhancement, altered protein content, altered starch content, altered carbohydrate content, altered sugar content, altered fiber content, altered oil content, increased biomass, increased shoot length, increased root length, improved root architecture, modulation of a metabolite, modulation of the proteome, increased seed weight, altered seed carbohydrate composition, altered seed oil composition, altered seed protein composition, altered seed nutrient composition, as compared to an isoline plant not comprising a modification derived from the methods or compositions herein.

[0413] 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 species, for example Triticum aestivum, Triticum monococcum), sugarcane (Saccharum spp.), oats (Avena), barley (Hordeum), switchgrass (Panicum virgatum), pineapple (Ananas comosus), banana (Musa spp.), palm, ornamentals, turfgrasses, and other grasses.

[0414] Examples of dicot plants that can be used include, but are not limited to, soybean (Glycine max), Brassica species (for example but not limited to: oilseed rape or Canola) (Brassica napus, B. campestris, Brassica rapa, Brassica juncea), alfalfa (Medicago sativa), tobacco (Nicotiana tabacum), Arabidopsis (Arabidopsis thaliana), sunflower (Helianthus annuus), cotton (Gossypium arboreum, Gossypium barbadense), and peanut (Arachis hypogaea), tomato (Solanum lycopersicum), potato (Solanum tuberosum).

[0415] Additional plants that can be used include safflower (Carthamus tinctorius), sweet potato (Ipomoea batatus), cassava (Manihot esculenta), coffee (Coffea spp.), coconut (Cocos nucifera), citrus trees (Citrus spp.), cocoa (Theobroma cacao), tea (Camellia sinensis), banana (Musa spp.), avocado (Persea americana), fig (Ficus casica), guava (Psidium guajava), mango (Mangifera indica), olive (Olea europaea), papaya (Carica papaya), cashew (Anacardium occidentale), macadamia (Macadamia integrifolia), almond (Prunus amygdalus), sugar beets (Beta vulgaris), vegetables, ornamentals, and conifers.

[0416] Vegetables that can be used include tomatoes (Lycopersicon esculentum), lettuce (e.g., Lactuca sativa), green beans (Phaseolus vulgaris), lima beans (Phaseolus limensis), peas (Lathyrus spp.), and members of the genus Cucumis such as cucumber (C. sativus), cantaloupe (C. cantalupensis), and musk melon (C. melo). Ornamentals include azalea (Rhododendron spp.), hydrangea (Macrophylla hydrangea), hibiscus (Hibiscus rosasanensis), roses (Rosa spp.), tulips (Tulipa spp.), daffodils (Narcissus spp.), petunias (Petunia hybrida), carnation (Dianthus caryophyllus), poinsettia (Euphorbia pulcherrima), and chrysanthemum.

[0417] Conifers that may be used include pines such as loblolly pine (Pinus taeda), slash pine (Pinus elliotii), ponderosa pine (Pinus ponderosa), lodgepole pine (Pinus contorta), and Monterey pine (Pinus radiata); Douglas fir (Pseudotsuga menziesii); Western hemlock (Tsuga canadensis); Sitka spruce (Picea glauca); redwood (Sequoia sempervirens); true firs such as silver fir (Abies amabilis) and balsam fir (Abies balsamea); and cedars such as Western red cedar (Thuja plicata) and Alaska yellow cedar (Chamaecyparis nootkatensis).

[0418] 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 comprised 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.

[0419] The present disclosure finds use in the breeding of plants comprising one or more edited alleles created by the methods or compositions disclosed herein. In some aspects, the edited alleles influence the phenotypic expression of one or more traits, such as plant health, growth, or yield. In some aspects, two plants may be crossed via sexual reproduction to create progeny plant(s) that comprise some or all of the edits from both parental plants.

[0420] ***Cells and Animals***

[0421] The presently disclosed polynucleotides and polypeptides can be introduced into an animal cell. Animal cells can include, but are not limited to: an organism of a phylum including chordates, arthropods, mollusks, annelids, cnidarians, or echinoderms; or an organism of a class including mammals, insects, birds, amphibians, reptiles, or fishes. In some aspects, the animal is human, mouse, C. elegans, rat, fruit fly (Drosophila spp.), zebrafish, chicken, dog, cat, guinea pig, hamster, chicken, Japanese ricefish, sea lamprey, pufferfish, tree frog (e.g., Xenopus spp.), monkey, or chimpanzee. Particular cell types that are contemplated include haploid cells, diploid cells, reproductive cells, neurons, muscle cells, endocrine or exocrine cells, epithelial cells, muscle cells, tumor cells, embryonic cells, hematopoietic cells, bone cells, germ cells, somatic cells, stem cells, pluripotent stem cells, induced pluripotent stem cells, progenitor cells, meiotic cells, and mitotic cells. In some aspects, a plurality of cells from an organism may be used.

[0422] The novel Cas endonucleases disclosed may be used to edit the genome of an animal cell in various ways. In some aspects, it may be desirable to delete one or more nucleotides. In another aspect, it may be desirable to insert one or more nucleotides. In some aspects, it may be desirable to replace one or more nucleotides. In another aspect, it may be desirable to modify one or more nucleotides via a covalent or non-covalent interaction with another atom or molecule.

[0423] Genome modification via a Cas endonuclease may be used to effect a genotypic and/or phenotypic change on the target organism. Such a change is preferably related to an improved phenotype of interest or a physiologically-important characteristic, the correction of an endogenous defect, or the expression of some type of expression marker. In some aspects, the phenotype of interest or physiologically-important characteristic is related to the overall health, fitness, or fertility of the animal, the ecological fitness of the animal, or the relationship or interaction of the animal with other organisms in its environment. In some aspects, the phenotype

of interest or physiologically-important characteristic is selected from the group consisting of: improved general health, disease reversal, disease modification, disease stabilization, disease prevention, treatment of parasitic infections, treatment of viral infections, treatment of retroviral infections, treatment of bacterial infections, treatment of neurological disorders (for example but not limited to: multiple sclerosis), correction of endogenous genetic defects (for example but not limited to: metabolic disorders, Achondroplasia, Alpha-1 Antitrypsin Deficiency, Antiphospholipid Syndrome, Autism, Autosomal Dominant Polycystic Kidney Disease, Barth syndrome, Breast cancer, Charcot-Marie-Tooth, Colon cancer, Cri du chat, Crohn's Disease, Cystic fibrosis, Dercum Disease, Down Syndrome, Duane Syndrome, Duchenne Muscular Dystrophy, Factor V Leiden Thrombophilia, Familial Hypercholesterolemia, Familial Mediterranean Fever, Fragile X Syndrome, Gaucher Disease, Hemochromatosis, Hemophilia, Holoprosencephaly, Huntington's disease, Klinefelter syndrome, Marfan syndrome, Myotonic Dystrophy, Neurofibromatosis, Noonan Syndrome, Osteogenesis Imperfecta, Parkinson's disease, Phenylketonuria, Poland Anomaly, Porphyria, Progeria, Prostate Cancer, Retinitis Pigmentosa, Severe Combined Immunodeficiency (SCID), Sickle cell disease, Skin Cancer, Spinal Muscular Atrophy, Tay-Sachs, Thalassemia, Trimethylaminuria, Turner Syndrome, Velocardiofacial Syndrome, WAGR Syndrome, and Wilson Disease), treatment of innate immune disorders (for example but not limited to: immunoglobulin subclass deficiencies), treatment of acquired immune disorders (for example but not limited to: AIDS and other HIV-related disorders), treatment of cancer, as well as treatment of diseases, including rare or "orphan" conditions, that have eluded effective treatment options with other methods.

[0424] Cells that have been genetically modified using the compositions or methods disclosed herein may be transplanted to a subject for purposes such as gene therapy, e.g. to treat a disease, or as an antiviral, antipathogenic, or anticancer therapeutic, for the production of genetically modified organisms in agriculture, or for biological research.

[0425] While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention. For instance, while the particular examples below may illustrate the methods and embodiments described herein using a specific plant, the principles in these examples may be applied to any plant. Therefore, it will be appreciated that the scope of this

invention is encompassed by the embodiments of the inventions recited herein and in the specification rather than the specific examples that are exemplified below. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety, for all purposes, to the same extent as if each were individually and specifically incorporated by reference.

## EXAMPLES

[0426] The following are examples of specific embodiments of some aspects of the invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the invention in any way.

[0427] **Table 1** details sequences used in the Examples.

**Table 1: Sequences**

| Sequence |
|---|
| TGAGAGTACAATGATGAACCTAGATTAATCAATGCCAAAGTCTGAAAAATGCACCCTCAGTCTATG<br>ATCCAGAAAATCAAGATTGCTTGAGGCCCTGTTCGGTTGTTCCGGATTAGAGCCCCGGATTAATTCC<br>TAGCCGGATTACTTCTCTAATTTATATAGATTTTGATGAGCTGGAATGAATCCTGGCTTATTCCGGTA<br>CAACCGAACAGGCCCTGAAGGATACCAGTAATCGCTGAGCTAAATTGGCATGCTGTCAGAGTGTCA<br>GTATTGCAGCAAGGTAGTGAGATAACCGGCATCATGGTGCCAGTTTGATGGCACCATTAGGGTTAG<br>AGATGGTGGCCATGGGCGCATGTCCTGGCCAACTTTGTATGATATATGGCAGGGTGAATAGGAAAG<br>TAAAATTGTATTGTAAAAAGGGATTTCTTCTGTTTGTTAGCGCATGTACAAGGAATGCAAGTTTTGA<br>GCGAGGGGGCATCAAAGATCTGGCTGTGTTTCCAGCTGTTTTTGTTAGCCCCATCGAATCCTTGACA<br>TAATGATCCCGCTTAAATAAGCAACCTCGCTTGTATAGTTCCTTGTGCTCTAACACACGATGATGAT<br>AAGTCGTAAAATAGTGGTGTCCAAAGAATTTCCAGGCCCAGTTGTAAAAGCTAAAATGCTATTCGA<br>ATTTCTACTAGCAGTAAGTCGTGTTTAGAAATTATTTTTTTATATACCTTTTTTCCTTCTATGTACAGT<br>AGGACACAGTGTCAGCGCCGCGTTGACGGAGAATATTTGCAAAAAAGTAAAAGAGAAAGTCATAG<br>CGGCGTATGTGCCAAAAACTTCGTCACAGAGAGGGCCATAAGAAACATGGCCCACGGCCCAATACG<br>AAGCACCGCGACGAAGCCCAAACAGCAGTCCGTAGGTGGAGCAAAGCGCTGGGTAATACGCAAAC<br>GTTTTGTCCCACCTTGACTAATCACAAGAGTGGAGCGTACCTTATAAACCGAGCCGCAAGCACCGA<br>ATT **(SEQ ID NO: 1)** |
| MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTAR<br>RRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRK<br>KLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKA<br>ILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLA<br>QIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIF<br>FDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAI<br>LRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIE<br>RMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTV<br>KQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIE<br>ERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSL<br>TFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQ<br>KGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVD<br>HIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGG<br>LSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREI<br>NNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFF |

| |
|---|
| KTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSD KLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKG YKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQK QLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYF DTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD **(SEQ ID NO: 2)** |
| CCCACGGCCCAATACGAAGCACCGCGACGAAGCCCAAACAGCAGTCCGTAGGTGGAGCAAAGCGC TGGGTAATACGCAAACGTTTTGTCCCACCTTGACTAATCACAAGAGTGGAGCGTACCTTATAAACCG AGCCGCAAGCACCGAATT **(SEQ ID NO: 3)** |
| TCTGCTTTTTCGCATGATATCTGGGCCGCACCAAAGAATCCAGCCCACGCGGCGTGGCGCCGTCGTT ACGGCTTGCGGGGGAAGGAAACGAGGGACGAACCGAGATTTAGCACCAGACCGGCCAGCGAGCAT TGCAGACACCGGCTTATAAGTTCAGCTGCGACTACCACTCC **(SEQ ID NO: 4)** |
| TCCATGGCCCATTATAAAGCACCGCCACAAAGCCCAAATAGCAGTTCGTCGGTGGAGCAAGCATCG CGCTAGGCAACAGGCAAACAGTTTGTCCCACCTCGTCCAGTCACAAAATAAAAGCACGAGTTATAA ACCAAACCGGAAGCACCGCATC **(SEQ ID NO: 5)** |
| CCCTGTTCCGCTTTGCTAGCTTGCGCCCTGACTGTCCAGCCCACGCGCTTCGGTCCGATTCACATGCT AGGCTGGTGCAAGCGAGCCGAGACTTTTTTTTAGAACCACCTTGCTCAGCAAACCTTAGGAACACC GGCTTATAAGTCGAAGCGAAGCGCTGTGCACT **(SEQ ID NO: 6)** |
| TCAATGGCCCATTATATAAAGCACCGCCACAAAGCCCAAATACCAGTTCGTCGGTGGAGCAAGTAA CGCGCTAGGCAACAGGCAAACAGTTTGTCCCACCTCGTCCAGTCACAAAGGCAAAGCGTGACTTAT AAGCCAGAGCGGAAGAACCATACC **(SEQ ID NO: 7)** |
| GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGC ACCGAGUCGGUGCU **(SEQ ID NO: 8)** |
| GUUUUAGAGCUGGAAACAGCGAGUUAAAAUAAGGCUUUGUCCGUACACAACUUGUAAAAGGGGC ACCCGAUUCGGGUGCAU **(SEQ ID NO: 9)** |
| GUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCGUACACAACUUGAAAAAGUGCG CACCGAUUCGGUGCU **(SEQ ID NO: 10)** |
| GUUUUAGAGCGAAAGCAAGUUAAAAUAAGGCUUUGUCCUUUAUCAUCUCGCUUAGCGAGUGGCG CUGUCUCGGCGCU **(SEQ ID NO: 11)** |
| GUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCGUUUCCAACAUUUGUGGCGCUG UCUCGGCGCU **(SEQ ID NO: 12)** |
| GUUUUAGAGCUAGAAAUAGCGAGUUAAAAUAAGGCUAGUCCGUAAUCAACUUGAAAAAGUGAG CACCGAGUCGGUGCU **(SEQ ID NO: 13)** |
| GAAGGGGCTTCGGAGGATCGG **(SEQ ID NO: 14)** |
| AAGTTCACGGCGTTCCAGGCGGG **(SEQ ID NO: 15)** |
| GGCCGAGGTCGACTACCGGCCGG **(SEQ ID NO: 16)** |
| GCATAATGAGGATCGAGGATGAGG **(SEQ ID NO: 17)** |
| GGCATCTACAGGGACGCAAAGG **(SEQ ID NO: 18)** |
| GAAGGGGCUUCGGAGGAUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGAAAAAGUGGCACCGAGUCGGUGCU **(SEQ ID NO: 19)** |
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGU UAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU **(SEQ ID NO: 20)** |
| GGCCGAGGUCGACUACCGGCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUU AUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU **(SEQ ID NO: 21)** |
| GCAUAAUGAGGAUCGAGGAUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCG UUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU **(SEQ ID NO: 22)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGAAAAAGUGGCACCGAGUCGGUGCU **(SEQ ID NO: 23)** |
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCUGGAAACAGCGAGUUAAAAUAAGGCUUUGUCCG UACACAACUUGUAAAAGGGGCACCCGAUUCGGGUGCAU **(SEQ ID NO: 24)** |

| |
|---|
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCG UACACAACUUGAAAAAGUGCGCACCGAUUCGGUGCU **(SEQ ID NO: 25)** |
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCGAAAGCAAGUUAAAAUAAGGCUUUGUCCUUUAU CAUCUCGCUUAGCGAGUGGCGCUGUCUCGGCGCU **(SEQ ID NO: 26)** |
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCG UUUCCAACAUUUGUGGCGCUGUCUCGGCGCU **(SEQ ID NO: 27)** |
| GAAGUUCACGGCGUUCCAGGCGUUUUAGAGCUAGAAAUAGCGAGUUAAAAUAAGGCUAGUCCGU AAUCAACUUGAAAAAGUGAGCACCGAGUCGGUGCU **(SEQ ID NO: 28)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCUGGAAACAGCGAGUUAAAAUAAGGCUUUGUCCGUA CACAACUUGUAAAAGGGGCACCCGAUUCGGGUGCAU **(SEQ ID NO: 29)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCGUA CACAACUUGAAAAAGUGCGCACCGAUUCGGUGCU **(SEQ ID NO: 30)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCGAAAGCAAGUUAAAAUAAGGCUUUGUCCUUUAUCA UCUCGCUUAGCGAGUGGCGCUGUCUCGGCGCU **(SEQ ID NO: 31)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCUGGAAACAGCAAGUUAAAAUAAGGCUUUGUCCGUU UCCAACAUUUGUGGCGCUGUCUCGGCGCU **(SEQ ID NO: 32)** |
| GGCAUCUACAGGGACGCAAGUUUUAGAGCUAGAAAUAGCGAGUUAAAAUAAGGCUAGUCCGUAA UCAACUUGAAAAAGUGAGCACCGAGUCGGUGCU **(SEQ ID NO: 33)** |
| MKELSTIQKREKLNTVERIGSEGPGGAYHEYVIKSNSMDSQGNYDVYETIKFQKGARKEEKSQHGVIDS DLLEIVRDRLKSFQAGPFSSRENACALTHVEEALMWMNRRVEDRIERNVLGTNTK **(SEQ ID NO: 34)** |
| MEIKNGLCTQKYTKVYAEDKEKWKFNAPHHFIVGKADCEDEYIEPIEYVNFQEGPIKEYGINGVNNEDLI LMVITRLQAFQDSPYKCRENAMAITKLQECLMWLGKRTLDREVKGIEGTSEI **(SEQ ID NO: 35)** |
| TTTTTTT **(SEQ ID NO: 36)** |

**Example 1: Construction of T-DNAs containing multiple guide RNA expression cassettes**

[0428] In this example, methods for constructing transfer (T)-DNAs encoding more than one guide RNA capable of directing a Cas effector polypeptide to multiple different DNA target sites in a plant cell are described.

[0429] In one method, the overall length and repetitiveness of a T-DNA harboring multiple guide RNA expression cassettes was reduced. This reduces complexity while also preserving sequence integrity by minimizing the probability of sequence loss due to recombination during synthesis, plasmid construction, and maintenance after delivery into a plant cell. First, a collection of sequence-diverse and compact U6 plant promoters were developed. As an example of a plant U6 promoter, a *Zea mays* U6 promoter previously demonstrated to drive guide RNA expression in maize (SEQ ID NO: 1) (Svitashev *et al.* (2015) *Plant Physiology*. 169, 931-945) was used initially. To reduce its length, it was first systematically truncated while monitoring the efficacy of *Streptococcus pyogenes* (Spy) Cas9 (SEQ ID NO: 2) targeted mutagenesis using transient Particle Gun (PG) transformation (Karvelis *et al.* (2015) *Genome Biology*. 16, 253). It was observed that a promoter length as short as 150 bp (SEQ ID NO: 3) provided editing frequencies similar to that of the original 1 kb length promoter. Next, the nucleotide sequence of a maize U6 gene (Veretnik and

Rubenstein (1990) *Nucleic Acid Research*. 18, 3661) was used as a query in BLAST (Altschul *et al.* (1990) *Journal of Molecular Biology*. 215, 403–410) and additional maize U6 genes identified. The 1 kb region immediately upstream of the newly identified genes was then isolated and aligned to the initial 1 kb length promoter using MUSCLE (Edgar (2004) *BMC Bioinformatics*. 5, 113). Next, using the functionally validated 150 bp promoter as a reference, new U6 promoters were trimmed down, realigned, and promoters differing in sequence composition by at least 10% selected. Using this alignment-based approach, a collection of 11 U6 promoters were identified that ranged in length from 144-175 bp (SEQ ID NOs: 4-7, 86-91) and varied in percent identity from 18.7-87.8%. When aligned to each other, they demonstrated several regions of conservation (**FIGS. 1A** and **1B**). To confirm functionality, four of the new promoters (SEQ ID Nos: 4-7) varying in percent identity and length from 35.8-87.8% and 154-173 bp, respectively, were selected and evaluated for their ability to support guide RNA expression by measuring SpyCas9 targeted mutagenesis frequencies in T0 plants produced from *Agrobacterium* (Agro) transformation relative to the original U6 promoter (minimized by 150 bp; SEQ ID NO: 3).

[0430] **Table 2** illustrates SpyCas9 targeted mutagenesis frequencies of guide RNAs expressed from compact and sequence diverse *Zea mays* U6 promoters. Each of the five U6 promoters was used to express a guide RNA targeting a different *Zea mays* genomic site NAC7 (SEQ ID NO: 14), MS26 (SEQ ID NO: 15), MS45 (SEQ ID NO: 16), TS45 (SEQ ID NO: 17), and WXY (SEQ ID NO: 18) for cleavage. All five guide RNA expression cassettes containing the respective U6 promoter (SEQ ID NOs: 3-7), guide RNA encoding sequence (SEQ ID NOs: 19-23), and U6 terminator (SEQ ID NO: 36) were concatenated, synthesized (GenScript, USA), and Gateway cloned into a single T-DNA, which further contained SpyCas9, BabyBoom (BBM), Wuschel2 (WUS2), and neomycin phosphotransferase II (NPTII) expression cassettes. Transformation, selection, and plant regeneration were subsequently performed (Lowe *et al.*, 2018). T0 plants containing a targeted insertion and/or deletion (indel) mutation were classified as containing a monoallelic or biallelic indel based on the total frequency of mutant sequence reads (Bigelyte *et al.*, 2021). Plants with greater than or equal to 30% but less than 80% targeted mutagenesis were categorized as monoallelic while those with greater than or equal to 80% indel frequencies deemed biallelic. As shown in **Table 2**, all four of the new U6 promoters supported robust targeted mutagenesis with greater than 85% of the regenerated plants containing biallelic target site

modifications resulting in efficiencies near or greater than that producing when using SEQ ID NO: 3.

[0431] Similar to that described above, a collection of compact and sequence diverse U6 promoters were also established for use in *Glycine max*. Here, 14 promoters were identified ranging in size from 218 to 309 bp and differed in homology from 22.9% to 81% (SEQ ID Nos: 96-109). As with the *Zea mays* U6 promoters, several conserved motifs were identified when aligned (**FIGS. 2A-2E**). To increase diversity further, U6 promoters from other legumes may also be used (e.g., *Medicago truncatula* (Kim and Nam (2013) Plant Mol Bio Rep. 31, 581-593; SEQ ID Nos:113-121).

**Table 2: Targeted Mutagenesis Frequencies**

| U6 promoter | SpyCas9 sgRNA | Genomic Target | % Monoallelic T0 Plants with Targeted Indel | % Biallelic T0 Plants with Targeted Indel |
|---|---|---|---|---|
| SEQ ID NO: 3 | SEQ ID NO: 19 | NAC7 | 1 | 91 |
| SEQ ID NO: 4 | SEQ ID NO: 20 | MS26 | 4 | 87 |
| SEQ ID NO: 5 | SEQ ID NO: 21 | MS45 | 3 | 94 |
| SEQ ID NO: 6 | SEQ ID NO: 22 | TS45 | 1 | 87 |
| SEQ ID NO: 7 | SEQ ID NO: 23 | WXY | 2 | 97 |

[0432] To further minimize repetitiveness, additional guide RNAs were identified from CRISPR systems homologous to SpyCas9 by using SpyCas9 polypeptide (SEQ ID NO: 2) as a query in BLAST (Altschul *et al.*, 1990) against the NCBI non-redundant (NR) protein sequence database. DNA loci encoding SpyCas9 orthologs were then retrieved and manually confirmed to contain an intact CRISPR system minimally comprising a *cas9* gene, a trans-activating CRISPR RNA (tracrRNA), and CRISPR array encoding a CRISPR RNA (crRNA) using methods described in the art (Karvelis *et al.*, 2015 and Dooley *et al.* (2021) *The CRISPR Journal.* 4, 438-447). The 3' end of the tracrRNA and 5' end of the crRNA were next linked together with a self-folding GAAA tetraloop into a single guide RNA (sgRNA) (Jinek *et al.* (2012) *Science.* 337, 816) backbone (sgRNA without targeting spacer sequence) and validated to support SpyCas9 editing in corn using Particle Gun transient testing (Karvelis *et al.*, 2015). In addition to the SpyCas9 sgRNA backbone (SEQ ID NO: 8), three new sgRNA backbones engineered from homologous CRISPR systems from *Streptococcus macacae* (Sma), *Facklamia hominis* (Fho), and *Streptococcus canis* (Sca) were

shown to support robust SpyCas9 editing (SEQ ID Nos: 9, 12, and 13). They ranged in size from 74-81 nts and varied in percent identity from 73.4-96.2%.

[0433] **Table 3** illustrates SpyCas9 targeted mutagenesis frequencies using guide RNA homologs. Using Particle Gun (PG) transformation, SpyCas9 and guide RNA expression cassettes were co-delivered into 9-to-10-day old immature maize embryos. Evenly transformed embryos (based on expression of an immunofluorescent protein) were then harvested two days after transformation and the frequency of targeted indels evaluated using Ampli-seq (Karvelis *et al.*, 2015). Experiments were performed in duplicate at two different SpyCas9 target sites, MS26 (SEQ ID NO: 15) and WXY (SEQ ID NO: 18). Indel frequencies across target sites were averaged and standard error measurement (SEM) was used to capture variation. The 150 bp truncated *Zea mays* U6 promoter 1 (SEQ ID NO: 3) was used to drive transcription of each guide RNA homolog targeting either MS26 or WXY (SEQ ID NOs: 24-33).

**Table 3: Targeted Mutagenesis Frequencies**

| sgRNA Backbone Source | sgRNA Targeting MS26 | sgRNA Targeting WXY | Average Indel % | SEM |
|---|---|---|---|---|
| Spy | SEQ ID NO: 20 | SEQ ID NO: 23 | 0.55 | 0.18 |
| Sma | SEQ ID NO: 24 | SEQ ID NO: 29 | 0.75 | 0.33 |
| Sor | SEQ ID NO: 25 | SEQ ID NO: 30 | 0.11 | 0.05 |
| Dpi | SEQ ID NO: 26 | SEQ ID NO: 31 | 0.29 | 0.15 |
| Fho | SEQ ID NO: 27 | SEQ ID NO: 32 | 0.47 | 0.06 |
| Sca | SEQ ID NO: 28 | SEQ ID NO: 33 | 0.56 | 0.09 |

[0434] Next, the T-DNA was constructed. For this, a Gateway compatible T-DNA plasmid destination vector was first built encoding the SpyCas9 DNA effector polypeptide, plant morphogenic factors Babyboom (BBM) and Wushcel2 (WUS2), and selectable marker neomycin phosphotransferase II (NPTII) as described in the art (Karimi *et al.* (2002) *Trends in Plant Science*. 7, 193-195). Protospacer targets in the vicinity of a suitable protospacer adjacent motif (PAM) for SpyCas9 (NGG) were then isolated and concatenated to the 5' end of the sequences encoding the functionally validated and sequence diverse sgRNAs at random *in silico*. Five of the compact and sequence diverse U6 promoters validated earlier were then operably linked to the 5' end of the resulting sequences incorporating a G bp to promote robust transcription. To ensure maximum sequence diversity, a different promoter was used for the first five sgRNAs and then recycled for the next five as needed across the collection of sgRNA encoding sequences. Promoter linked

sequences were next appended with a U6 terminator (5'-TTTTTTTT-3'(SEQ ID NO: 36)) at their 3' end and synthesized (GenScript, USA) into one, two, or three gateway entry plasmids. Then using Gateway cloning (Hartley *et al.* (2007) *Genome Research.* 10, 1788-1795), introduced into the destination cassette. Colonies positive for recombination with the destination vector were then confirmed by restriction enzyme digestion, transformed into LBA4404 (a thymine auxotrophic strain of *Agrobacterium* (Agro) tumefaciens) containing Vir9 (a separate plasmid encoding the Bo542 virulence genes (US20170121722A1 and WO 2017/078836)), and sequence confirmed. An example of a T-DNA containing 48 sgRNA expression cassettes constructed using this method is shown in **FIG. 3**.

**Example 2: Delivery of T-DNA encoding 48 sgRNAs**

[0435] An *Agrobacterium* strain harboring a T-DNA encoding 48 sgRNAs and a SpyCas9 nuclease (Example 1) was used to transform nine-to-ten-day old immature embryos (approximately 2 mm in size) as described earlier (Lowe et al. (2018) In vitro cellular & developmental biology-Plant. 54, 240-252). Briefly, immature embryos (IEs) were submersed in 700A liquid media containing the strain at an optical density (OD) of 0.4 to 2.0 at 550 nm for 5 min., removed from the media, and placed on solid co-cultivation medium overnight at 21°C in the dark. Following T-DNA delivery, embryos were transferred to resting media, 13266R, and grown in the dark at 28-37°C for ~3 days and then moved to 28°C (if incubated at a higher temperature) until day 7. Coleoptiles were subsequently removed, the embryos transferred to selection media for 11-16 days, and then kept in the dark at 28°C. Embryos were then moved to maturation media, incubated for 14-25 days in the dark at 28°C, and then subject to light for 2-5 days. Embryos were then moved to rooting media and incubated at 26-28°C under light for 14-28 days refreshing media as needed.

[0436] Following transformation, plants were regenerated, DNA extracted from leaf tissue samples, and each genomic site evaluated for the presence of a target modification using Illumina amplicon sequencing as described earlier (Svitashev et al. (2015) Plant Physiology. 169, 931-945 and Karvelis et al. (2015) Genome Biology. 16, 253). To confirm that targeted mutations were likely to be germline, only regenerated maize plants containing targeted mutations frequencies of 30% or greater relative to wildtype reads were counted (Bigelyte et al. (2021) Nature Communications. 12, 6191). To distinguish between transient and stable delivery of the sgRNA

expression cassettes, the region of the T-DNA encoding the targeting region of each guide RNA, the spacer, was also subject to Illumina sequencing. In this way, if a targeted mutation was recovered but the sequence encoding the respective sgRNA was absent, the plant was classified as having transient sgRNA delivery. In contrast, if the sequence encoding the respective guide RNA was present (at least 1,000 Illumina reads), the plant was labeled as having a sgRNA stably integrated into its genome.

[0437] As shown in **FIG. 6**, 72 out of the 94 (77%) T0 corn plants regenerated contained all forty-eight sgRNA expression cassettes stably integrated in the genome. Moreover, 47 of the 94 (50%) plants exhibited targeted alteration(s) at thirty or more sites (**FIG. 7**) with many sites demonstrating bi-allelic targeted modification (**FIG. 8**). Taken together, the vector construction methods described herein permitted the stable delivery of forty-eight sgRNA expression cassettes enabling highly efficient multiplexed DNA target editing in a plant cell.

**Example 3: Co-delivery of T-DNAs into a plant cell**

[0438] In this example, methods for introducing multiple different T-DNAs into a plant cell are described.

[0439] In a first method, *Agrobacterium* transformation was utilized to deliver two or more different T-DNAs into the same plant cell (**FIG. 4**). For this, transformation was setup as described earlier (Lowe *et al.*, 2018) except different *Agrobacterium* strains, each containing a different T-DNA, were mixed in equal concentrations (based on optical density (OD) readings) and used to infect maize immature embryos. To measure T-DNA co-delivery into a single cell, five different T-DNAs were initially constructed each encoding a SpyCas9 nuclease (SEQ ID NO: 2) and a unique sgRNA designed to direct double-stranded DNA target cleavage in unique genomic locations. Targets included NAC7, MS26, MS45, TS45, and WXY (SEQ ID NOs: 14-18). Following transformation, plants were regenerated, DNA extracted from leaf tissue samples, and each target site evaluated for the presence of a targeted mutation as described above. To distinguish between transient and stable T-DNA delivery, the region of the T-DNA encoding the targeting region of each guide RNA, the spacer, was also subject to Illumina sequencing as described above. In this way, if a targeted mutation was recovered but the sequencing encoding the respective guide RNA was not present, the plant was classified as having transient T-DNA delivery. In contrast, if

the sequence encoding the respective guide RNA was abundantly present (at least 1,000 Illumina reads), the plant was labeled as having a T-DNA stably integrated into its genome.

**Example 4: Co-delivery of T-DNAs into a plant cell with increased *Agrobacterium* concentration**

[0440] To determine if T-DNA co-delivery could be enhanced, the method of Example 3 was combined with an additional treatment. In a first treatment, the relative abundance of each of the five *Agrobacterium* strains was increased from an $OD_{600}$ of 0.4 to 2.0 before mixing. Immature embryos were also subjected to a five-minute incubation at 45°C, and subsequently cooled to room temperature just prior to *Agrobacterium* infection.

[0441] As shown in **Table 4** and **Table 5**, elevated *Agrobacterium* concentrations decreased transient delivery and increased stable delivery allowing up to five different T-DNAs to be stably co-delivered into the genome of a single plant. When each of the five *Agrobacterium* strains was grown to an $OD_{600}$ of 0.4, mixed, and used to infect immature embryos, 29% of the 188 regenerated plants contained two or more T-DNA species stably integrated into their genome while the transient delivery of one or more different T-DNAs occurred in 54% of the plants. At an $OD_{600}$ of 1.2, transient delivery was identical (54%), but the percentage of plants demonstrated to harbor two or more T-DNA species in their genome increased to 49%. Finally, an $OD_{600}$ of 2.0, reduced transient delivery down to 23% and permitted the stable delivery of two or more different T-DNAs at a frequency of 47%. Additionally, an $OD_{600}$ of 2.0 resulted in elevated frequencies of stable delivery for four and five different T-DNAs compared with 0.4 and 1.2 $OD_{600}$ concentrations.

**Table 4: Transient T-DNA co-delivery as a function of *Agrobacterium* concentration**

| No. of T-DNAs Delivered Transiently | % of T0 Plants | | |
|---|---|---|---|
| | $OD_{600}$ of 0.4 | $OD_{600}$ of 1.2 | $OD_{600}$ of 2.0 |
| 0 | 46 | 46 | 77 |
| 1 | 43 | 41 | 19 |
| 2 | 11 | 11 | 4 |
| 3 | 1 | 2 | 1 |
| 4 | 0 | 1 | 0 |
| 5 | 0 | 0 | 0 |

**Table 5: Stable T-DNA co-delivery as a function of *Agrobacterium* concentration**

| No. of T-DNAs Delivered Stably | % of T0 Plants | | |
|---|---|---|---|
| | $OD_{600}$ of 0.4 | $OD_{600}$ of 1.2 | $OD_{600}$ of 2.0 |

| | | | |
|---|---|---|---|
| 0 | 5 | 3 | 5 |
| 1 | 66 | 48 | 48 |
| 2 | 24 | 36 | 35 |
| 3 | 3 | 12 | 8 |
| 4 | 1 | 1 | 4 |
| 5 | 0 | 0 | 1 |

**Example 5: Co-delivery of T-DNAs into a plant cell with immune blocker**

[0442] To determine if T-DNA co-delivery can be enhanced, the method of Example 3 is combined with genes encoding proteins demonstrated to block immune response(s) in heterologous organisms. These genes are codon optimized and incorporated into the T-DNA and/or in associated *Agrobacterium* virulence plasmids to promote multiple rounds of *Agrobacterium* infection and the co-delivery of different T-DNAs. An example of this, is a Thoeris anti-defense 1 (Tad1) protein (SEQ ID NOs: 34 and 35) from bacteriophages shown to block immune response signaling molecules (variants and isomers of cyclic adenine diphosphate ribose (cADPR)) produced by Toll/interleukin-1 receptors in bacteria (Leavitt *et al.* (2022) Nature. https://doi.org/10.1038/s41586-022-05375-9).

**Example 6: Highly multiplexed guide RNA delivery using *Agrobacterium***

[0443] In this example, methods for introducing more than one different transfer (T)-DNAs each encoding more than one guide RNA using *Agrobacterium* into a plant cell are described. The methods of Examples 1-5 are combined to deliver tens or hundreds of guide RNAs into a plant cell using *Agrobacterium* transformation (**FIG. 5**).

**Example 7: Highly multiplexed guide RNA delivery using *Ochrobactrum***

[0444] In this example, methods for introducing more than one different transfer (T)-DNAs each encoding more than one guide RNA using *Ochrobactrum*-mediated transformation of plants are provided. Methods include, but are not limited to, using an *Ochrobactrum* strain to transfer a plurality of guide polynucleotides of interest to a plant cell. These methods include VirD2-dependent methods. Compositions include an *Ochrobactrum* strain, transfer DNAs, and constructs and/or plasmids. These compositions include *Ochrobactrum* strains having a plasmid comprising one or more virulence gene(s), border region, and/or origin of replication. Plant cells, tissues,

plants, and seeds comprising a polynucleotide of interest produced by the methods are also provided.

## CLAIMS

1. A method of delivering guide polynucleotides to a plant cell, the method comprising:

transforming the plant cell with a first T-DNA, the first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the plant cell; and

transforming the plant cell with a second T-DNA, the second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell;

wherein the optical density of the first and second T-DNAs ranges from about 0.2 to about 3.0.

2. The method of claim 1, wherein the first and second T-DNAs are *Agrobacterium.*
3. The method of claim 1, wherein the first and second T-DNAs are *Ochrobacterium*.
4. The method of any one of claims 1-3, wherein the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.
5. The method of any one of claims 1-4, wherein the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the first and second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
6. The method of any one of claims 1-5, further comprising pre-treating the plant cell prior to transforming with the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell for about 2 minutes to about 10 minutes at a temperature above about 28°C.
7. The method of claim 6, wherein the temperature ranges from about 28°C to about 50°C.

8. The method of any one of claims 1-5, further comprising pre-treating the plant cell prior to transforming with the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell at about 45°C for about 5 minutes.
9. The method of any one of claims 1-8, further comprising inhibiting an immune response in the plant cell.
10. The method of any one of claims 1-8, further comprising inhibiting cyclic adenine diphosphate ribose, or a variant or isomer thereof, in the plant cell.
11. The method of claim 10, wherein inhibiting cyclic adenine diphosphate ribose, or the variant or isomer thereof, comprises providing to the plant cell a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide.
12. The method of claim 11, wherein the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell prior to transforming the plant cell with the first and second T-DNAs.
13. The method of claim 11, wherein the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell simultaneously with the first and second T-DNAs.
14. The method of any one of claims 1-13, further comprising transforming the plant cell with a third T-DNA, the third T-DNA expressing a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
15. The method of any one of claims 1-14, further comprising transforming the plant cell with a fourth T-DNA, the fourth T-DNA expressing a fourth plurality of guide polynucleotides for multiplexed genome editing of a fourth set of target nucleotide

sequences in the plant cell, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

16. The method of any one of claims 1-15, further comprising transforming the plant cell with a fifth T-DNA, the fifth T-DNA expressing a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
17. The method of any one of claims 1-4, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.
18. The method of any one of claims 14-16, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of

guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

19. The method of any one of claims 1-18, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.
20. The method of any one of claims 1-18, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.
21. The method of any one of claims 1-19, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.
22. The method of claim 20, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.
23. The method of any one of claims 1-18 or 20, wherein each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.
24. The method of any one of claims 1-18, wherein each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.
25. The method of any one of claims 14-16, wherein each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising:
    a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;

b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;

c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

d. at least 95% sequence identity to SEQ ID Nos: 96-109; or

e. at least 95% sequence identity to SEQ ID Nos: 113-121.

26. The method of any one of claims 19-25, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises:

a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and

e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,

wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

27. The method of claim 25, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises:

a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and

e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,

wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

28. The method of any one of claims 1-27, wherein each guide polynucleotide is expressed by a separate promoter.

29. The method of claim 28, wherein the separate promoter of each guide polynucleotide is a U6 promoter comprising:

a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;

b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;

c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

d. at least 95% sequence identity to SEQ ID Nos: 96-109; or

e. at least 95% sequence identity to SEQ ID Nos: 113-121.

30. A composition for delivering guide polynucleotides for multiplexed genome editing in a plant cell, the composition comprising:

(a) a first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the first plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence;

(b) a second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; and

(c) optionally an inhibitor of cyclic adenine diphosphate ribose, or a variant or isomer thereof.

31. The composition of claim 30, wherein the inhibitor is a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide.

32. The composition of claim 30 or claim 31, wherein the first and second T-DNAs are *Agrobacterium*.

33. The composition of claim 30 or claim 31, wherein the first and second T-DNAs are *Ochrobacterium*.
34. The composition of any one of claims 30-33, wherein the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.
35. The composition of any one of claims 30-34, wherein the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides.
36. The composition of any one of claims 30-35, further comprising a third T-DNA comprising a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
37. The composition of any one of claims 30-36, further comprising a fourth T-DNA comprising a fourth plurality of guide polynucleotides for multiplexed genome editing of a fourth set of target nucleotide sequences in the plant cell, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least

seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

38. The composition of any one of claims 30-37, further comprising a fifth T-DNA comprising a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
39. The composition of any one of claims 30-34, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.
40. The composition of any one of claims 36-38, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

41. The composition of any one of claims 30-40, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.
42. The composition of any one of claims 30-40, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.
43. The composition of any one of claims 30-41, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.
44. The composition of claim 43, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.
45. The composition of any one of claims 30-40 or 42, wherein each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.
46. The composition of any one of claims 30-40, wherein each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.
47. The composition of any one of claims 36-38, wherein each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising:
    a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;
    b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;
    c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;
    d. at least 95% sequence identity to SEQ ID Nos: 96-109; or
    e. at least 95% sequence identity to SEQ ID Nos: 113-121.

48. The composition of any one of claims 30-47, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises:

a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and

e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,

wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

49. The composition of claim 47, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises:

a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and

e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,

wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

50. The composition of any one of claims 30-49, wherein each guide polynucleotide is expressed by a separate promoter.

51. The composition of claim 50, wherein the separate promoter of each guide polynucleotide is a U6 promoter comprising:
    a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;
    b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;
    c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;
    d. at least 95% sequence identity to SEQ ID Nos: 96-109; or
    e. at least 95% sequence identity to SEQ ID Nos: 113-121.
52. The composition of any one of claims 30-51, further comprising a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide.
53. The composition of claim 52, wherein the Cas polypeptide has endonuclease activity.
54. The composition of claim 51 or claim 52, further comprising a donor DNA or a polynucleotide modification template.
55. The composition of any one of claims 52-54, wherein the Cas polypeptide is Cas12a, Cas12f, or Cas9.
56. The composition of claim 52, wherein the Cas polypeptide comprises a deactivated Cas endonuclease (dCas).
57. The composition of claim 56, wherein the dCas is dCas12f or dCas9.
58. The composition of claim 52 or claim 56, wherein the dCas endonuclease is operably associated with a deaminase.
59. The composition of claim 58, wherein the deaminase is a cytosine deaminase or an adenine deaminase.
60. The composition of claim 52, wherein the Cas polypeptide has nickase activity and is operably associated with or co-expressed with a reverse transcriptase.
61. A method for editing a plant genome at a plurality of target sites in the genome, the method comprising:
    providing a plant cell with:
    (a) a Cas polypeptide or a polynucleotide sequence encoding a Cas polypeptide;
    (b) a first T-DNA expressing a first plurality of guide polynucleotides for multiplexed genome editing of a first set of target nucleotide sequences in the

plant cell, wherein each guide polynucleotide of the first plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; and

(c) a second T-DNA expressing a second plurality of guide polynucleotides for multiplexed genome editing of a second set of target nucleotide sequences in the plant cell, wherein each guide polynucleotide of the second plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence; wherein the optical density of the first and second T-DNAs ranges from about 0.2 to about 3.0;

introducing site-specific modifications in the first set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the first plurality of guide polynucleotides; and

introducing site-specific modifications in the second set of target nucleotide sequences in the plant cell when the Cas polypeptide complexes with each guide polynucleotide in the second plurality of guide polynucleotides.

62. The method of claim 61, wherein the first and second T-DNAs are *Agrobacterium.*
63. The method of claim 61, wherein the first and second T-DNAs are *Ochrobacterium*.
64. The method of any one of claims 61-63, wherein the optical density of the first and second T-DNAs ranges from about 0.8 to about 2.0.
65. The method of any one of claims 61-64, wherein the first plurality of guide polynucleotides, the second plurality of guide polynucleotides, or both the first plurality and second plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides.
66. The method of any one of claims 61-65, further comprising pre-treating the plant cell prior to providing the Cas polypeptide and the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell for about 2 minutes to about 10 minutes at a temperature above about 28°C.

67. The method of claim 66, wherein the temperature ranges from about 28°C to about 50°C.
68. The method of any one of claims 61-65, further comprising pre-treating the plant cell prior to providing the Cas polypeptide and the first and second T-DNAs, wherein pre-treating the plant cell comprises incubating the plant cell at about 45°C for about 5 minutes.
69. The method of any one of claims 61-68, further comprising inhibiting an immune response in the plant cell.
70. The method of any one of claims 61-68, further comprising inhibiting cyclic adenine diphosphate ribose, or a variant or isomer thereof, in the plant cell.
71. The method of claim 70, wherein inhibiting cyclic adenine diphosphate ribose, or the variant or isomer thereof, comprises providing to the plant cell a Tad1 polypeptide or a polynucleotide sequence encoding a Tad1 polypeptide.
72. The method of claim 71, wherein the Tad1 polypeptide or the polynucleotide sequence encoding the Tad1 polypeptide is provided to the plant cell simultaneously with the first and second T-DNAs.
73. The method of any one of claims 61-72, further comprising providing the plant cell with a third T-DNA, the third T-DNA expressing a third plurality of guide polynucleotides for multiplexed genome editing of a third set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the third set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the third plurality of guide polynucleotides, wherein the third plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the third plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.
74. The method of any one of claims 61-73, further comprising providing the plant cell with a fourth T-DNA, the fourth T-DNA expressing a fourth plurality of guide polynucleotides

for multiplexed genome editing of a fourth set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the fourth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fourth plurality of guide polynucleotides, wherein the fourth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fourth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

75. The method of any one of claims 61-74, further comprising providing the plant cell with a fifth T-DNA, the fifth T-DNA expressing a fifth plurality of guide polynucleotides for multiplexed genome editing of a fifth set of target nucleotide sequences in the plant cell, and introducing site-specific modifications in the fifth set of target nucleotide sequences when the Cas polypeptide complexes with each guide polynucleotide in the fifth plurality of guide polynucleotides, wherein the fifth plurality of guide polynucleotides comprises at least ten guide polynucleotides, alternatively at least twenty guide polynucleotides, alternatively at least thirty guide polynucleotides, alternatively at least forty guide polynucleotides, alternatively at least fifty guide polynucleotides, alternatively at least sixty guide polynucleotides, alternatively at least seventy guide polynucleotides, alternatively at least eighty guide polynucleotides, alternatively at least ninety guide polynucleotides, alternatively at least one hundred guide polynucleotides, wherein each guide polynucleotide of the fifth plurality of guide polynucleotides is distinct and has a unique target nucleotide sequence.

76. The method of any one of claims 61-75, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to

100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.

77. The method of any one of claims 73-76, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises up to 5 guide polynucleotides, alternatively up to 10 guide polynucleotides, alternatively up to 15 guide polynucleotides, alternatively up to 20 guide polynucleotides, alternatively up to 25 guide polynucleotides, alternatively up to 50 guide polynucleotides, alternatively up to 100 guide polynucleotides, alternatively up to 250 guide polynucleotides, alternatively up to 500 guide polynucleotides, alternatively up to 750 guide polynucleotides, alternatively up to 1000 guide polynucleotides.
78. The method of any one of claims 61-77, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94.
79. The method of any one of claims 61-77, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter comprising a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112.
80. The method of any one of claims 61-78, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 1-7 or 86-91.
81. The method of claim 80, wherein each guide polynucleotide from the first and second plurality of guide polynucleotides is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91.
82. The method of any one of claims 61-77 or 79, wherein each guide polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 96-109.
83. The method of any one of claims 61-77, wherein each polynucleotide is operably linked to a U6 promoter having at least 95% sequence identity to SEQ ID Nos: 113-121.

84. The method of any one of claims 73-75, wherein each guide polynucleotide of the third plurality of guide polynucleotides, each guide polynucleotide of the fourth plurality of guide polynucleotides, and/or each guide polynucleotide of the fifth plurality of guide polynucleotides is operably linked to a U6 promoter, the U6 promoter comprising:
    a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;
    b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;
    c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;
    d. at least 95% sequence identity to SEQ ID Nos: 96-109; or
    e. at least 95% sequence identity to SEQ ID Nos: 113-121.
85. The method of any one of claims 61-84, wherein the first plurality of guide polynucleotides and/or the second plurality of guide polynucleotides comprises:
    a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;
    b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;
    c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;
    d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and
    e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,
    wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.
86. The method of claim 85, wherein the third plurality of guide polynucleotides, the fourth plurality of guide polynucleotides, and/or the fifth plurality of guide polynucleotides comprises:
    a. a first guide polynucleotide operably linked to a U6 promoter comprising at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

b. a second guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

c. a third guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91;

d. a fourth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91; and

e. a fifth guide polynucleotide operably linked to a U6 promoter comprising at least 95% identity to SEQ ID Nos: 4-7 or 86-91,

wherein the SEQ ID NO of the U6 promoter of the first, second, third, fourth, and fifth guide polynucleotide is unique.

87. The method of any one of claims 61-77, wherein each guide polynucleotide is expressed by a separate promoter.

88. The method of claim 87, wherein the separate promoter of each guide polynucleotide is a U6 promoter comprising:

a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;

b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;

c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

d. at least 95% sequence identity to SEQ ID Nos: 96-109; or

e. at least 95% sequence identity to SEQ ID Nos: 113-121.

89. The method of any one of claims 61-88, wherein the Cas polypeptide has endonuclease activity.

90. The method of claim 89, further comprising providing the plant cell with a donor DNA or a polynucleotide modification template.

91. The method of any one of claims 61-90, wherein the Cas polypeptide is Cas12f or Cas9.

92. The method of any one of claims 61-88, wherein the Cas polypeptide comprises a deactivated Cas endonuclease (dCas).

93. The method of claim 92, wherein the dCas is dCas12f or dCas9.

94. The method of claim 92 or claim 93, wherein the dCas endonuclease is operably associated with a deaminase.

95. The method of claim 94, wherein the deaminase is a cytosine deaminase or an adenine deaminase.
96. The method of any one of claims 61-95, further comprising providing one or more developmental genes to the plant cell.
97. The method of claim 96, wherein the one or more developmental genes is BBM and/or WUS.
98. The method of any one of claims 61-97, wherein the first T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.
99. The method of any one of claims 61-98, wherein the second T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.
100. The method of any one of claims 61-99, wherein the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.
101. The method of any one of claims 61-100, wherein the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.
102. The method of any one of claims 61-100, wherein introducing site-specific modifications in the first and/or second set of target nucleotide sequences in the plant cell comprises bi-allelic site-specific modification of the target nucleotide sequences.
103. The method of any one of claims 1-29, further comprising providing one or more developmental genes to the plant cell.
104. The method of claim 102, wherein the one or more developmental genes is BBM and/or WUS.
105. The method of any one of claims 1-29, wherein the first T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.

106. The method of any one of claims 1-29, wherein the second T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.

107. The method of any one of claims 1-29, wherein the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

108. The method of any one of claims 1-29, wherein the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

109. The composition of any one of claims 30-60, further comprising an expression cassette comprising one or more developmental genes.

110. The composition of claim 108, wherein the one or more developmental genes is BBM and/or WUS.

111. The composition of claim 108 or claim 109, wherein the expression cassette comprising the one or more developmental genes is expressed by the first T-DNA and/or the second T-DNA.

112. The composition of any one of claims 30-60, wherein the first T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the first plurality of guide polynucleotides.

113. The composition of any one of claims 30-60, wherein the second T-DNA comprises a plurality of expression cassettes, each expression cassette expressing a guide polynucleotide of the second plurality of guide polynucleotides.

114. The composition of any one of claims 30-60, wherein the first plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

115. The composition of any one of claims 30-60, wherein the second plurality of guide polynucleotides comprises 5 guide polynucleotides, wherein each of the 5 guide

polynucleotides is expressed by a unique expression cassette and each expression cassette comprises a unique promoter sequence.

116. A recombinant DNA construct comprising a U6 promoter, wherein the U6 promoter comprises:

a. a first motif having SEQ ID NO: 92, a second motif having SEQ ID NO: 93, and a third motif having SEQ ID NO: 94;

b. a first motif having SEQ ID NO: 110, a second motif having SEQ ID NO: 111, and a third motif having SEQ ID NO: 112;

c. at least 95% sequence identity to SEQ ID Nos: 4-7 or 86-91;

d. at least 95% sequence identity to SEQ ID Nos: 96-109;

e. at least 95% sequence identity to SEQ ID Nos: 113-121;

f. a polynucleotide sequence of SEQ ID Nos: 4-7 or 86-91;

g. a polynucleotide sequence of SEQ ID Nos: 96-109; or

h. a polynucleotide sequence of SEQ ID Nos: 113-121.

117. The recombinant DNA construct of claim 116, further comprising a guide polynucleotide operably linked to the U6 promoter.

FIG. 1A

```
          :                                                    :
i)  -150 NMHDB----------------------NDNHNNNNN--BNHHBMNNNN -127
ii)      CCCAC----------------------GGCCCAATA--CGAAGCA-CC
iii)     TCTGCTTTTTCGCATGATATCTGGGCCGCACCAAAGAA--TCCAGCCCAC
iv)      GCCGC----------------------GCCTAAGCA--TCCAGCCCAC
v)       TCCAT----------------------GGCCCATTA--TAAAGCA-CC
vi)      TCCAC----------------------TGAT-------CCACCCCCAC
vii)     GCCTC----------------------GTTCCACTTT-GCTAGCTTGC
viii)    CCCTG----------------------TTCC-GCTTT-GCTAGCTTGC
ix)      TCCGT----------------------GGCCCAATA--CAAAGCA-CC
x)       TCAAT----------------------GGCCCATTATATAAAGCA-CC
xi)      ACTTC----------------------GATGTAATTT-TTTTTCTAGA
xii)     GACAT----------------------TATTGCAATC-GCAAGAG---

               ::          :     :
i)  -126 NNNNN-RW-NBNNB---MNHBNNSVB---------BBNNNNDNB------  -97
ii)      GCGAC-GA-AGCCC---AAACAGCAG---------TCCGTAGGT------
iii)     GCGGC-GT-GGCGCCGTCGTTACGGC---------TT----GCG------
iv)      GCGGGCGTGCGCGTCGTCGCTACGGC---------TT----GCG------
v)       GCCAC-AA-AGCCC---AAATAGCAG---------TTCGTCGGT------
vi)      GCGGC-GT-GGCGTCGTCATTAACGG---------CT----TGT------
vii)     ACCTT-GA-CT--T---CCATCCCATG--------CGCTTCGATCCGATT
viii)    GCCCT-GA-CT-GT---CCAGCCCACG--------CGCTTCGGTCCGATT
ix)      GCAAC-AA-AGCCC---AAATAACAG---------TTCGTCGGT------
x)       GCCAC-AA-AGCCC---AAATACCAG---------TTCGTCGGT------
xi)      AAATG-GA-TC-AG---ATATGTCATGTTGAGTGTGGCTTCGGTCCGTGT
xii)     ------AA-GTCCG---AGACTGCCGC--------CGCTTCATC------

           :    : ::         : :                   :   :*:
i)   -96 --SND-RVWR----VVNDSDRB-------NV----D-YNVRCM------N  -72
ii)      --GGA-GCAA----AGCGCTGGG------TA----A-TACGCA------A
iii)     --GGG-GAAG----GAAACGAG-------GG----A-CGAACC------G
iv)      --GGG-GAAG----GGATCAAG-------GG----A-CGAACC------G
v)       --GGA-GCAAGCATCGCGCTAGG------CA----A-CAGGCA------A
vi)      --GGG-GAAG----GGAACGAG-------CA----A-CTAACC------G
vii)     CACAA-GCTAG---GCTGGTGC-------AA----G-CGAGCCGAGA--C
viii)    CACAT-GCTAG---GCTGGTGC-------AA----G-CGAGCCGAGACTT
ix)      --GGA-GCAA----CGCGCAACG------CACTAGG-CAAGCA------A
x)       --GGA-GCAAGTAACGCGCTAGG------CA----A-CAGGCA------A
xi)      CAGTGCGGTGC---GAGACTAT-------GA----G-CCAGCCGAGT--T
xii)     --GCA-AAAACTCACGCACAAGTGCATGCAC----TCTAGGCC---A--C
```

# FIG. 1B

```
                :*:: ::   ::: ::: ::::  :         :    :  ::**
i)   -71 NNDDYTWKNMY---SWSHYYRNYYRRHNRNVNDDNNVNRNNNBRNNKWAT -25
ii)      ACGTTTTGTCC---CACCTTGACTAATCACAAGAGTGGAGCGTACCTTAT
iii)     AGATTTAGCAC---CAGACCGGCCAGCGAGCATTGCAGACACCGGCTTAT
iv)      AGAACTAGTAC---CAGACCGGCCAGCGAGCATTGCAGACACCGGCTTAT
v)       ACAGTTTGTCC---CACCTCGTCCAGTCACAAAATAAAAGCACGAGTTAT
vi)      ATAATTAGTAC---CAGACCGGCCAGTGAACGATGCCAAAACCGGCTTAT
vii)     TTTTTTAGAAC---CACCTCGCTCAGCAAACTTGAGGAACACCAGCTTAT
viii)    TTTTTTAGAAC---CACCTTGCTCAGCAAACCTTAGGAACACCGGCTTAT
ix)      ATAGTTTGTCC---CACCTCGTCCAGTCACGGAACCAAAGTTGAAGTTAT
x)       ACAGTTTGTCC---CACCTCGTCCAGTCACAAAGGCAAAGCGTGACTTAT
xi)      --TGTTTGTACCACCTCATCACTCGGCTAACAATAGACATGCCAGCTTAT
xii)     TTATTTATGAT---GTGTTTAGTTAGATGTATAAGAA-GGAATA--GAAT


         **: : :   ::           ::
i)   -24 AARHYNRDB-YRNDDN-HHBHDHWYB -1  +1
ii)      AAACCGAGC-CGCAAG-CACCGAATT
iii)     AAGTTCAGC-TGCGAC-TACCACTCC
iv)      AAGTTCAGC-TGCGAC-CACCGCTCC
v)       AAACCAAAC-CGGAAG-CACCGCATC
vi)      AAGCTCAGC-TGCGAC-AACCGTTTT
vii)     AAGTCAAAG-CGTAGCGTTGTGCTCT
viii)    AAGTCGAAG-CGAAGCGCTGTGCACT
ix)      AAACCTGAC-CGAAGA-CACCACATC
x)       AAGCCAGAG-CGGAAG-AACCATACC
xi)      AAGCCTGTG-TGTAGCCACTATTACT
xii)     AAAACGGTTATATTTT-TTTATATTG


i)    SEQ ID NO:85     Motif 1: YTWKNMY (SEQ ID NO:92)
ii)   SEQ ID NO:3      Motif 2: SWSHYYRNYYRR (SEQ ID NO:93)
iii)  SEQ ID NO:4      Motif 3: KWATAAR (SEQ ID NO:94)
iv)   SEQ ID NO:86
v)    SEQ ID NO:5
vi)   SEQ ID NO:87
vii)  SEQ ID NO:88
viii) SEQ ID NO:6
ix)   SEQ ID NO:89
x)    SEQ ID NO:7
xi)   SEQ ID NO:90
xii)  SEQ ID NO:91
```

# FIG. 2A

```
                        :   :
i) -250 NHNHNDNNNNNHRNNDWNNNDNND-------N-DNNDDH-NNHMNNNNNN -210
ii)     GCTAGAAAGGCTACCGATAGATAAACTATAGT-TAATTA-AATACATTAA
iii)    ACTCCTGGATTTACCAAATTTTGG-------T-T-CGTC-CCTATACTCG
iv)     TTTATTTAGAATAATATTTTTTG-------G-GTAAGA-GTTATAAAAT
v)      ACTACTTACTACACCGATTCTTGT-------G-TGCAGA-AAAATATGTT
vi)     TCTACTTACTACACCGATTCTTGT-------G-TACATA-AAAATATTTT
vii)    ACTTCTGGATTTACCAAACTTCTG-------T-T-TGTC-CCCATACTCC
viii)   AAAACTTGAATTGACTATTTTTTG-------C-T-CTTT-ACTCTGCACC
ix)     CACCTTGGCCGTATAAAATTTTGA-------A-T-TTAA-CTCAAAAGTT
x)      GAAACGGAAGACATATA--CGTCT-------A-AACGGA-GAAATTTCAA
xi)     AATCTTAGCCATACAAAATATATT-------T-T-ATTA-AAACCAAGCA
xii)    TCAACATATAGCACCTATTCATTG-------T-TCCTAA-AACATAATTT
xiii)   AAGTCTCCGTCAAAGGTGTCTGTG-------T-A-GGTA-AGAACCCTTA
xiv)    AATCTTAGCCATACAAAATATATT-------T-T-CTTTAAAACGAAGCA
xv)     ACTAATTTGTTAGGTGATTGGTTG-------GCTGCATA-TAAAT-----


i) -209 NN--NNNNNNNN----------------------------------NNNNN -195
ii)     A---AAATACTT-------------------------------------
iii)    AA--AAATAAAA-CAA-----------------------AATAAATTT
iv)     A---AAATACAA-------------------------------------
v)      A---AAATAATT-------------------------------------
vi)     A---AAATAATT-------------------------------------
vii)    AA--AAACAAAA-CCA----------------------------TTTTT
viii)   AA--CTTTAC-------------------------------------
ix)     AC--GCAAAATT-AAT--------------TCT----------AACACAA
x)      AAAACAAAAATT-------------------------------------
xi)     TG--AAAAAAGT-CAC--------------------------TAAAGAG
xii)    A---AGAACAAAAACTTAAACTTAAATAATAATAATAAAAGAGTACATCG
xiii)   TC--TTTCAA-------------------------------------
xiv)    TG--AAAAAAGT-CTC--------------------------TATAGGC
xv)     ----------------------------GATAATAATAGAATACATTG
```

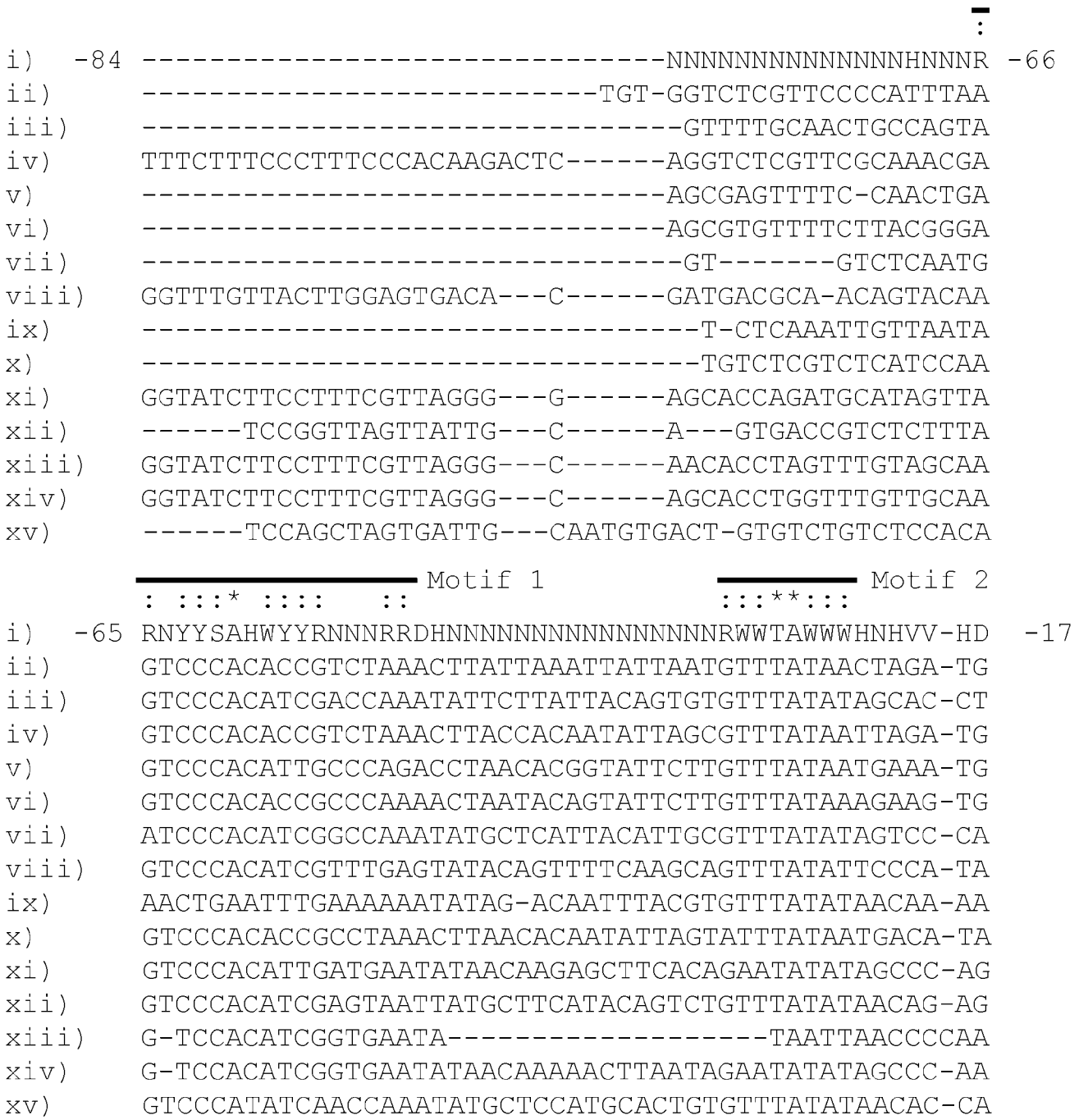
FIG. 2B

```
                                    :       :
i) -194 ----------NN--------NWNNN-NNYBDNNN--YNNNNNNNNNNN-- -168
ii)     ----------GG--------ATCTT-TCTCTTAC--C-CTGTTTATAT--
iii)    CA--------GT--------ATCTT-CGTTTTTG--TATGCTTTGACT--
iv)     ----------AA--------AAAAA-ACCTAATA--T-CAATTTTTCA--
v)      ----------GA--------ATCTT-TCTCTAGC--C-AAATTTGACA--
vi)     ----------GA--------ATCTT-TCTTTAGC--C-AGCTTTGACA--
vii)    TT--------TT--------ATCTT-CGTTTTTG--TTTGCTTTGACT--
viii)   ----------TA--------TTCCT-TCTTTTAG--TGTGAGC-------
ix)     AAATAATTCTGA--------ATTTT-TCTCTCAT-GTGAAATTAAACA--
x)      ----------GG--------ATCATTTCTCGATT--T-GTGGGTGTC---
xi)     CT------------------ATATA-ACTCATGCAGCTAGAAATGAAG--
xii)    AAGTA--TCTGT--------GT------TCTCTA--T------------
xiii)   --------------------GTGTT-CGTTTTCC—TTCGATGTGGGTAC
xiv)    CAGCACCTTTGAATCTTTAGCTATA-ACTCATGCAGCTAGAAATGAGG--
xv)     AAATA--TCTGT--------ATCTT-TCTGTGTA--T------------


i) -167 -NNNNNNN-----N-NNNNNNNNNN-------NNNNNNNN---ND----- -140
ii)     -TGAGACC-----T-GAAACTTGAG-------AGAGA-TA---CA-----
iii)    -GTGAGGC-GAGGC-CAACTTTCTT-------CTTCTGTC---TG-----
iv)     -----CTG-----A-CTCCGTTTATATTG-AGACTTG-AG---AA-----
v)      -ACAATGT-----A-CACCGTTCAT-------ATTGA-GA---GA-----
vi)     -ACAATGT-----A-CACCGTTCGTACTTCTTACTGG-TA---GG-----
vii)    -GTGAGTT-GAGGCCCAACTTTCTG-------CTTCTGTC---CGACTCT
viii)   ------------------------------------------T-----
ix)     -TCTAAAAATATAT-TCAAAATCAA-------TTTCAGAC---TT-----
x)      -----------------ATCTTGTG-------CAGGG-CA---TG-----
xi)     -TGAAGGG----AA-TCCAGTTTGT-------TCTCAGTC---GAAA---
xii)    ------------------------C-------CTTCTGAC---TA-----
xiii)   AATGGTTG----AA-TCCAGTTTGT-------TCTCAGTC---TAAA---
xiv)    -AGAAGGG----AA-TCCAGTTTGT-------TCTCAGTC---GAAA---
xv)     ------------------------C-------ATTCCATCTTTCA-----
```

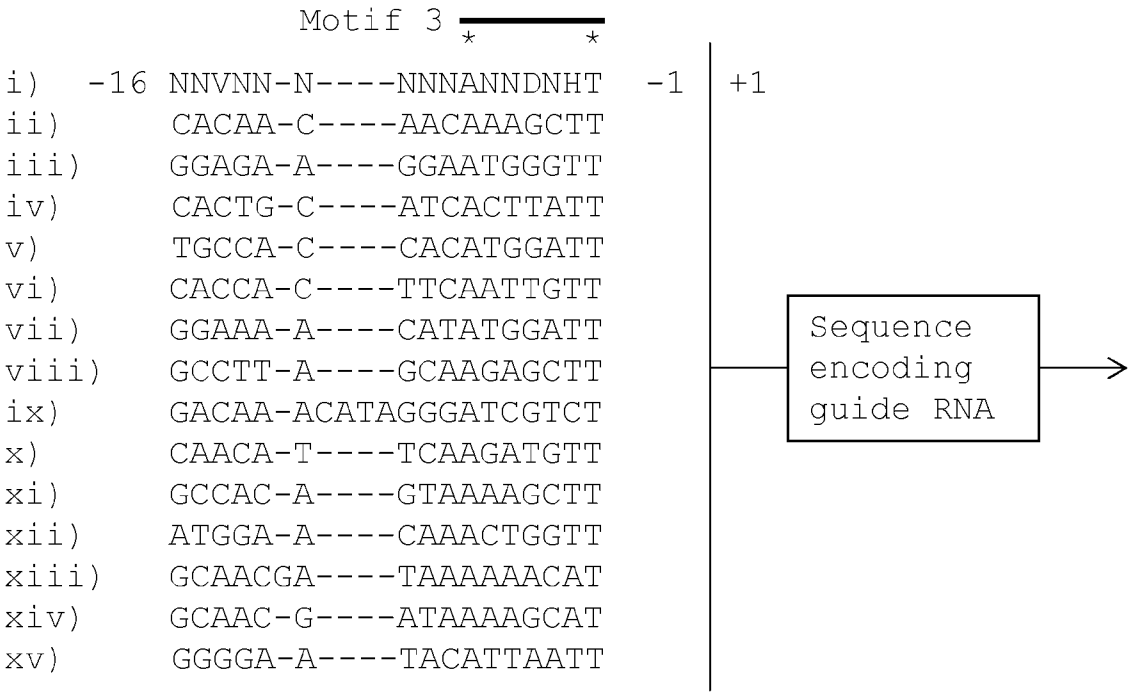
FIG. 2C

```
                ::    : ::::          ::    :
i) -139 ---NNDWKNH--WNYYKK---------KWNBNHYNNNN-N-----NNNNN -110
ii)     ---CTAATCT--TGCCTT---------GTTGTTTCATTCC-----CTAAC
iii)    ---AGATGAA--TTTTGT---------TTGCCTCCTGT-G-----AAGGA
iv)     ---AGATGGT--TCCCGT---------TTGCTCCCGGT-G-----GAGGC
v)      ---CGATGCT--TCTTGT---------TTGCTTTCGGT-G-----GAAGC
vi)     ---CAATGCT--TCTTGT---------TTGCTTTCGGT-G-----GAAGG
vii)    ATTTGATGAA--TTTTGT---------TTGCCTCCTGT-GATGTGAAGGA
viii)   ---TCATGCA--TCTTGTTCACCGCAATTCCGCTCGGT-G-----AAAGT
ix)     ---AGAATCA--AATTTT---------TAACACCAAAT-C-----AA---
x)      ---CTAATCT--TCTCTT---------TACCCTT--------------TC
xi)     ---GAGTGTCTATCTTTG---------TTCTTTTCTGC-A-----ACCGA
xii)    ---ACATTCA--TGTTGT---------TTGTATTCAGC-A-----AAGGG
xiii)   ---GGGTGCT--TATTTG---------TTCTTTTCTAC-A-----ACCGA
xiv)    ---GGGTGTC--TCTTTG---------TTCTATTCTGC-A-----ACCGA
xv)     ---TCTTGCA--TGTTGT---------TTGCCACCAAT-G-----AAGGG

                                  : :
i) -109 N-----------NNNN---NNNBYBRNVVNNHNBNN----------NNN-  -85
ii)     T-----------TACA---GGACTCAGCGCATGTCA--------------
iii)    T-----------GTAT---C-ATTCAAAGTGAAT----------------
iii)    TCCGAGGC-TGTGTAT---ATACTCGACATTACTTTAGCTTGTTTTGTTG
iv)     T-----------GCAT---ATACTCAACATTACTCC----------TTC-
vi)     T-----------GTAT---ATACTCAACATTACTTC-------TTTTTC-
vii)    T-----------GTAT---C-ATTGAAAGGGAAC----------------
viii)   T-----------GCACAATTCACTCACAATCTGTTTCT---GGTCTGTTA
ix)     ------------ACCG---AAACCCAAGA-TACTT--------------
x)      C-----------CACA---AGACTCAGCGCATGT----------------
xi)     ----------------------GTTAAGCAAAATGG------GAATGCGA
xii)    C--------C--GTGC--AGGATTTGTGCGTCGCGC--------------
xiii)   G-----TGAA--GAGA--TCGAGTTAAGCTTAATGG------AAATGTGA
xiv)    G-----CGAA--GAGA--CCCAGTTAAGCTAAATGG------GAATGTGA
xv)     C--------T--GTGC--AGGACTCAAGCATTTGGC--------------
```

# FIG. 2D


i) -84 ----------NNNNNNNNNNNNNNNHNNNR -66
ii) ----------TGT-GGTCTCGTTCCCCATTTAA
iii) ----------GTTTTGCAACTGCCAGTA
iv) TTTCTTTCCCTTTCCCACAAGACTC------AGGTCTCGTTCGCAAACGA
v) ----------AGCGAGTTTTC-CAACTGA
vi) ----------AGCGTGTTTTCTTACGGGA
vii) ----------GT-------GTCTCAATG
viii) GGTTTGTTACTTGGAGTGACA---C------GATGACGCA-ACAGTACAA
ix) ----------T-CTCAAATTGTTAATA
x) ----------TGTCTCGTCTCATCCAA
xi) GGTATCTTCCTTTCGTTAGGG---G------AGCACCAGATGCATAGTTA
xii) ------TCCGGTTAGTTATTG---C------A---GTGACCGTCTCTTTA
xiii) GGTATCTTCCTTTCGTTAGGG---C------AACACCTAGTTTGTAGCAA
xiv) GGTATCTTCCTTTCGTTAGGG---C------AGCACCTGGTTTGTTGCAA
xv) ------TCCAGCTAGTGATTG---CAATGTGACT-GTGTCTGTCTCCACA
Motif 1
Motif 2
i) -65 RNYYSAHWYYRNNNRRDHNNNNNNNNNNNNNNNNRWWTAWWWHNHVV-HD -17
ii) GTCCCACACCGTCTAAACTTATTAAATTATTAATGTTTATAACTAGA-TG
iii) GTCCCACATCGACCAAATATTCTTATTACAGTGTGTTTATATAGCAC-CT
iv) GTCCCACACCGTCTAAACTTACCACAATATTAGCGTTTATAATTAGA-TG
v) GTCCCACATTGCCCAGACCTAACACGGTATTCTTGTTTATAATGAAA-TG
vi) GTCCCACACCGCCCAAAACTAATACAGTATTCTTGTTTATAAAGAAG-TG
vii) ATCCCACATCGGCCAAATATGCTCATTACATTGCGTTTATATAGTCC-CA
viii) GTCCCACATCGTTTGAGTATACAGTTTTCAAGCAGTTTATATTCCCA-TA
ix) AACTGAATTTGAAAAAATATAG-ACAATTTACGTGTTTATATAACAA-AA
x) GTCCCACACCGCCTAAACTTAACACAATATTAGTATTTATAATGACA-TA
xi) GTCCCACATTGATGAATATAACAAGAGCTTCACAGAATATATAGCCC-AG
xii) GTCCCACATCGAGTAATTATGCTTCATACAGTCTGTTTATATAACAG-AG
xiii) G-TCCACATCGGTGAATA----------TAATTAACCCCAA
xiv) G-TCCACATCGGTGAATATAACAAAAACTTAATAGAATATATAGCCC-AA
xv) GTCCCATATCAACCAAATATGCTCCATGCACTGTGTTTATATAACAC-CA

# FIG. 2E




i) SEQ ID NO:95 Motif 1: RRNYYSAHWYYRNNNRR (SEQ ID NO:110)
ii) SEQ ID NO:96 Motif 2: RWWTAWWW (SEQ ID NO:111)
iii) SEQ ID NO:97 Motif 3: ANNDNHT (SEQ ID NO:112)
iv) SEQ ID NO:98
v) SEQ ID NO:99
vi) SEQ ID NO:100
vii) SEQ ID NO:101
viii) SEQ ID NO:102
ix) SEQ ID NO:103
x) SEQ ID NO:104
xi) SEQ ID NO:105
xii) SEQ ID NO:106
xiii) SEQ ID NO:107
xiv) SEQ ID NO:108
xv) SEQ ID NO:109

FIG. 3

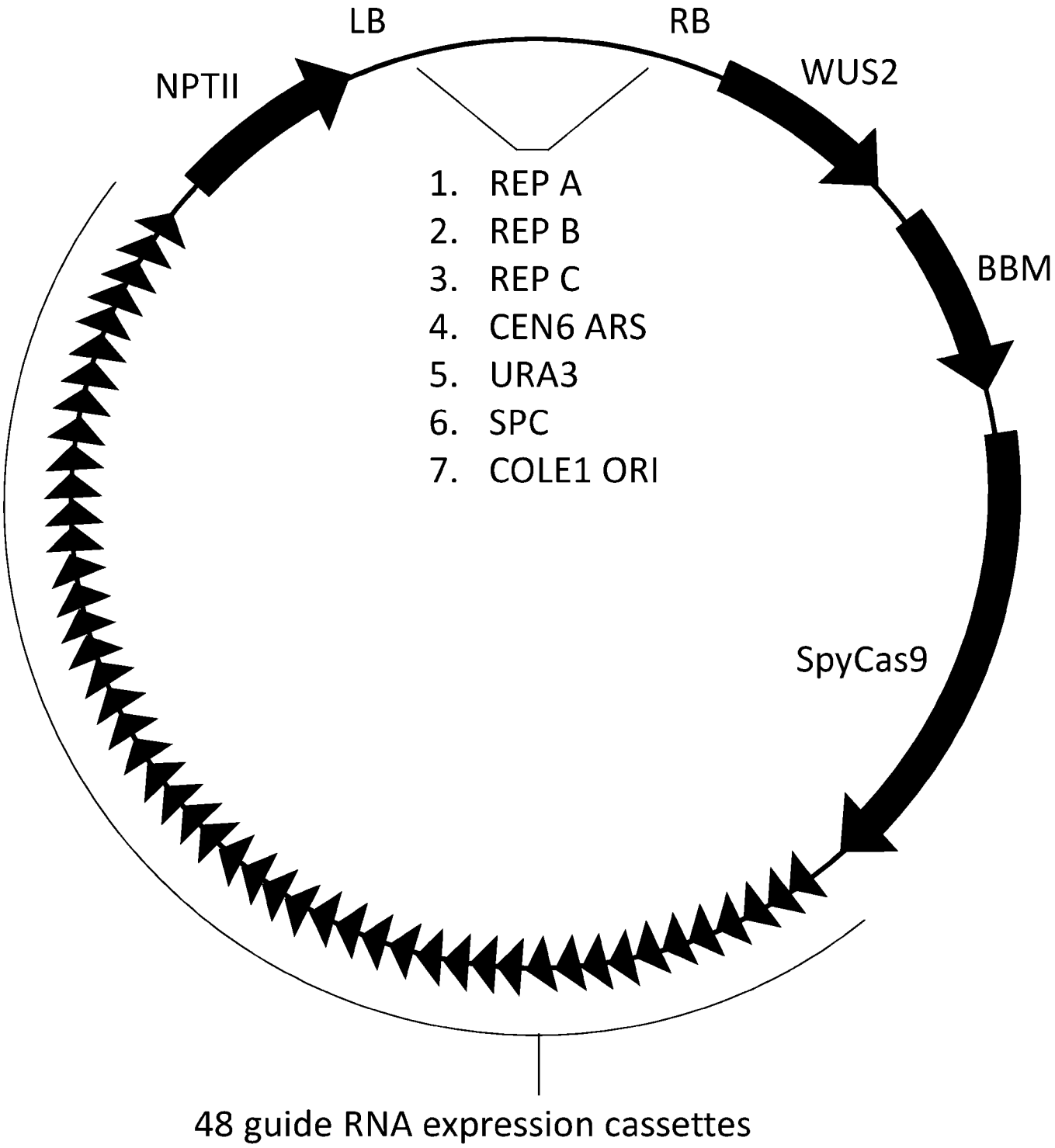

LB
RB
NPTII
WUS2
1. REP A
2. REP B
3. REP C
4. CEN6 ARS
5. URA3
6. SPC
7. COLE1 ORI
BBM
SpyCas9
48 guide RNA expression cassettes

FIG. 4

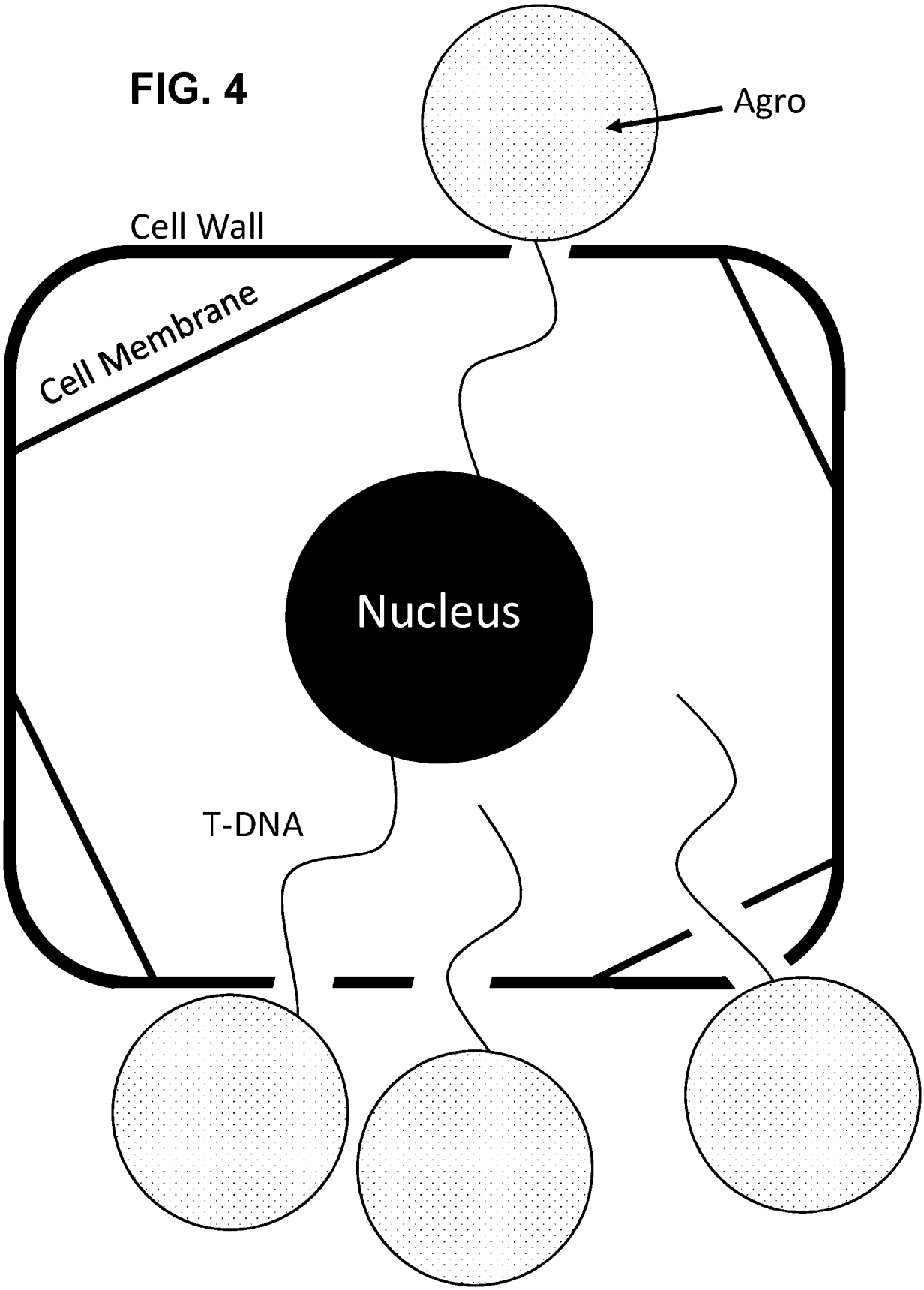


FIG. 5

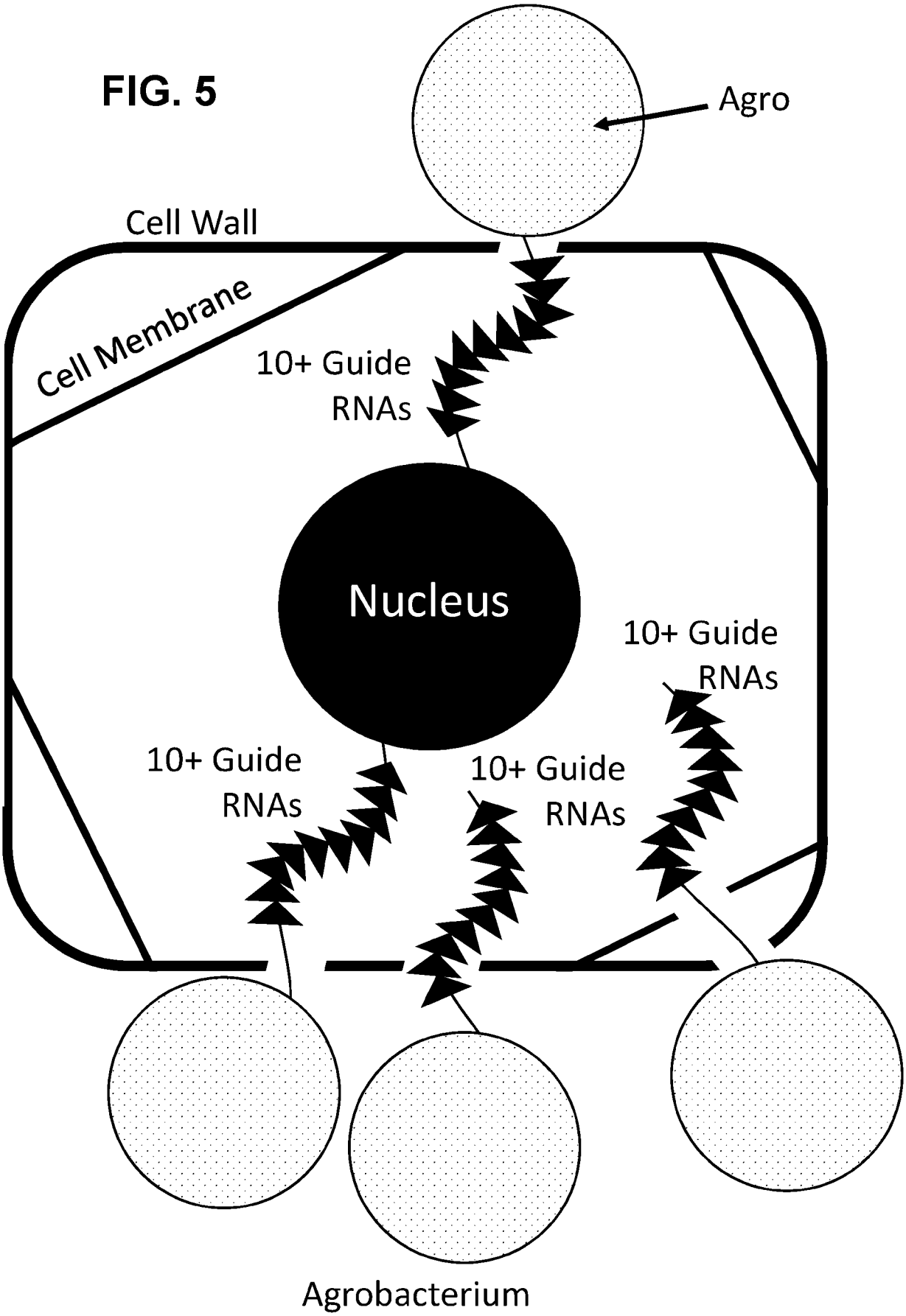

Agro
Cell Wall
Cell Membrane
10+ Guide RNAs
Nucleus
10+ Guide RNAs
10+ Guide RNAs
10+ Guide RNAs
Agrobacterium

FIG. 6

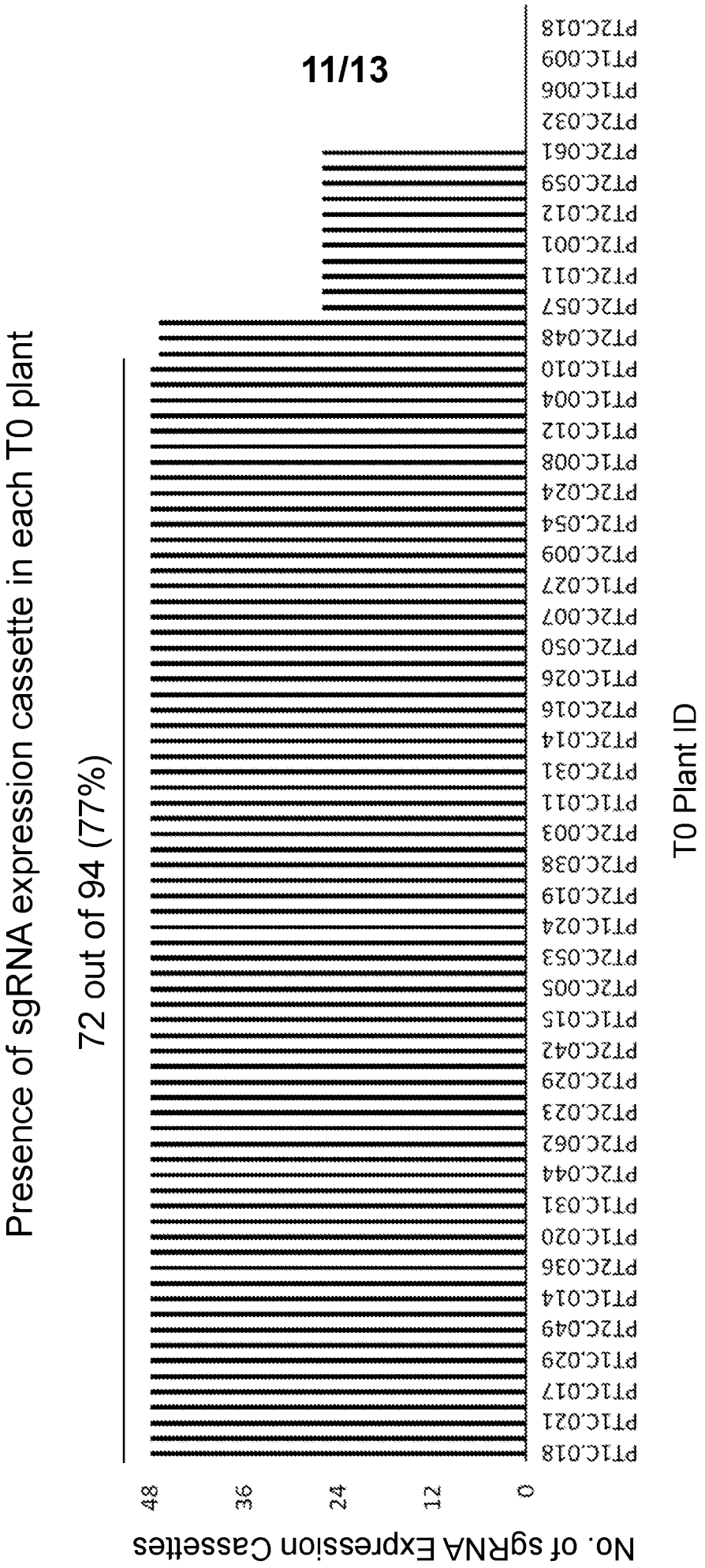

Presence of sgRNA expression cassette in each T0 plant
72 out of 94 (77%)
No. of sgRNA Expression Cassettes
0
12
24
36
48
T0 Plant ID
PTC.018
PTC.021
PTC.017
PTC.029
PTC.049
PTC.014
PTC.036
PTC.020
PTC.031
PTC.044
PTC.062
PTC.023
PTC.029
PTC.042
PTC.015
PTC.005
PTC.053
PTC.024
PTC.019
PTC.038
PTC.003
PTC.011
PTC.031
PTC.014
PTC.016
PTC.026
PTC.050
PTC.007
PTC.027
PTC.009
PTC.054
PTC.024
PTC.008
PTC.012
PTC.004
PTC.010
PTC.048
PTC.057
PTC.011
PTC.001
PTC.012
PTC.059
PTC.061
PTC.032
PTC.006
PTC.009
PTC.018

FIG. 7

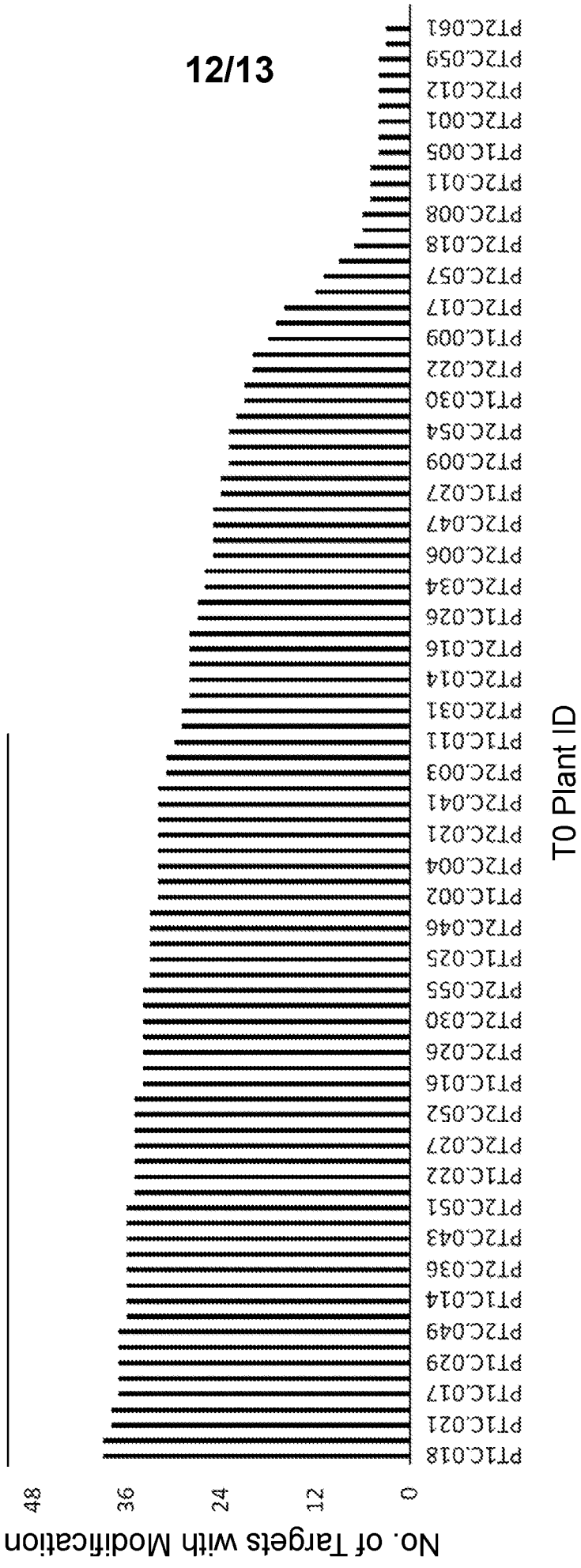
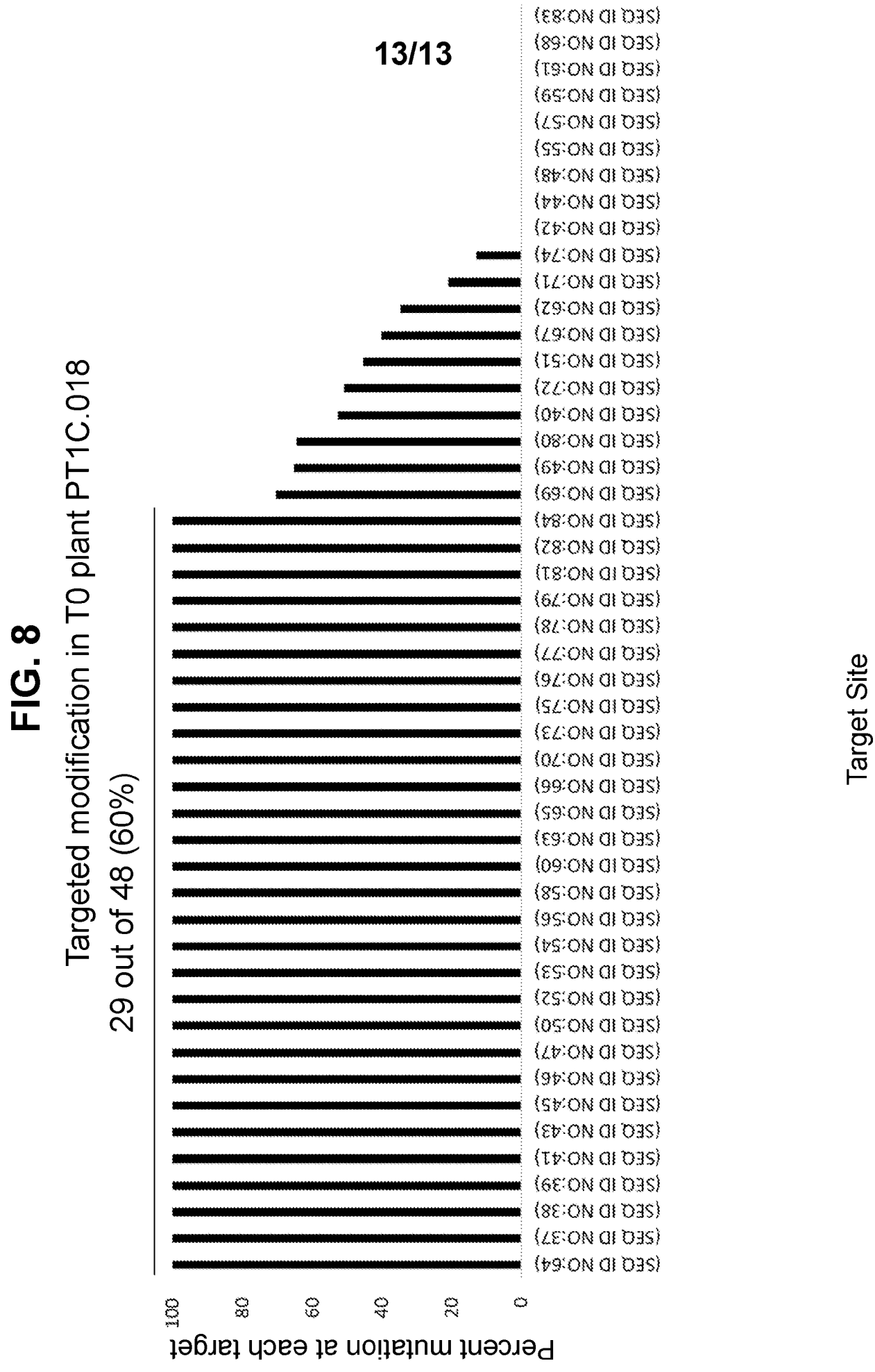

Sites with targeted modification in each T0 plant
47 out of 94 (50%)
No. of Targets with Modification
0
12
24
36
48
T0 Plant ID
PTC.018
PTC.021
PTC.017
PTC.029
PTC.049
PTC.014
PTC.036
PTC.043
PTC.051
PTC.022
PTC.027
PTC.052
PTC.016
PTC.026
PTC.030
PTC.055
PTC.025
PTC.046
PTC.002
PTC.004
PTC.021
PTC.041
PTC.003
PTC.011
PTC.031
PTC.014
PTC.016
PTC.026
PTC.034
PTC.006
PTC.047
PTC.027
PTC.054
PTC.030
PTC.022
PTC.009
PTC.017
PTC.057
PTC.018
PTC.008
PTC.011
PTC.005
PTC.001
PTC.012
PTC.059
PTC.061

FIG. 8


Targeted modification in T0 plant PT1C.018
29 out of 48 (60%)
Percent mutation at each target
100
80
60
40
20
0
Target Site
(SEQ ID NO:64)
(SEQ ID NO:37)
(SEQ ID NO:38)
(SEQ ID NO:39)
(SEQ ID NO:41)
(SEQ ID NO:43)
(SEQ ID NO:45)
(SEQ ID NO:46)
(SEQ ID NO:47)
(SEQ ID NO:50)
(SEQ ID NO:52)
(SEQ ID NO:53)
(SEQ ID NO:54)
(SEQ ID NO:56)
(SEQ ID NO:58)
(SEQ ID NO:60)
(SEQ ID NO:63)
(SEQ ID NO:65)
(SEQ ID NO:66)
(SEQ ID NO:70)
(SEQ ID NO:73)
(SEQ ID NO:75)
(SEQ ID NO:76)
(SEQ ID NO:77)
(SEQ ID NO:78)
(SEQ ID NO:79)
(SEQ ID NO:81)
(SEQ ID NO:82)
(SEQ ID NO:84)
(SEQ ID NO:69)
(SEQ ID NO:49)
(SEQ ID NO:80)
(SEQ ID NO:40)
(SEQ ID NO:72)
(SEQ ID NO:51)
(SEQ ID NO:67)
(SEQ ID NO:62)
(SEQ ID NO:71)
(SEQ ID NO:74)
(SEQ ID NO:42)
(SEQ ID NO:44)
(SEQ ID NO:48)
(SEQ ID NO:55)
(SEQ ID NO:57)
(SEQ ID NO:59)
(SEQ ID NO:61)
(SEQ ID NO:68)
(SEQ ID NO:83)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/082540

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/22 C12N15/82
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2021/162909 A1 (PIONEER HI BRED INT [US]) 19 August 2021 (2021-08-19) pages 16, 72-74, 76, 77; Seq. ID Nos 17-22 | 1-117 |
| X | WO 2016/160389 A1 (DU PONT [US]) 6 October 2016 (2016-10-06) claims 1-18; Examples 2 and 5; pages 3 and 26; Seq. Id Nos 8, 10, 24, 30, 34 | 116,117 |

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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

"L" document which may throw doubts on priority claim(s) 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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 18 March 2024 | 05/04/2024 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Kurz, Birgit |

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Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/082540

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | STUTTMANN JOHANNES ET AL: "Highly efficient multiplex editing: one-shot generation of 8x Nicotiana benthamiana and 12x Arabidopsis mutants", THE PLANT JOURNAL, vol. 106, no. 1, 25 March 2021 (2021-03-25), pages 8-22, XP093107894, GB ISSN: 0960-7412, DOI: 10.1111/tpj.15197 Retrieved from the Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1111/tpj.15197> abstract; Figures 1, 3 and 4; pages 9, 10, 13, 16-18 | 1-117 |
| A | MCKNIGHT T D ET AL: "SEGREGATION OF GENES TRANSFERRED TO ONE PLANT CELL FORM TWO SEPARATE AGROBACTERIUM STRAINS", PLANT MOLECULAR BIOLOGY, SPRINGER, DORDRECHT, NL, vol. 8, 1 January 1987 (1987-01-01), pages 439-445, XP000886564, ISSN: 0167-4412, DOI: 10.1007/BF00017989 abstract | 1-115 |
| A | LOWE KEITH ET AL: "Rapid genotype "independent"Zea maysL. (maize) transformation via direct somatic embryogenesis", IN VITRO CELLULAR & DEVELOPMENT BIOLOGY. PLANT, GAITHERSBURG, MD, US, vol. 54, no. 3, 30 April 2018 (2018-04-30), pages 240-252, XP036504790, ISSN: 1054-5476, DOI: 10.1007/S11627-018-9905-2 [retrieved on 2018-04-30] abstract; pages 240 and 242; Table 1 | 1-115 |
| A | Yadav Sushil Kumar ET AL: "Optimization of Agrobacterium mediated genetic transformation of cotyledonary node explants of Vigna radiata", SpringerPlus, 10 December 2012 (2012-12-10), pages 1-8, XP093142026, Cham DOI: 10.1186/2193-1801-1-59 Retrieved from the Internet: URL:https://springerplus.springeropen.com/articles/10.1186/2193-1801-1-59 [retrieved on 2024-03-15] abstract; passage bridging pages 2 and 3 | 1-115 |

-/--

2

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2023/082540

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | LI SHUXUAN ET AL: "Optimization of Agrobacterium-Mediated Transformation in Soybean", FRONTIERS IN PLANT SCIENCE, vol. 8, 24 February 2017 (2017-02-24), XP093142027, CH ISSN: 1664-462X, DOI: 10.3389/fpls.2017.00246 abstract; pages 2-4 ----- | 1-115 |
| A | GUREL SONGUL ET AL: "Efficient, reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos", PLANT CELL REPORTS, vol. 28, no. 3, 30 December 2008 (2008-12-30), pages 429-444, XP093142021, Berlin/Heidelberg ISSN: 0721-7714, DOI: 10.1007/s00299-008-0655-1 Retrieved from the Internet: URL:http://link.springer.com/article/10.1007/s00299-008-0655-1/fulltext.html> abstract; page 431; Figure 3 ----- | 6-8, 66-68 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2023/082540

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2021162909 A1 | 19-08-2021 | BR 112022015830 A2 | 25-10-2022 |
| | | CA 3169636 A1 | 19-08-2021 |
| | | CN 115135143 A | 30-09-2022 |
| | | EP 4102961 A1 | 21-12-2022 |
| | | US 2023183724 A1 | 15-06-2023 |
| | | WO 2021162909 A1 | 19-08-2021 |
| WO 2016160389 A1 | 06-10-2016 | BR 112017017260 A2 | 17-04-2018 |
| | | CA 2976387 A1 | 06-10-2016 |
| | | CN 107567499 A | 09-01-2018 |
| | | EP 3274460 A1 | 31-01-2018 |
| | | EP 3553178 A1 | 16-10-2019 |
| | | US 2018057832 A1 | 01-03-2018 |
| | | WO 2016160389 A1 | 06-10-2016 |