

NOVEL PLANT CELLS, PLANTS, AND SEEDS**PRIORITY CLAIMS AND RELATED APPLICATIONS**

[0001] This application claims the benefit of priority to U. S. Provisional Patent Applications 62/418,078, filed on 4 November 2016, and 62/442,601, filed on 5 January 2017, which are incorporated by reference in their entirety herein.

INCORPORATION OF SEQUENCE LISTING

[0002] The sequence listings contained in the files named “10001P1_ST25.txt” (which is 98 kilobytes measured in operating system Windows 7 x64, created on 4 November 2016 and electronically filed via EFS-Web on 4 November 2016) and “98062-02_ST25.txt” (which is ~100 kilobytes measured in operating system Windows 8.1 x64, created on 4 January 2017 and electronically filed via EFS-Web on 5 January 2017), are incorporated herein by reference in their entirety. The sequence listing contained in the file named “10001WO1_ST25.txt”, which is 176 kilobytes measured in operating system Windows 7 x64, created on 3 November 2017, is electronically filed herewith via EFS-Web and incorporated herein by reference in its entirety.

FIELD

[0003] Aspects of this invention relate to agricultural biotechnology. Disclosed herein are novel plant cells, plants and seeds derived from such plant cells and having enhanced traits, and methods of making and using such plant cells and derived plants and seeds.

BACKGROUND

[0004] Recent advances in genome editing technologies have provided opportunities for precise modification of the genome in many types of organisms, including plants and animals. For example, technologies based on genome editing proteins, such as zinc finger nucleases, TALENs, and CRISPR systems are advancing rapidly and it is now possible to target genetic changes to specific DNA sequences in the genome.

[0005] CRISPR technology for editing the genes of eukaryotes is disclosed in US Patent Application Publications 2016/0138008A1 and US2015/0344912A1, and in US Patents 8,697,359, 8,771,945, 8,945,839, 8,999,641, 8,993,233, 8,895,308, 8,865,406, 8,889,418, 8,871,445, 8,889,356, 8,932,814, 8,795,965, and 8,906,616. Cpf1 endonuclease and corresponding guide RNAs and PAM sites are disclosed in US Patent Application Publication 2016/0208243 A1. Other CRISPR nucleases useful for editing genomes include C2c1 and C2c3 (see Shmakov *et al.* (2015) *Mol. Cell*, 60:385 – 397) and CasX and CasY (see Burstein *et al.* (2016) *Nature*, doi:10.1038/nature21059). Plant RNA promoters for expressing CRISPR guide RNA and plant codon-optimized CRISPR Cas9 endonuclease are disclosed in International Patent Application PCT/US2015/018104 (published as WO 2015/131101 and claiming priority to US

Provisional Patent Application 61/945,700). Methods of using CRISPR technology for genome editing in plants are disclosed in in US Patent Application Publications US 2015/0082478A1 and US 2015/0059010A1 and in International Patent Application PCT/US2015/038767 A1 (published as WO 2016/007347 and claiming priority to US Provisional Patent Application 62/023,246).

SUMMARY

[0006] Disclosed herein are methods for providing novel plant cells, plants, and seeds having one or more altered genetic sequences.

[0007] In one aspect, the invention provides a method of delivering a guide RNA (gRNA) (or other sequence-editing guide nucleic acid capable of directing a nuclease to a specific target sequence) to a non-epidermal plant cell in a plant or part of a plant. The gRNA can be provided as a CRISPR RNA (crRNA) or as a single guide RNA (sgRNA) or as a polynucleotide that encodes or is processed to a crRNA or sgRNA, wherein the gRNA has a nucleotide sequence designed to alter a target nucleotide sequence in the non-epidermal plant cell. In embodiments, the non-epidermal cell is a cell capable of division and differentiation, such as a meristem cell or a cell in a plant embryo or seed or seedling. In embodiments, the non-epidermal plant cell is in a monocot plant or in a dicot plant, and can be haploid or diploid. In embodiments, the non-epidermal plant cell contains a nuclease, such as a Cas9 nuclease or other RNA-guided nuclease, that is capable of altering the target nucleotide sequence; in other embodiments the nuclease is provided to the non-epidermal plant cell, either together with the crRNA (or other genome-editing polynucleotide) or separately. The nuclease can be provided as a functional enzyme (*e. g.*, as a ribonucleoprotein or micelle or other molecular or supramolecular complex), or as a polynucleotide that encodes the functional nuclease. The target nucleotide sequence is one or more nucleotide sequences, including protein-coding sequence or non-coding sequence or a combination thereof. Embodiments include a plant nuclear sequence, a plant plastid sequence, a plant mitochondrial sequence, a sequence of a symbiont, pest, or pathogen of a plant, and combinations thereof. The crRNA (or other sequence-editing polynucleotide) and the RNA-guided nuclease are provided separately (*e. g.*, in discrete compositions or in discrete steps), or alternatively are provided simultaneously (*e. g.*, combined in a single composition, or in a single step or treatment). Embodiments of the method include one or more delivery steps or treatments, including treatment with at least one chemical, enzymatic, or physical agent or use of techniques such as application of heat or cold, ultrasonication, centrifugation, and electroporation, whereby the gRNA is delivered to the non-epidermal plant cell. In embodiments, the method further includes growing or regeneration of a seedling, plantlet, or plant from the non-epidermal plant cell having the altered target nucleotide sequence. Related aspects include: the non-epidermal plant cell with the altered target nucleotide sequence provided by the method; pluralities, arrays, and heterogeneous populations of such non-epidermal plant cells; and callus, seedlings, plantlets, and plants and their seeds, grown or regenerated from the non-epidermal plant cell and having the altered target nucleotide sequence, and pluralities, arrays, and heterogeneous populations thereof.

[0008] In another aspect, the invention provides a method of providing a plant having a genetic alteration, including the step of delivering an effector molecule such as a sequence-specific nuclease or a guide nucleic acid to a plant cell capable of division and differentiation, resulting in a genetic alteration of the plant cell, and growing or regenerating a plant from the resulting genetically altered plant cell, wherein the plant includes differentiated cells or tissues having the genetic alteration. In embodiments, the plant cell is in a plant or part of a plant, is monocot or dicot, is haploid or diploid, and is capable of division and differentiation or capable of growth or regeneration into callus, a seedling, a plantlet, or a plant. Embodiments include those wherein the effector molecule is at least one selected from the group consisting of: a polynucleotide, a ribonucleoprotein, a polypeptide (for example, a protein, an enzyme, or a nuclease), and a polynucleotide encoding a polypeptide; or a combination thereof. Embodiments of the method include one or more delivery steps or treatments, including treatment with chemical or physical agents or use of techniques such as application of heat or cold, ultrasonication, centrifugation, and electroporation. Related aspects include plants having a genetic alteration provided by the method, heterogeneous populations or libraries of such plants, succeeding generations or seeds of such plants, parts of the plants, or products made from the plants or their seeds.

[0009] In another aspect, the invention provides a method of identifying a nucleotide sequence (or alteration of a nucleotide sequence) associated with a phenotype of interest, including altering the genome of a population of plant cells or protoplasts, optionally growing or regenerating a population of calli, seedlings, plantlets, or plants from the population of plant cells or protoplasts, and selecting the plant cells or protoplasts (or calli, seedlings, plantlets, or plants) exhibiting the phenotype of interest. Embodiments of the method include culturing or growing the plant cells or protoplasts (or calli, seedlings, plantlets, or plants) under conditions that permit expression of the phenotype of interest.

DETAILED DESCRIPTION OF THE INVENTION

[0010] Unless otherwise stated, nucleic acid sequences in the text of this specification are given, when read from left to right, in the 5' to 3' direction. Nucleic acid sequences may be provided as DNA or as RNA, as specified; disclosure of one necessarily defines the other, as well as necessarily defines the exact complements, as is known to one of ordinary skill in the art. Where a term is provided in the singular, the inventors also contemplate aspects of the invention described by the plural of that term.

[0011] By "polynucleotide" is meant a nucleic acid molecule containing multiple nucleotides and refers to "oligonucleotides" (defined here as a polynucleotide molecule of between 2 – 25 nucleotides in length) and polynucleotides of 26 or more nucleotides. Polynucleotides are generally described as single- or double-stranded. Where a polynucleotide contains double-stranded regions formed by intra- or intermolecular hybridization, the length of each double-stranded region is conveniently described in terms of the number of base pairs. Aspects of this invention include the use of polynucleotides or compositions containing polynucleotides; embodiments include one or more oligonucleotides or polynucleotides or a mixture of both, including single- or double-stranded RNA or single- or double-stranded DNA or double-

stranded DNA/RNA hybrids or chemically modified analogues or a mixture thereof. In various embodiments, the polynucleotide includes a combination of ribonucleotides and deoxyribonucleotides (*e. g.*, synthetic polynucleotides consisting mainly of ribonucleotides but with one or more terminal deoxyribonucleotides or synthetic polynucleotides consisting mainly of deoxyribonucleotides but with one or more terminal dideoxyribonucleotides), or includes non-canonical nucleotides such as inosine, thioridine, or pseudouridine. In embodiments, the polynucleotide includes chemically modified nucleotides (see, *e. g.*, Verma and Eckstein (1998) *Annu. Rev. Biochem.*, 67:99-134); for example, the naturally occurring phosphodiester backbone of an oligonucleotide or polynucleotide can be partially or completely modified with phosphorothioate, phosphorodithioate, or methylphosphonate internucleotide linkage modifications, modified nucleoside bases or modified sugars can be used in oligonucleotide or polynucleotide synthesis, and oligonucleotides or polynucleotides can be labelled with a fluorescent moiety (*e. g.*, fluorescein or rhodamine) or other label (*e. g.*, biotin). Modified nucleic acids, particularly modified RNAs, are disclosed in US Patent 9,464,124, incorporated by reference in its entirety herein.

[0012] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) systems, or CRISPR systems, are adaptive defense systems originally discovered in bacteria and archaea. CRISPR systems use RNA-guided nucleases termed CRISPR-associated or “Cas” endonucleases (*e. g.*, Cas9 or Cpf1) to cleave foreign DNA. In a typical CRISPR/Cas system, a Cas endonuclease is directed to a target nucleotide sequence (*e. g.*, a site in the genome that is to be sequence-edited) by sequence-specific, non-coding “guide RNAs” that target single- or double-stranded DNA sequences. In microbial hosts, CRISPR loci encode both Cas endonucleases and “CRISPR arrays” of the non-coding RNA elements that determine the specificity of the CRISPR-mediated nucleic acid cleavage.

[0013] Three classes (I-III) of CRISPR systems have been identified across a wide range of bacterial hosts. The well characterized class II CRISPR systems use a single Cas endonuclease (rather than multiple Cas proteins). One class II CRISPR system includes a type II Cas endonuclease such as Cas9, a CRISPR RNA (“crRNA”), and a trans-activating crRNA (“tracrRNA”). The crRNA contains a “guide RNA”, typically a 20-nucleotide RNA sequence that corresponds to (*i. e.*, is identical or nearly identical to, or alternatively is complementary or nearly complementary to) a 20-nucleotide target DNA sequence. The crRNA also contains a region that binds to the tracrRNA to form a partially double-stranded structure which is cleaved by RNase III, resulting in a crRNA/tracrRNA hybrid. The crRNA/tracrRNA hybrid then directs the Cas9 endonuclease to recognize and cleave the target DNA sequence.

[0014] The target DNA sequence must generally be adjacent to a “protospacer adjacent motif” (“PAM”) that is specific for a given Cas endonuclease; however, PAM sequences are short and relatively non-specific, appearing throughout a given genome. CRISPR endonucleases identified from various prokaryotic species have unique PAM sequence requirements; examples of PAM sequences include 5’-NGG (*Streptococcus pyogenes*), 5’-NNAGAA (*Streptococcus thermophilus* CRISPR1), 5’-NGGNG (*Streptococcus thermophilus* CRISPR3), and 5’-NNNGATT (*Neisseria meningitidis*). Some endonucleases,

e. g., Cas9 endonucleases, are associated with G-rich PAM sites, *e. g.*, 5'-NGG, and perform blunt-end cleaving of the target DNA at a location 3 nucleotides upstream from (5' from) the PAM site.

[0015] Another class II CRISPR system includes the type V endonuclease Cpf1, which is a smaller endonuclease than is Cas9; examples include AsCpf1 (from *Acidaminococcus* sp.) and LbCpf1 (from *Lachnospiraceae* sp.). Cpf1-associated CRISPR arrays are processed into mature crRNAs without the requirement of a tracrRNA; in other words, a Cpf1 system requires only the Cpf1 nuclease and a crRNA to cleave the target DNA sequence. Cpf1 endonucleases, are associated with T-rich PAM sites, *e. g.*, 5'-TTN. Cpf1 can also recognize a 5'-CTA PAM motif. Cpf1 cleaves the target DNA by introducing an offset or staggered double-strand break with a 4- or 5-nucleotide 5' overhang, for example, cleaving a target DNA with a 5-nucleotide offset or staggered cut located 18 nucleotides downstream from (3' from) from the PAM site on the coding strand and 23 nucleotides downstream from the PAM site on the complimentary strand; the 5-nucleotide overhang that results from such offset cleavage allows more precise genome editing by DNA insertion by homologous recombination than by insertion at blunt-end cleaved DNA. See, *e. g.*, Zetsche *et al.* (2015) *Cell*, 163:759 – 771. Other CRISPR nucleases useful in methods and compositions of the invention include C2c1 and C2c3 (see Shmakov *et al.* (2015) *Mol. Cell*, 60:385 – 397) and CasX and CasY (see Burstein *et al.* (2016) *Nature*, doi:10.1038/nature21059). Like other CRISPR nucleases, C2c1 from *Alicyclobacillus acidoterrestris* (AacC2c1) requires a guide RNA and PAM recognition site; C2c1 cleavage results in a staggered seven-nucleotide DSB in the target DNA (see Yang *et al.* (2016) *Cell*, 167:1814 – 1828.e12) and is reported to have high mismatch sensitivity, thus reducing off-target effects (see Liu *et al.* (2016) *Mol. Cell*, available on line at [dx\[dot\]doi\[dot\]org/10\[dot\]1016/j\[dot\]molcel\[dot\]2016\[dot\]11.040](https://doi.org/10.1016/j.molcel.2016.11.040)). Yet other CRISPR nucleases include nucleases identified from the genomes of uncultivated microbes, such as CasX and CasY; see Burstein *et al.* (2016) *Nature*, doi:10.1038/nature21059.

[0016] For the purposes of gene editing, CRISPR arrays can be designed to contain one or multiple guide RNA sequences corresponding to a desired target DNA sequence; see, for example, Cong *et al.* (2013) *Science*, 339:819–823; Ran *et al.* (2013) *Nature Protocols*, 8:2281 – 2308. At least 16 or 17 nucleotides of gRNA sequence are required by Cas9 for DNA cleavage to occur; for Cpf1 at least 16 nucleotides of gRNA sequence are needed to achieve detectable DNA cleavage and at least 18 nucleotides of gRNA sequence were reported necessary for efficient DNA cleavage *in vitro*; see Zetsche *et al.* (2015) *Cell*, 163:759 – 771. In practice, guide RNA sequences are generally designed to have a length of between 17 – 24 nucleotides (frequently 19, 20, or 21 nucleotides) and exact complementarity (*i. e.*, perfect base-pairing) to the targeted gene or nucleic acid sequence; guide RNAs having less than 100% complementarity to the target sequence can be used (*e. g.*, a gRNA with a length of 20 nucleotides and between 1 – 4 mismatches to the target sequence) but can increase the potential for off-target effects. The design of effective guide RNAs for use in plant genome editing is disclosed in US Patent Application Publication 2015/0082478 A1, the entire specification of which is incorporated herein by reference. More recently, efficient gene editing has been achieved using a chimeric “single guide RNA” (“sgRNA”), an engineered (synthetic) single RNA molecule

that mimics a naturally occurring crRNA-tracrRNA complex and contains both a tracrRNA (for binding the nuclease) and at least one crRNA (to guide the nuclease to the sequence targeted for editing); see, for example, Cong *et al.* (2013) *Science*, 339:819 – 823; Xing *et al.* (2014) *BMC Plant Biol.*, 14:327 – 340. Chemically modified sgRNAs have been demonstrated to be effective in genome editing; see, for example, Hendel *et al.* (2015) *Nature Biotechnol.*, 985 – 991.

[0017] CRISPR-type genome editing has value in various aspects of agriculture research and development. CRISPR elements, *i. e.*, CRISPR endonucleases and CRISPR single-guide RNAs, are useful in effecting genome editing without remnants of the CRISPR elements or selective genetic markers occurring in progeny. Alternatively, genome-inserted CRISPR elements are useful in plant lines adapted for multiplex genetic screening and breeding. For instance, a plant species can be created to express one or more of a CRISPR endonuclease such as a Cas9- or a Cpf1-type endonuclease or combinations with unique PAM recognition sites. Cpf1 endonuclease and corresponding guide RNAs and PAM sites are disclosed in US Patent Application Publication 2016/0208243 A1, which is incorporated herein by reference for its disclosure of DNA encoding Cpf1 endonucleases and guide RNAs and PAM sites. Introduction of one or more of a wide variety of CRISPR guide RNAs that interact with CRISPR endonucleases integrated into a plant genome or otherwise provided to a plant is useful for genetic editing for providing desired phenotypes or traits, for trait screening, or for trait introgression. Multiple endonucleases can be provided in expression cassettes with the appropriate promoters to allow multiple genome editing in a spatially or temporally separated fashion in either in chromosome DNA or episome DNA.

[0018] Whereas wild-type Cas9 generates double-strand breaks (DSBs) at specific DNA sequences targeted by a gRNA, a number of CRISPR endonucleases having modified functionalities are available, for example: (1) a “nickase” version of Cas9 generates only a single-strand break; (2) a catalytically inactive Cas9 (“dCas9”) does not cut the target DNA but interferes with transcription; dCas9 can further be fused with a repressor peptide; (3) a catalytically inactive Cas9 (“dCas9”) fused to an activator peptide can activate or increase gene expression; (4) a catalytically inactive Cas9 (dCas9) fused to FokI nuclease (“dCas9-FokI”) can be used to generate DSBs at target sequences homologous to two gRNAs. See, *e. g.*, the numerous CRISPR/Cas9 plasmids disclosed in and publicly available from the Addgene repository (Addgene, 75 Sidney St., Suite 550A, Cambridge, MA 02139; [addgene\[dot\]org/crispr/](http://addgene.org/crispr/)). A “double nickase” Cas9 that introduces two separate double-strand breaks, each directed by a separate guide RNA, is described as achieving more accurate genome editing by Ran *et al.* (2013) *Cell*, 154:1380 – 1389.

[0019] CRISPR technology for editing the genes of eukaryotes is disclosed in US Patent Application Publications 2016/0138008A1 and US2015/0344912A1, and in US Patents 8,697,359, 8,771,945, 8,945,839, 8,999,641, 8,993,233, 8,895,308, 8,865,406, 8,889,418, 8,871,445, 8,889,356, 8,932,814, 8,795,965, and 8,906,616. Cpf1 endonuclease and corresponding guide RNAs and PAM sites are disclosed in US Patent Application Publication 2016/0208243 A1. Plant RNA promoters for expressing CRISPR guide RNA and plant codon-optimized CRISPR Cas9 endonuclease are disclosed in International Patent Application PCT/US2015/018104 (published as WO 2015/131101 and claiming priority to US

Provisional Patent Application 61/945,700). Methods of using CRISPR technology for genome editing in plants are disclosed in in US Patent Application Publications US 2015/0082478A1 and US 2015/0059010A1 and in International Patent Application PCT/US2015/038767 A1 (published as WO 2016/007347 and claiming priority to US Provisional Patent Application 62/023,246). All of the patent publications referenced in this paragraph are incorporated herein by reference in their entirety.

[0020] In some embodiments, one or more vectors driving expression of one or more polynucleotides encoding elements of a genome-editing system (*e. g.*, encoding a guide RNA or a nuclease) are introduced into a plant cell, whereby these elements, when expressed, result in alteration of a target nucleotide sequence. In embodiments, a vector includes a regulatory element such as a promoter operably linked to one or more polynucleotides encoding elements of a genome-editing system. In such embodiments, expression of these polynucleotides can be controlled by selection of the appropriate promoter, particularly promoters functional in a plant cell; useful promoters include constitutive, conditional, inducible, and temporally or spatially specific promoters (*e. g.*, a tissue specific promoter, a developmentally regulated promoter, or a cell cycle regulated promoter). In embodiments the promoter is operably linked to nucleotide sequences encoding multiple guide RNAs, wherein the sequences encoding guide RNAs are separated by a cleavage site such as a nucleotide sequence encoding a microRNA recognition/cleavage site or a self-cleaving ribozyme (see, *e. g.*, Ferré-D'Amaré and Scott (2014) *Cold Spring Harbor Perspectives Biol.*, 2:a003574). In embodiments, the promoter is a pol II promoter operably linked to a nucleotide sequence encoding one or more guide RNAs. In embodiments, the promoter operably linked to one or more polynucleotides encoding elements of a genome-editing system is a constitutive promoter that drives DNA expression in plant cells including in the nucleus or in an organelle such as a chloroplast or mitochondrion. Examples of constitutive promoters include a CaMV 35S promoter as disclosed in US Patents 5,858,742 and 5,322,938, a rice actin promoter as disclosed in US Patent 5,641,876, a maize chloroplast aldolase promoter as disclosed in US Patent 7,151,204, and a opaline synthase (NOS) and octapine synthase (OCS) promoter from *Agrobacterium tumefaciens*. In embodiments, the promoter operably linked to one or more polynucleotides encoding elements of a genome-editing system is a promoter from figwort mosaic virus (FMV), a RUBISCO promoter, or a pyruvate phosphate dikinase (PDK) promoter, which is active in the chloroplasts of mesophyll cells. Other contemplated promoters include cell-specific or tissue-specific or developmentally regulated promoters, for example, a promoter that limits the expression of the nucleic acid targeting system to germline or reproductive cells (*e. g.*, promoters of genes encoding DNA ligases, recombinases, replicases, or other genes specifically expressed in germline or reproductive cells); in such embodiments, the nuclease-mediated genetic modification (*e. g.*, chromosomal or episomal double-stranded DNA cleavage) is limited only those cells from which DNA is inherited in subsequent generations, which is advantageous where it is desirable that expression of the genome-editing system be limited in order to avoid genotoxicity or other unwanted effects. All of the patent publications referenced in this paragraph are incorporated herein by reference in their entirety.

[0021] In some embodiments, elements of a genome-editing system (*e. g.*, an RNA-guided nuclease and a guide RNA) are operably linked to separate regulatory elements on separate vectors. In other embodiments, two or more elements of a genome-editing system expressed from the same or different regulatory elements or promoters are combined in a single vector, optionally with one or more additional vectors providing any additional necessary elements of a genome-editing system not included in the first vector. For example, multiple guide RNAs can be expressed from one vector, with the appropriate RNA-guided nuclease expressed from a second vector. In another example, one or more vectors for the expression of one or more guide RNAs (*e. g.*, crRNAs or sgRNAs) are delivered to a plant cell that expresses the appropriate RNA-guided nuclease, or to a plant cell that otherwise contains the nuclease, such as by way of prior administration thereto of a vector for *in vivo* expression of the nuclease.

[0022] Genome-editing system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In embodiments, the endonuclease and the nucleic acid-targeting guide RNA may be operably linked to and expressed from the same promoter. In embodiments, a single promoter drives expression of a transcript encoding an endonuclease and the guide RNA, embedded within one or more intron sequences (*e. g.*, each in a different intron, two or more in at least one intron, or all in a single intron), which can be plant-derived; such use of introns is especially contemplated when the expression vector is being transformed or transfected into a monocot cell.

[0023] Expression vectors provided herein may contain a DNA segment near the 3' end of an expression cassette that acts as a signal to terminate transcription and directs polyadenylation of the resultant mRNA. These are commonly referred to as "3'-untranslated regions" or "3'-UTRs" or "polyadenylation signals". Useful 3' elements include: *Agrobacterium tumefaciens* nos 3', tml 3', tmr 3', tms 3', ocs 3', and tr7 3' elements disclosed in U.S. Pat. No. 6,090,627, incorporated herein by reference, and 3' elements from plant genes such as the heat shock protein 17, ubiquitin, and fructose-1,6-biphosphatase genes from wheat (*Triticum aestivum*), and the glutelin, lactate dehydrogenase, and beta-tubulin genes from rice (*Oryza sativa*), disclosed in US Patent Application Publication 2002/0192813 A1, incorporated herein by reference.

[0024] In certain embodiments, a vector or an expression cassette includes additional components, *e. g.*, a polynucleotide encoding a drug resistance or herbicide gene or a polynucleotide encoding a detectable marker such as green fluorescent protein (GFP) or *beta*-glucuronidase (*gus*) to allow convenient screening or selection of cells expressing the vector. In embodiments, the vector or expression cassette includes additional elements for improving delivery to the plant cell or for directing or modifying expression of one or more genome-editing system elements, for example, fusing a sequence encoding a cell-penetrating peptide, localization signal, transit, or targeting peptide to the RNA-guided nuclease, or adding a nucleotide sequence to stabilize a guide RNA; such fusion proteins (and the polypeptides encoding such fusion proteins) or combination polypeptides, as well as expression cassettes and vectors for their expression in a

cell, are specifically claimed. In embodiments, an RNA-guided nuclease (*e. g.*, Cas9, Cpf1, CasY, CasX, C2c1, or C2c3) is fused to a localization signal, transit, or targeting peptide, *e. g.*, a nuclear localization signal (NLS), a chloroplast transit peptide (CTP), or a mitochondrial targeting peptide (MTP); in a vector or an expression cassette, the nucleotide sequence encoding any of these can be located either 5' and/or 3' to the DNA encoding the nuclease. For example, a plant-codon-optimized Cas9 (pco-Cas9) from *Streptococcus pyogenes* and *S. thermophilus* containing nuclear localization signals and codon-optimized for expression in maize is disclosed in PCT/US2015/018104 (published as WO/2015/131101 and claiming priority to US Provisional Patent Application 61/945,700), incorporated herein by reference. In another example, a chloroplast-targeting RNA is appended to the 5' end of an mRNA encoding an endonuclease to drive the accumulation of the mRNA in chloroplasts; see Gomez, *et al.* (2010) *Plant Signal Behav.*, 5: 1517 – 1519. In an embodiment, a Cas9 from *Streptococcus pyogenes* is fused to a nuclear localization signal (NLS), such as the NLS from SV40. In an embodiment, a Cas9 from *Streptococcus pyogenes* is fused to a cell-penetrating peptide (CPP), such as octa-arginine or nona-arginine or a homoarginine 12-mer oligopeptide, or a CPP disclosed in the database of cell-penetrating peptides CPPsite 2.0, publicly available at [crdd\[dot\]osdd\[dot\]net/raghava/cppsite/](http://crdd[dot]osdd[dot]net/raghava/cppsite/). In an embodiment, a Cas9 from *Streptococcus pyogenes* is fused to a chloroplast transit peptide (CTP) sequence. In embodiments, a CTP sequence is obtained from any nuclear gene that encodes a protein that targets a chloroplast, and the isolated or synthesized CTP DNA is appended to the 5' end of the DNA that encodes a nuclease targeted for use in a chloroplast. Chloroplast transit peptides and their use are described in US Patents 5,188,642, 5,728,925, and 8,420,888, all of which are incorporated herein by reference in their entirety. Specifically, the CTP nucleotide sequences provided with the sequence identifier (SEQ ID) numbers 12 – 15 and 17 – 22 of US Patent 8,420,888 are incorporated herein by reference. In an embodiment, a Cas9 from *Streptococcus pyogenes* is fused to a mitochondrial targeting peptide (MTP), such as a plant MTP sequence; see, *e. g.*, Jores *et al.* (2016) *Nature Communications*, 7:12036 – 12051.

[0025] Plasmids designed for use in plants and encoding CRISPR genome editing elements (CRISPR nucleases and guide RNAs) are publicly available from plasmid repositories such as Addgene (Cambridge, Massachusetts; also see “addgene[dot]com”). In embodiments, such plasmids are used to co-express both CRISPR nuclease mRNA and guide RNA(s); in other embodiments, CRISPR endonuclease mRNA and guide RNA are delivered from separate plasmids. In embodiments, the plasmids are *Agrobacterium* TI plasmids. Materials and methods for preparing expression cassettes and vectors for CRISPR endonuclease and guide RNA for stably integrated and/or transient plant transformation are disclosed in PCT/US2015/018104 (published as WO/2015/131101 and claiming priority to US Provisional Patent Application 61/945,700), US Patent Application Publication 2015/0082478 A1, and PCT/US2015/038767 (published as WO/2016/007347 and claiming priority to US Provisional Patent Application 62/023,246), all of which are incorporated herein by reference in their entirety. In embodiments, such expression cassettes are isolated linear fragments, or are part of a larger construct that includes bacterial replication elements and selectable markers; such embodiments are useful, *e. g.*, for

particle bombardment or nanoparticle delivery or protoplast transformation. In embodiments, the expression cassette is adjacent to or located between T-DNA borders or contained within a binary vector, *e. g.*, for *Agrobacterium*-mediated transformation. In embodiments, a plasmid encoding a CRISPR nuclease is delivered to a plant cell for stable integration of the CRISPR nuclease into the plant cell's genome, or alternatively for transient expression of the CRISPR nuclease. In embodiments, plasmids encoding a CRISPR nuclease are delivered to a plant cell to achieve stable or transient expression of the CRISPR nuclease, and one or multiple guide RNAs (such as a library of individual guide RNAs or multiple pooled guide RNAs) or plasmids encoding the guide RNAs are delivered to the plant cell individually or in combinations, thus providing libraries or arrays of plant cells, plant parts or tissues, embryos, seeds, or intact plants, in which a variety of genome edits are provided by the different guide RNAs.

[0026] In certain embodiments where the genome-editing system is a CRISPR system, expression of the guide RNA is driven by a plant U6 spliceosomal RNA promoter, which can be native to the plant being edited or from a different plant, *e. g.*, a U6 promoter from maize, tomato, or soybean such as those disclosed in PCT/US2015/018104 (published as WO 2015/131101 and claiming priority to US Provisional Patent Application 61/945,700), incorporated herein by reference, or a homologue thereof; such a promoter is operably linked to DNA encoding the guide RNA for directing an endonuclease, followed by a suitable 3' element such as a U6 poly-T terminator. In another embodiment, an expression cassette for expressing guide RNAs in plants is used, wherein the promoter is a plant U3, 7SL (signal recognition particle RNA), U2, or U5 promoter, or chimerics thereof, *e. g.*, as described in PCT/US2015/018104 (published as WO 2015/131101 and claiming priority to US Provisional Patent Application 61/945,700), incorporated herein by reference. When multiple or different guide RNA sequences are used, a single expression construct may be used to correspondingly direct the genome editing activity to the multiple or different target sequences in a cell. In various embodiments, a single vector includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, about 15, about 20, or more guide RNA sequences; in other embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, about 15, about 20, or more guide RNA sequences are provided on multiple vectors, which can be delivered to one or multiple cells (*e. g.*, delivered to an array of plant cells, plant parts or tissues, embryos, seeds, or plants).

[0027] In embodiments, one or more guide RNAs and the corresponding RNA-guided nuclease are delivered together or simultaneously. In other embodiments, one or more guide RNAs and the corresponding RNA-guided nuclease are delivered separately; these can be delivered in separate, discrete steps and using the same or different delivery techniques. In an example, an RNA-guided nuclease is delivered to a plant cell by particle bombardment, on carbon nanotubes, or by *Agrobacterium*-mediated transformation, and one or more guide RNAs is delivered to the plant cell in a separate step using the same or different delivery technique. In embodiments, an RNA-guided nuclease encoded by a DNA molecule or an mRNA is delivered to a plant cell with enough time prior to delivery of the guide RNA to permit expression of the nuclease in the plant cell; for example, an RNA-guided nuclease encoded by a DNA molecule or an mRNA is delivered to a plant cell between 1 – 12 hours (*e. g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 hours, or between about 1 – 6 hours or between about 2 – 6 hours) prior to the delivery of the

guide RNA to the plant cell. In embodiments, whether the RNA-guided nuclease is delivered simultaneously with or separately from an initial dose of guide RNA, succeeding “booster” doses of guide RNA are delivered subsequent to the delivery of the initial dose; for example, a second “booster” dose of guide RNA is delivered to a plant cell between 1 – 12 hours (*e. g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 hours, or between about 1 – 6 hours or between about 2 – 6 hours) subsequent to the delivery of the initial dose of guide RNA to the plant cell. Similarly, in some embodiments, multiple deliveries of an RNA-guided nuclease or of a DNA molecule or an mRNA encoding an RNA-guided nuclease are used to increase efficiency of the genome modification.

[0028] In embodiments, the desired genome modification involves homologous recombination, wherein one or more double-stranded DNA break in the target nucleotide sequence is generated by the RNA-guided nuclease and guide RNA(s), followed by repair of the break(s) using a homologous recombination mechanism (“homology-directed repair”). In such embodiments, a donor template that encodes the desired nucleotide sequence to be inserted or knocked-in at the double-stranded break is provided to the cell; examples of suitable templates include single-stranded DNA templates and double-stranded DNA templates (*e. g.*, in the form of a plasmid). In general, a donor template encoding a nucleotide change over a region of less than about 50 nucleotides is conveniently provided in the form of single-stranded DNA; larger donor templates (*e. g.*, more than 100 nucleotides) are often conveniently provided as double-stranded DNA plasmids. In embodiments, the various compositions and methods described herein for delivering guide RNAs and nucleases are also generally useful for delivering the donor template polynucleotide to the plant cell; this delivery can be simultaneous with, or separate from (generally after) delivery of the nuclease and guide RNA to the cell. For example, a donor template can be transiently introduced into a plant cell, optionally with the nuclease and/or gRNA; in embodiments, the donor template is provided to the cell in a quantity that is sufficient to achieve the desired homology-directed repair but that does not persist in the cell after a given period of time (*e. g.*, after one or more cell division cycles). In embodiments, a donor template has a core nucleotide sequence that differs from the target nucleotide sequence (*e. g.*, a homologous endogenous genomic region) by at least 1, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, or more nucleotides. This core sequence is flanked by “homology arms” or regions of high sequence identity with the targeted nucleotide sequence; in embodiments, the regions of high identity include at least 10, at least 50, at least 100, at least 150, at least 200, at least 300, at least 400, at least 500, at least 600, at least 750, or at least 1000 nucleotides on each side of the core sequence. In embodiments where the donor template is in the form of a single-stranded DNA, the core sequence is flanked by homology arms including at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 100 nucleotides on each side of the core sequence. In embodiments where the donor template is in the form of a double-stranded DNA plasmid, the core sequence is flanked by homology arms including at least 500, at least 600, at least 700, at least 800, at least 900, or at least 1000 nucleotides on each side of the core sequence. In an embodiment, two separate double-strand breaks are introduced into

the cell's target nucleotide sequence with a "double nickase" Cas9 (see Ran *et al.* (2013) *Cell*, 154:1380 – 1389), followed by delivery of the donor template.

Methods of altering a target nucleotide sequence in a plant cell

[0029] In one aspect the invention provides a method of delivering a guide RNA (gRNA) to a plant cell, particularly a non-epidermal plant cell, wherein the non-epidermal plant cell is in a plant or part of a plant, wherein the gRNA has a nucleotide sequence designed to alter a target nucleotide sequence in the non-epidermal plant cell, wherein the gRNA is provided as a polynucleotide composition including: (i) a CRISPR RNA (crRNA) that includes the gRNA, or a polynucleotide that encodes a crRNA, or a polynucleotide that is processed into a crRNA; or (ii) a single guide RNA (sgRNA) that includes the gRNA, or a polynucleotide that encodes an sgRNA, or a polynucleotide that is processed into an sgRNA; wherein the delivery of the polynucleotide composition includes at least one treatment selected from the group consisting of: direct application; soaking or imbibition; vacuum infiltration; application of negative or positive pressure; introduction into the vascular system; microinjection; application of ultrasound or vibration; application of hydrodynamic pressure, friction, cavitation or shear stress; vortexing; centrifugation; mechanical cell wall or cell membrane deformation or breakage; enzymatic cell wall or cell membrane breakage or permeabilization; abrasion; electroporation; and treatment with at least one chemical, enzymatic, or physical agent; whereby the gRNA is delivered to the non-epidermal plant cell. In embodiments, delivery of the gRNA results in alteration of the target nucleotide sequence in the non-epidermal plant cell.

[0030] The target nucleotide sequence is one or more nucleotide sequences, including protein-coding sequence or non-coding sequence or a combination thereof. Embodiments include a plant nuclear sequence, a plant plastid sequence, a plant mitochondrial sequence, a sequence of a symbiont, pest, or pathogen of a plant, and combinations thereof. Embodiments include exons, introns, regulatory sequences including promoters, other 5' elements and 3' elements, and genomic loci encoding non-coding RNAs including long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and *trans*-acting siRNAs (ta-siRNAs). In embodiments, multiple target nucleotide sequences are altered, for example, by delivery of multiple gRNAs to the non-epidermal plant cell; the multiple target nucleotide sequences can be part of the same gene (*e. g.*, different locations in a single coding region or in different exons of a protein-coding gene) or different genes.

[0031] In embodiments, the guide RNA (gRNA) has a sequence of between 16 – 24 nucleotides in length (*e. g.*, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length). Specific embodiments include gRNAs of 19, 20, or 21 nucleotides in length and having 100% complementarity to the target nucleotide sequence. In many embodiments the gRNA has exact complementarity (*i. e.*, perfect base-pairing) to the target nucleotide sequence; in certain other embodiments the gRNA has less than 100% complementarity to the target nucleotide sequence. The design of effective gRNAs for use in plant genome editing is disclosed in US Patent Application Publication 2015/0082478 A1, the entire specification of which is incorporated

herein by reference. Efficient Cas9-mediated gene editing has been achieved using a chimeric “single guide RNA” (“sgRNA”), an engineered (synthetic) single RNA molecule that mimics a naturally occurring crRNA-tracrRNA complex and contains both a tracrRNA (for binding the nuclease) and at least one crRNA (to guide the nuclease to the sequence targeted for editing).

[0032] Thus, in certain embodiments wherein the nuclease is a Cas9-type nuclease, the gRNA can be provided as a polynucleotide composition including: (a) a CRISPR RNA (crRNA) that includes the gRNA together with a separate tracrRNA, or (b) at least one polynucleotide that encodes a crRNA and a tracrRNA (on a single polynucleotide or on separate polynucleotides), or (c) at least one polynucleotide that is processed into one or more crRNAs and a tracrRNA. In other embodiments wherein the nuclease is a Cas9-type nuclease, the gRNA can be provided as a polynucleotide composition including a CRISPR RNA (crRNA) that includes the gRNA, and the required tracrRNA is provided in a separate composition or in a separate step, or is otherwise provided to the plant cell (for example, to a plant cell located in a plant part or tissue, embryo, seed, or plants stably or transiently expressing the tracrRNA from a polynucleotide encoding the tracrRNA). In other embodiments wherein the nuclease is a Cas9-type nuclease, the gRNA can be provided as a polynucleotide composition including: (a) a single guide RNA (sgRNA) that includes the gRNA, or (b) a polynucleotide that encodes an sgRNA, or (c) a polynucleotide that is processed into an sgRNA. Cpf1-mediated gene editing does not require a tracrRNA; thus, in embodiments wherein the nuclease is a Cpf1-type nuclease, the gRNA is provided as a polynucleotide composition including (a) a CRISPR RNA (crRNA) that includes the gRNA, or (b) a polynucleotide that encodes a crRNA, or (c) a polynucleotide that is processed into a crRNA.

[0033] In embodiments of the method, the polynucleotide composition optionally includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease. In other embodiments of the method, the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease. In other embodiments of the method, the non-epidermal plant cell includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; in an example the non-epidermal plant cell is in or from a transgenic plant that expresses the RNA-guided nuclease. In embodiments, the RNA-guided nuclease is selected from the group consisting of an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered RNA-guided nuclease, and a codon-optimized RNA-guided nuclease. In embodiments, the polynucleotide that encodes the RNA-guided nuclease is, for example, DNA that encodes the RNA-guided nuclease and is stably integrated in the genome of the non-epidermal plant cell, DNA or RNA that encodes the RNA-guided nuclease and is transiently present in or introduced into the non-epidermal plant cell; such DNA or RNA can be introduced, *e. g.*, by using a vector such as a plasmid or viral vector or as an mRNA.

[0034] In embodiments of the method that further include the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease, the RNA-guided nuclease is provided simultaneously with the polynucleotide composition that includes the

gRNA, or in a separate step that precedes or follows the step of providing the polynucleotide composition. In embodiments, the polynucleotide composition that includes the gRNA further includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease. In other embodiments, there is provided a separate composition that includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease. In embodiments, the RNA-guided nuclease is provided as a ribonucleoprotein (RNP) complex, *e. g.*, a preassembled RNP that includes the RNA-guided nuclease complexed with a polynucleotide including the gRNA or encoding a gRNA, or a preassembled RNP that includes a polynucleotide that encodes the RNA-guided nuclease (and optionally encodes the gRNA, or is provided with a separate polynucleotide including the gRNA or encoding a gRNA), complexed with a protein. In embodiments, the RNA-guided nuclease is a fusion protein, *i. e.*, wherein the RNA-guided nuclease is covalently bound through a peptide bond to a cell-penetrating peptide, a nuclear localization signal peptide, a chloroplast transit peptide, or a mitochondrial targeting peptide; such fusion proteins are conveniently encoded in a single nucleotide sequence, optionally including codons for linking amino acids. In embodiments, the RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease is provided as a complex with a cell-penetrating peptide or other transfecting agent. In embodiments, the RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease is complexed with, or covalently or non-covalently bound to, a further element, *e. g.*, a carrier molecule, an antibody, an antigen, a viral movement protein, a polymer, a detectable label (*e. g.*, a moiety detectable by fluorescence, radioactivity, or enzymatic or immunochemical reaction), a quantum dot, or a particulate or nanoparticulate. In embodiments, the RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease is provided in a solution, or is provided in a liposome, micelle, emulsion, reverse emulsion, suspension, or other mixed-phase composition.

[0035] The RNA-guided nuclease is provided to the non-epidermal plant cell by any suitable technique. In embodiments, the RNA-guided nuclease is provided by directly contacting the non-epidermal plant cell with the RNA-guided nuclease or the polynucleotide that encodes the RNA-guided nuclease. In embodiments, the RNA-guided nuclease is provided by transporting the RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease into the non-epidermal plant cell using a chemical, enzymatic, or physical agent as provided in detail below in the paragraphs following the heading “Delivery Agents”. In embodiments, the RNA-guided nuclease is provided by bacterially mediated (*e. g.*, *Agrobacterium* sp., *Rhizobium* sp., *Sinorhizobium* sp., *Mesorhizobium* sp., *Bradyrhizobium* sp., *Azobacter* sp., *Phyllobacterium* sp.) transfection of the non-epidermal plant cell with a polynucleotide encoding the RNA-guided nuclease; see, *e. g.*, Broothaerts *et al.* (2005) *Nature*, 433:629 – 633. In an embodiment, the RNA-guided nuclease is provided by transcription in the non-epidermal plant cell of a DNA that encodes the RNA-guided nuclease and is stably integrated in the genome of the non-epidermal plant cell or that is provided to the non-epidermal plant cell in the form of a plasmid or expression vector (*e. g.*, a viral vector) that encodes the RNA-guided nuclease (and optionally encodes one or more gRNAs, crRNAs, or sgRNAs, or is optionally provided with a separate plasmid or vector that encodes one or more gRNAs, crRNAs, or

sgRNAs). In embodiments, the RNA-guided nuclease is provided to the non-epidermal plant cell as a polynucleotide that encodes the RNA-guided nuclease, *e. g.*, in the form of an mRNA encoding the nuclease.

[0036] Where a polynucleotide is concerned (*e. g.*, a crRNA that includes the gRNA together with a separate tracrRNA, or at least one polynucleotide that encodes a crRNA and a tracrRNA (on a single polynucleotide or on separate polynucleotides), or at least one polynucleotide that is processed into one or more crRNAs and a tracrRNA, or an sgRNA that includes the gRNA, or a polynucleotide that encodes an sgRNA, or a polynucleotide that is processed into an sgRNA, or a polynucleotide that encodes the RNA-guided nuclease), embodiments of the polynucleotide include: (a) double-stranded RNA; (b) single-stranded RNA; (c) chemically modified RNA; (d) double-stranded DNA; (e) single-stranded DNA; (f) chemically modified DNA; or (g) a combination of (a) – (f). Where expression of a polynucleotide is involved (*e. g.*, expression of a crRNA from a DNA encoding the crRNA, or expression and translation of a RNA-guided nuclease from a DNA encoding the nuclease), in some embodiments it is sufficient that expression be transient, *i. e.*, not necessarily permanent or stable in the plant cell. Certain embodiments of the polynucleotide further include additional nucleotide sequences that provide useful functionality; non-limiting examples of such additional nucleotide sequences include an aptamer or riboswitch sequence, nucleotide sequence that provides secondary structure such as stem-loops or that provides a sequence-specific site for an enzyme (*e. g.*, a sequence-specific recombinase or endonuclease site), T-DNA (*e. g.*, DNA sequence encoding a gRNA, crRNA, tracrRNA, or sgRNA is enclosed between left and right T-DNA borders from *Agrobacterium* spp. or from other bacteria that infect or induce tumours in plants), a DNA nuclear-targeting sequence, a regulatory sequence such as a promoter sequence, and a transcript-stabilizing sequence. Certain embodiments of the polynucleotide include those wherein the polynucleotide is complexed with, or covalently or non-covalently bound to, a non-nucleic acid element, *e. g.*, a carrier molecule, an antibody, an antigen, a viral movement protein, a cell-penetrating or pore-forming peptide, a polymer, a detectable label, a quantum dot, or a particulate or nanoparticulate.

[0037] Generally, the non-epidermal plant cell is not an isolated plant cell (*e. g.*, not a plant cell or protoplast isolated from plant tissue or in suspension or plate culture) and is a cell located in an intact or growing plant or in a plant part or tissue. In embodiments, the non-epidermal plant cell is capable of division and further differentiation. In embodiments, the non-epidermal plant cell is located in a plant or part of a plant selected from the group consisting of a plant tissue, a whole plant, an intact nodal bud, a shoot apex or shoot apical meristem, a root apex or root apical meristem, lateral meristem, intercalary meristem, a seedling (*e. g.*, a germinating seed or small seedling or a larger seedling with one or more true leaves), a whole seed (*e. g.*, an intact seed, or a seed with part or all of its seed coat removed or treated to make permeable), a halved seed or other seed fragment, an embryo (*e. g.*, a mature dissected zygotic embryo, a developing embryo, a dry or rehydrated or freshly excised embryo), and callus.

[0038] In embodiments, the non-epidermal cell is diploid or polyploid. In embodiments, the non-epidermal plant cell is haploid or can be induced to become haploid; techniques for making and using haploid plants and plant cells are known in the art, see, *e. g.*, methods for generating haploids in *Arabidopsis*

thaliana by crossing of a wild-type strain to a haploid-inducing strain that expresses altered forms of the centromere-specific histone CENH3, as described by Maruthachalam and Chan in “How to make haploid *Arabidopsis thaliana*”, a protocol publicly available at [www\[dot\]openwetware\[dot\]org/images/d/d3/Haploid_Arabidopsis_protocol\[dot\]pdf](http://www.openwetware.org/images/d/d3/Haploid_Arabidopsis_protocol.pdf); Ravi *et al.* (2014) *Nature Communications*, 5:5334, doi: 10.1038/ncomms6334); . Examples of haploid cells include but are not limited to plant cells in haploid plants and plant cells in reproductive tissues, *e. g.*, flowers, developing flowers or flower buds, ovaries, ovules, megaspores, anthers, pollen, and microspores. In embodiments where the non-epidermal plant cell is haploid, the method can further include the step of chromosome doubling (*e. g.*, by spontaneous chromosomal doubling by meiotic non-reduction, or by using a chromosome doubling agent such as colchicine, oryzalin, or trifluralin) in the non-epidermal plant cell including the altered target nucleotide sequence to produce a doubled haploid cell that is homozygous for the altered target nucleotide sequence; yet other embodiments include regeneration of a doubled haploid plant from the doubled haploid cell, wherein the regenerated doubled haploid plant is homozygous for the altered target nucleotide sequence. Thus, aspects of the invention are related to the haploid cell having the altered target nucleotide sequence as well as a doubled haploid cell or a doubled haploid plant that is homozygous for the altered target nucleotide sequence. Another aspect of the invention is related to a hybrid plant having at least one parent plant that is a doubled haploid plant provided by the method. Production of doubled haploid plants by these methods provides homozygosity in one generation, instead of requiring several generations of self-crossing to obtain homozygous plants; this may be particularly advantageous in slow-growing plants, such as fruit and other trees, or for producing hybrid plants that are offspring of at least one doubled-haploid plant.

[0039] In embodiments, the plant is a dicot or a monocot. In embodiments, the plant is a gymnosperm, such as a conifer. Plants of interest include row crop plants, fruit-producing plants and trees, vegetables, trees, and ornamental plants including ornamental flowers, shrubs, trees, groundcovers, and turf grasses. Examples of commercially important cultivated crops, trees, and plants include: alfalfa (*Medicago sativa*), almonds (*Prunus dulcis*), apples (*Malus x domestica*), apricots (*Prunus armeniaca*, *P. brigantine*, *P. mandshurica*, *P. mume*, *P. sibirica*), asparagus (*Asparagus officinalis*), bananas (*Musa* spp.), barley (*Hordeum vulgare*), beans (*Phaseolus* spp.), blueberries and cranberries (*Vaccinium* spp.), cacao (*Theobroma cacao*), canola and rapeseed or oilseed rape, (*Brassica napus*), carnation (*Dianthus caryophyllus*), carrots (*Daucus carota sativus*), cassava (*Manihot esculentum*), cherry (*Prunus avium*), chickpea (*Cicer arietinum*), chicory (*Cichorium intybus*), chili peppers and other capsicum peppers (*Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, *C. baccatum*), chrysanthemums (*Chrysanthemum* spp.), coconut (*Cocos nucifera*), coffee (*Coffea* spp. including *Coffea arabica* and *Coffea canephora*), cotton (*Gossypium hirsutum* L.), cowpea (*Vigna unguiculata*), cucumber (*Cucumis sativus*), currants and gooseberries (*Ribes* spp.), eggplant or aubergine (*Solanum melongena*), eucalyptus (*Eucalyptus* spp.), flax (*Linum usitatissimum* L.), geraniums (*Pelargonium* spp.), grapefruit (*Citrus x paradisi*), grapes (*Vitis* spp.) including wine grapes (*Vitis vinifera*), guava (*Psidium guajava*), irises (*Iris* spp.), lemon (*Citrus*

limon), lettuce (*Lactuca sativa*), limes (*Citrus* spp.), maize (*Zea mays* L.), mango (*Mangifera indica*), mangosteen (*Garcinia mangostana*), melon (*Cucumis melo*), millets (*Setaria* spp, *Echinochloa* spp, *Eleusine* spp, *Panicum* spp., *Pennisetum* spp.), oats (*Avena sativa*), oil palm (*Ellis quineensis*), olive (*Olea europaea*), onion (*Allium cepa*), orange (*Citrus sinensis*), papaya (*Carica papaya*), peaches and nectarines (*Prunus persica*), pear (*Pyrus* spp.), pea (*Pisa sativum*), peanut (*Arachis hypogaea*), peonies (*Paeonia* spp.), petunias (*Petunia* spp.), pineapple (*Ananas comosus*), plantains (*Musa* spp.), plum (*Prunus domestica*), poinsettia (*Euphorbia pulcherrima*), Polish canola (*Brassica rapa*), poplar (*Populus* spp.), potato (*Solanum tuberosum*), pumpkin (*Cucurbita pepo*), rice (*Oryza sativa* L.), roses (*Rosa* spp.), rubber (*Hevea brasiliensis*), rye (*Secale cereale*), safflower (*Carthamus tinctorius* L), sesame seed (*Sesame indium*), sorghum (*Sorghum bicolor*), soybean (*Glycine max* L.), squash (*Cucurbita pepo*), strawberries (*Fragaria* spp., *Fragaria x ananassa*), sugar beet (*Beta vulgaris*), sugarcane (*Saccharum* spp.), sunflower (*Helianthus annuus*), sweet potato (*Ipomoea batatas*), tangerine (*Citrus tangerina*), tea (*Camellia sinensis*), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum*), tulips (*Tulipa* spp.), turnip (*Brassica rapa rapa*), walnuts (*Juglans* spp. L.), watermelon (*Citrulus lanatus*), wheat (*Tritium aestivum*), and yams (*Discorea* spp.).

[0040] Embodiments of the method involve various treatments employed to deliver the polynucleotide composition to the non-epidermal plant cell. In embodiments, one or more treatments is employed to deliver the polynucleotide composition into the non-epidermal plant cell, *e. g.*, through barriers such as a seed coat, a cell wall, a plasma membrane or nuclear envelope or other lipid bilayer, or through multiple cell layers or tissues. In an embodiment, the polynucleotide composition is delivered directly, for example by direct contact of the polynucleotide composition with the non-epidermal plant cell. Polynucleotide compositions in the form of a liquid, a solution, a suspension, an emulsion, a reverse emulsion, a colloid, a dispersion, a gel, liposomes, micelles, an injectable material, an aerosol, a solid, a powder, a particulate, a nanoparticle, or a combination thereof can be applied directly to a plant or plant part, to the surface or to the interior (*e. g.*, through an incision, abrasion, or puncture, by spraying or dipping or soaking or otherwise directly contacting, by injection or microinjection). For example, a seed or seed fragment or embryo is soaked in or imbibes a liquid polynucleotide composition, whereby the gRNA is delivered to non-epidermal cells in the seed or seed fragment or embryo. In embodiments, the polynucleotide composition is delivered using negative or positive pressure, for example, using vacuum infiltration or application of hydrodynamic or fluid pressure. In embodiments, the polynucleotide composition is introduced into the vascular system of a plant or plant part, *e. g.*, by injection or microinjection into the phloem, or by vascular uptake through a stem or petiole; see, *e. g.*, Sun *et al.* (2005) *Plant J.*, 44:128 – 138. In embodiments, the polynucleotide composition is introduced into non-vascular tissues by injection or microinjection or through the application of negative or positive pressure. Other techniques useful for delivering the polynucleotide composition to a non-epidermal plant cell include: ultrasound or sonication; vibration, friction, shear stress, vortexing, cavitation; centrifugation or application of mechanical force; mechanical cell wall or cell membrane deformation or breakage; enzymatic cell wall or

cell membrane breakage or permeabilization; abrasion or mechanical scarification (*e. g.*, abrasion with carborundum or other particulate abrasive or scarification with a file or sandpaper) or chemical scarification (*e. g.*, treatment with an acid or caustic agent); and electroporation. In embodiments, the polynucleotide composition is provided by bacterially mediated (*e. g.*, *Agrobacterium* sp., *Rhizobium* sp., *Sinorhizobium* sp., *Mesorhizobium* sp., *Bradyrhizobium* sp., *Azobacter* sp., *Phyllobacterium* sp.) transfection of the non-epidermal plant cell with a polynucleotide encoding the gRNA; see, *e. g.*, Broothaerts *et al.* (2005) *Nature*, 433:629 – 633.

[0041] In embodiments, a treatment employed in delivery of the polynucleotide composition to the non-epidermal plant cell is carried out under a specific thermal regime, which can involve one or more appropriate temperatures, *e. g.*, chilling or cold stress (exposure to temperatures below that at which normal plant growth occurs), or heating or heat stress (exposure to temperatures above that at which normal plant growth occurs), or treating at a combination of different temperatures. In embodiments, a specific thermal regime is carried out on the non-epidermal plant cell, or on the plant or plant part in which the non-epidermal plant cell is located, in one or more steps separate from the polynucleotide composition delivery.

[0042] Delivery Agents: Embodiments of the method include treatment of the non-epidermal plant cell, or the plant or plant part in which the non-epidermal plant cell is located, with one or more delivery agents which can include at least one chemical, enzymatic, or physical agent, or a combination thereof. In embodiments, the polynucleotide composition further includes one or more one chemical, enzymatic, or physical agent for delivery. In embodiments of the method that further include the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease, a composition including the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease further includes one or more one chemical, enzymatic, or physical agent for delivery. Treatment with the chemical, enzymatic or physical agent can be carried out simultaneously with the polynucleotide composition delivery, with the RNA-guided nuclease delivery, or in one or more separate steps that precede or follow the polynucleotide composition delivery or the RNA-guided nuclease delivery. In embodiments, a chemical, enzymatic, or physical agent, or a combination of these, is associated or complexed with the polynucleotide composition, with the gRNA or polynucleotide that encodes or is processed to the gRNA, or with the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease; examples of such associations or complexes include those involving non-covalent interactions (*e. g.*, ionic or electrostatic interactions, hydrophobic or hydrophilic interactions, formation of liposomes, micelles, or other heterogeneous composition) and covalent interactions (*e. g.*, peptide bonds, bonds formed using cross-linking agents). In non-limiting examples, a gRNA or polynucleotide that encodes or is processed to the gRNA is provided as a liposomal complex with a cationic lipid; a gRNA or polynucleotide that encodes or is processed to the gRNA is provided as a complex with a carbon nanotube; and an RNA-guided nuclease is provided as a fusion protein between the nuclease and a cell-penetrating peptide. Examples of agents useful for delivering a gRNA or polynucleotide that encodes or is processed to the gRNA or a nuclease or polynucleotide that encodes the nuclease include the various cationic liposomes and polymer nanoparticles

reviewed by Zhang *et al.* (2007) *J. Controlled Release*, 123:1 – 10, and the cross-linked multilamellar liposomes described in US Patent Application Publication 2014/0356414 A1, incorporated by reference in its entirety herein.

[0043] In embodiments, the chemical agent is at least one selected from the group consisting of:

- (a) solvents (*e. g.*, water, dimethylsulfoxide, dimethylformamide, acetonitrile, *N*-pyrrolidine, pyridine, hexamethylphosphoramide, alcohols, alkanes, alkenes, dioxanes, polyethylene glycol, and other solvents miscible or emulsifiable with water or that will dissolve phosphonucleotides in non-aqueous systems);
- (b) fluorocarbons (*e. g.*, perfluorodecalin, perfluoromethyldecalin);
- (c) glycols or polyols (*e. g.*, propylene glycol, polyethylene glycol);
- (d) surfactants, including cationic surfactants, anionic surfactants, non-ionic surfactants, and amphiphilic surfactants, *e. g.*, alkyl or aryl sulfates, phosphates, sulfonates, or carboxylates; primary, secondary, or tertiary amines; quaternary ammonium salts; sultaines, betaines; cationic lipids; phospholipids; tallowamine; bile acids such as cholic acid; saponins or glycosylated triterpenoids or glycosylated sterols (*e. g.*, saponin commercially available as catalogue number 47036-50g-F, Sigma-Aldrich, St. Louis, MO); long chain alcohols; organosilicone surfactants including nonionic organosilicone surfactants such as trisiloxane ethoxylate surfactants or a silicone polyether copolymer such as a copolymer of polyalkylene oxide modified heptamethyl trisiloxane and allyloxypolypropylene glycol methylether (commercially available as SILWET L-77TM brand surfactant having CAS Number 27306-78-1 and EPA Number CAL. REG. NO. 5905-50073-AA, Momentive Performance Materials, Inc., Albany, N.Y.); specific examples of useful surfactants include sodium lauryl sulfate, the Tween series of surfactants, Triton-X100, Triton-X114, CHAPS and CHAPSO, Tergitol-type NP-40, Nonidet P-40;
- (e) lipids, lipoproteins, lipopolysaccharides;
- (f) acids, bases, caustic agents;
- (g) peptides, proteins, or enzymes (*e. g.*, cellulase, pectolyase, maceroenzyme, pectinase), including cell-penetrating or pore-forming peptides (*e. g.*, (BO100)2K8, Genscript; poly-lysine, poly-arginine, or poly-homoarginine peptides; gamma zein, see US Patent Application publication 2011/0247100, incorporated herein by reference in its entirety; transcription activator of human immunodeficiency virus type 1 (“HIV-1 Tat”) and other Tat proteins, see, *e. g.*, [www\[dot\]lifetein\[dot\]com/Cell_Penetrating_Peptides\[dot\]html](http://www.lifetein.com/Cell_Penetrating_Peptides.html) and Järver (2012) *Mol. Therapy–Nucleic Acids*, 1:e27,1 – 17); octa-arginine or nona-arginine; poly-homoarginine (see Unnamalai *et al.* (2004) *FEBS Letters*, 566:307 – 310); see also the database of cell-penetrating peptides CPPsite 2.0 publicly available at [crdd\[dot\]osdd\[dot\]net/raghava/cppsite/](http://crdd[dot]osdd[dot]net/raghava/cppsite/)
- (h) RNase inhibitors;
- (i) cationic branched or linear polymers such as chitosan, poly-lysine, DEAE-dextran, polyvinylpyrrolidone (“PVP”), or polyethylenimine (“PEI”, *e. g.*, PEI, branched, MW 25,000, CAS# 9002-98-6; PEI, linear, MW 5000, CAS# 9002-98-6; PEI linear, MW 2500, CAS# 9002-98-6);
- (j) dendrimers (see, *e. g.*, US Patent Application Publication 2011/0093982, incorporated herein by reference in its entirety);

(k) counter-ions, amines or polyamines (*e. g.*, spermine, spermidine, putrescine), osmolytes, buffers, and salts (*e. g.*, calcium phosphate, ammonium phosphate);

(l) polynucleotides (*e. g.*, non-specific double-stranded DNA, salmon sperm DNA);

(m) transfection agents (*e. g.*, Lipofectin®, Lipofectamine®, and Oligofectamine®, and InvivoFectamine® (all from Thermo Fisher Scientific, Waltham, MA), PepFect (see Ezzat *et al.* (2011) *Nucleic Acids Res.*, 39:5284 – 5298), TransIt® transfection reagents (Mirus Bio, LLC, Madison, WI), and poly-lysine, poly-homoarginine, and poly-arginine molecules including octo-arginine and nono-arginine as described in Lu *et al.* (2010) *J. Agric. Food Chem.*, 58:2288 – 2294);

(n) antibiotics, including non-specific DNA double-strand-break-inducing agents (*e. g.*, phleomycin, bleomycin, talisomycin);

(o) antioxidants (*e. g.*, glutathione, dithiothreitol, ascorbate); and

(p) chelating agents (*e. g.*, EDTA, EGTA).

[0044] In embodiments, the chemical agent is provided simultaneously with the gRNA (or polynucleotide encoding the gRNA or that is processed to the gRNA), for example, the polynucleotide composition including the gRNA further includes one or more chemical agent. In embodiments, the gRNA or polynucleotide encoding the gRNA or that is processed to the gRNA is covalently or non-covalently linked or complexed with one or more chemical agent; for example, the gRNA or polynucleotide encoding the gRNA or that is processed to the gRNA can be covalently linked to a peptide or protein (*e. g.*, a cell-penetrating peptide or a pore-forming peptide) or non-covalently complexed with cationic lipids, polycations (*e. g.*, polyamines), or cationic polymers (*e. g.*, PEI). In embodiments, the gRNA or polynucleotide encoding the gRNA or that is processed to the gRNA is complexed with one or more chemical agents to form, *e. g.*, a solution, liposome, micelle, emulsion, reverse emulsion, suspension, colloid, or gel.

[0045] In embodiments, the physical agent is at least one selected from the group consisting of particles or nanoparticles (*e. g.*, particles or nanoparticles made of materials such as carbon, silicon, silicon carbide, gold, tungsten, polymers, or ceramics) in various size ranges and shapes, magnetic particles or nanoparticles (*e. g.*, silenceMag Magnetotransfection™ agent, OZ Biosciences, San Diego, CA), abrasive or scarifying agents, needles or microneedles, matrices, and grids. In embodiments, particulates and nanoparticulates are useful in delivery of the polynucleotide composition or the nuclease or both. Useful particulates and nanoparticles include those made of metals (*e. g.*, gold, silver, tungsten, iron, cerium), ceramics (*e. g.*, aluminum oxide, silicon carbide, silicon nitride, tungsten carbide), polymers (*e. g.*, polystyrene, polydiacetylene, and poly(3,4-ethylenedioxythiophene) hydrate), semiconductors (*e. g.*, quantum dots), silicon (*e. g.*, silicon carbide), carbon (*e. g.*, graphite, graphene, graphene oxide, or carbon nanosheets, nanocomplexes, or nanotubes), and composites (*e. g.*, polyvinylcarbazole/graphene, polystyrene/graphene, platinum/graphene, palladium/graphene nanocomposites). In embodiments, such particulates and nanoparticulates are further covalently or non-covalently functionalized, or further include modifiers or cross-linked materials such as polymers (*e. g.*, linear or branched polyethylenimine, poly-lysine), polynucleotides (*e. g.*, DNA or RNA), polysaccharides, lipids, polyglycols (*e. g.*, polyethylene

glycol, thiolated polyethylene glycol), polypeptides or proteins, and detectable labels (*e. g.*, a fluorophore, an antigen, an antibody, or a quantum dot). In various embodiments, such particulates and nanoparticles are neutral, or carry a positive charge, or carry a negative charge. Embodiments of compositions including particulates include those formulated, *e. g.*, as liquids, colloids, dispersions, suspensions, aerosols, gels, and solids. Embodiments include nanoparticles affixed to a surface or support, *e. g.*, an array of carbon nanotubes vertically aligned on a silicon or copper wafer substrate. Embodiments include polynucleotide compositions including particulates (*e. g.*, gold or tungsten or magnetic microparticles or nanoparticles) delivered by a Biolistic-type technique or with magnetic force. The size of the particles used in Biolistics is generally in the “microparticle” range, for example, gold microcarriers in the 0.6, 1.0, and 1.6 micrometer size ranges (see, *e. g.*, instruction manual for the Helios® Gene Gun System, Bio-Rad, Hercules, CA; Randolph-Anderson *et al.* (2015) “Sub-micron gold particles are superior to larger particles for efficient Biolistic® transformation of organelles and some cell types”, Bio-Rad US/EG Bulletin 2015), but successful Biolistics delivery using larger (40 nanometer) nanoparticles has been reported in cultured animal cells; see O’Brian and Lummis (2011) *BMC Biotechnol.*, 11:66 – 71. Other embodiments of useful particulates are nanoparticles, which are generally in the nanometer (nm) size range or less than 1 micrometer, *e. g.*, with a diameter of less than about 1 nm, less than about 3 nm, less than about 5 nm, less than about 10 nm, less than about 20 nm, less than about 40 nm, less than about 60 nm, less than about 80 nm, and less than about 100 nm. Specific, non-limiting embodiments of nanoparticles commercially available (all from Sigma-Aldrich Corp., St. Louis, MO) include gold nanoparticles with diameters of 5, 10, or 15 nm; silver nanoparticles with particle sizes of 10, 20, 40, 60, or 100 nm; palladium “nanopowder” of less than 25 nm particle size; single-, double-, and multi-walled carbon nanotubes, *e. g.*, with diameters of 0.7 – 1.1, 1.3 – 2.3, 0.7 – 0.9, or 0.7 – 1.3 nm, or with nanotube bundle dimensions of 2 – 10 nm by 1- 5 micrometers, 6 – 9 nm by 5 micrometers, 7 – 15 nm by 0.5 – 10 micrometers, 7 – 12 nm by 0.5 – 10 micrometers, 110 – 170 nm by 5 – 9 micrometers, 6 – 13 nm by 2.5 – 20 micrometers. Embodiments include polynucleotide compositions including materials such as gold, silicon, cerium, or carbon, *e. g.*, gold or gold-coated nanoparticles, silicon carbide whiskers, carborundum, porous silica nanoparticles, gelatin/silica nanoparticles, nanoceria or cerium oxide nanoparticles (CNPs), carbon nanotubes (CNTs) such as single-, double-, or multi-walled carbon nanotubes and their chemically functionalized versions (*e. g.*, carbon nanotubes functionalized with amide, amino, carboxylic acid, sulfonic acid, or polyethylene glycol moieties), and graphene or graphene oxide or graphene complexes; see, for example, Wong *et al.* (2016) *Nano Lett.*, 16:1161 – 1172; Giraldo *et al.* (2014) *Nature Materials*, 13:400-409; Shen *et al.* (2012) *Theranostics*, 2:283 – 294; Kim *et al.* (2011) *Bioconjugate Chem.*, 22:2558 – 2567; Wang *et al.* (2010) *J. Am. Chem. Soc. Comm.*, 132:9274 – 9276; Zhao *et al.* (2016) *Nanoscale Res. Lett.*, 11:195 – 203; Choi *et al.* (2016) *J. Controlled Release*, 235:222 – 235; and Zhai *et al.* (2014) *Environ. Sci. Technol. Lett.*, 1:146–151. See also, for example, the various types of particles and nanoparticles, their preparation, and methods for their use, *e. g.*, in delivering polynucleotides and polypeptides to cells, disclosed in US Patent Application Publications 2010/0311168, 2012/0023619, 2012/0244569, 2013/0145488, 2013/0185823, 2014/0096284,

2015/0040268, 2015/0047074, and 2015/0208663, all of which are incorporated herein by reference in their entirety.

[0046] In embodiments wherein the polynucleotide composition includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease, or wherein the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease, one or more one chemical, enzymatic, or physical agent can similarly be employed. In embodiments, the RNA-guided nuclease (or polynucleotide encoding the RNA-guided nuclease) is provided separately, *e. g.*, in a separate composition including the RNA-guided nuclease or polynucleotide encoding the RNA-guided nuclease. Such compositions can include other chemical or physical agents (*e. g.*, solvents, surfactants, proteins or enzymes, transfection agents, particulates or nanoparticles), such as those described above as useful in the polynucleotide composition used to provide the gRNA. For example, porous silica nanoparticles are useful for delivering a DNA recombinase into maize cells; see, *e. g.*, Martin-Ortigosa *et al.* (2015) *Plant Physiol.*, 164:537 – 547. In an embodiment, the polynucleotide composition includes a gRNA and Cas9 nuclease, and further includes a surfactant and a cell-penetrating peptide. In an embodiment, the polynucleotide composition includes a plasmid that encodes both an RNA-guided nuclease and at least one gRNA, and further includes a surfactant and carbon nanotubes. In an embodiment, the polynucleotide composition includes multiple gRNAs and an mRNA encoding the RNA-guided nuclease, and further includes gold particles, and the polynucleotide composition is delivered to the plant cell by Biolistics.

[0047] In related embodiments, one or more one chemical, enzymatic, or physical agent can be used in one or more steps separate from (preceding or following) that in which the polynucleotide composition is provided to the non-epidermal plant cell. In an embodiment, the plant or plant part in which the non-epidermal plant cell is located is treated with an agent to assist in access to the interior of the plant or plant part, for example, with an abrasive, a caustic agent, a surfactant, or an enzyme, followed by application of the polynucleotide composition (and optionally the nuclease). In an embodiment, a halved seed or dissected embryo is treated with a surfactant such as Silwet L-77, followed by application of the polynucleotide composition (and optionally the nuclease). In an embodiment, the shoot apical meristem of a plant is treated with cellulase, followed by application of the polynucleotide composition.

[0048] In embodiments, the polynucleotide composition is provided/applied at a location in the plant or plant part other than the non-epidermal plant cell. In embodiments, the polynucleotide composition is applied to adjacent or distal non-meristematic cells and is transported (*e. g.*, through the vascular system or by cell-to-cell movement) to meristematic non-epidermal plant cell. In embodiments, the polynucleotide composition is applied by soaking a seed or seed fragment or embryo in the polynucleotide composition, whereby the gRNA is delivered to non-epidermal cells in the seed or seed fragment or embryo. In embodiments, a flower bud or shoot tip is contacted with the polynucleotide composition, whereby the gRNA is delivered to non-epidermal cells in the flower bud or shoot tip, or to other non-epidermal cells in the plant. In embodiments, the polynucleotide composition is applied to the surface of a plant or of a part of

a plant (*e. g.*, a leaf surface), whereby the gRNA is delivered to non-epidermal cells in the plant. In embodiments a whole plant or plant tissue is subjected to particle- or nanoparticle-mediated delivery (*e. g.*, Biolistics or carbon nanotube or nanoparticle delivery) of the polynucleotide composition, whereby the gRNA is delivered to non-epidermal plant cells.

[0049] Delivery of a gRNA by the method of the invention results in alteration of the target nucleotide sequence in the non-epidermal plant cell. In embodiments, the altered target nucleotide sequence includes at least one sequence modification selected from the group consisting of insertion of a nucleotide, deletion of a nucleotide, and replacement of a nucleotide. In embodiments, insertion of a nucleotide includes insertion of additional heterologous sequence. In embodiments, alteration of the target nucleotide sequence results in a change (*e. g.*, increase or decrease or change in temporal or spatial specificity) in expression of the target nucleotide sequence, methylation or demethylation of the target nucleotide sequence (*e. g.*, resulting in an epigenetic change), or a phenotype, or a combination of these. In embodiments, alteration of the target nucleotide sequence results in a phenotype or trait of interest observable in a seedling or plant grown or regenerated from the non-epidermal plant cell; in some embodiments the phenotype or trait is heritable to succeeding generations of plants. Thus, related embodiments include such succeeding generations of plants or their seeds having inherited the altered target nucleotide sequence.

[0050] A related aspect of the invention is directed to the non-epidermal plant cell including an altered target nucleotide sequence, provided by the method. Embodiments of the method further include one or more steps of growing or regenerating a plant from the non-epidermal plant cell including an altered target nucleotide sequence, wherein the grown or regenerated plant contains at least some cells or tissues having the altered target nucleotide sequence. In embodiments, callus is produced from the non-epidermal plant cell, and plantlets and plants produced from such callus. In other embodiments, whole seedlings or plants are grown directly from the non-epidermal plant cell without a callus stage. Thus, additional related aspects are directed to whole seedlings and plants grown or regenerated from the non-epidermal plant cell including an altered target nucleotide sequence, as well as the seeds of such plants. In embodiments, the grown or regenerated plant exhibits a phenotype associated with the altered target nucleotide sequence. In embodiments, the grown or regenerated plant includes in its genome two or more genetic modifications that in combination provide at least one phenotype of interest, wherein at least one genetic modification includes the altered target nucleotide sequence in the non-epidermal plant cell. In embodiments, a heterogeneous population of non-epidermal plant cells, at least some of which include one or more altered target nucleotide sequences, is provided by the method; related aspects include a plant having a phenotype of interest associated with the altered target nucleotide sequence, provided by either regeneration of a plant having the phenotype of interest from a cell selected from the heterogeneous population of non-epidermal plant cells, or by selection of a plant having the phenotype of interest from a heterogeneous population of plants grown or regenerated from the population of non-epidermal plant cells. Examples of phenotypes of interest include herbicide resistance, improved tolerance of abiotic stress (*e. g.*, tolerance of temperature extremes, drought, or salt) or biotic stress (*e. g.*, resistance to bacterial or fungal pathogens), improved utilization of nutrients or

water, modified lipid, carbohydrate, or protein composition, improved flavour or appearance, improved storage characteristics (*e. g.*, resistance to bruising, browning, or softening), increased yield, altered morphology (*e. g.*, floral architecture or colour, plant height, branching, root structure). In an embodiment, a heterogeneous population of non-epidermal plant cells (or seedlings or plants grown or regenerated from the cells) is exposed to conditions permitting expression of the phenotype of interest; *e. g.*, selection for herbicide resistance can include exposing the population of cells (or seedlings or plants) to an amount of herbicide or other substance that inhibits growth or is toxic, allowing identification and selection of those resistant cells (or seedlings or plants) that survive treatment. Also contemplated are heterogeneous populations, arrays, or libraries of such plants, succeeding generations or seeds of such plants, parts of the plants (including plant parts used in grafting as scions or rootstocks), or products (*e. g.*, fruits or other edible plant parts, cleaned grains or seeds, edible oils, flours or starches, proteins, and other processed products) made from the plants or their seeds. Embodiments include plants that contain cells or tissues that do not have the altered nucleotide sequence, *e. g.*, grafted plants in which the scion or rootstock contains the altered nucleotide sequence, or chimeric plants in which some but not all cells or tissues contain the altered nucleotide sequence. Plants in which grafting is commonly useful include many fruit trees and plants such as many citrus trees, apples, stone fruit (*e. g.*, peaches, apricots, cherries, and plums), avocados, tomatoes, eggplant, cucumber, melons, watermelons, and grapes as well as various ornamental plants such as roses. Grafted plants can be grafts between the same or different (generally related) species. Additional related aspects include a hybrid plant provided by crossing a first plant grown or regenerated from a non-epidermal plant cell with an altered target nucleotide sequence, with a second plant, wherein the hybrid plant contains the altered target nucleotide sequence; also contemplated is seed produced by the hybrid plant.

Delivery of effector molecules to a plant cell

[0051] In related aspects, the delivery techniques, delivery agents, and compositions disclosed above under the heading “Methods of altering a target nucleotide sequence in a non-epidermal plant cell” are useful in general for delivering other molecules to effect an alteration in a nucleotide sequence in a plant cell capable of division and differentiation. Such “effector molecules” include other nucleases or polynucleotides encoding a nuclease capable of effecting site-specific alteration of a target nucleotide sequence, and guide polynucleotides that guide nucleases in a sequence-specific manner to a target nucleotide sequence.

[0052] Thus, a related aspect of the invention is a method of providing a plant having a genetic alteration, including: (a) delivery of at least one effector molecule to a plant cell capable of division and differentiation, resulting in a genetic alteration of the plant cell, wherein the plant cell is a cell in a plant or part of a plant selected from the group consisting of a plant tissue, a whole plant, an intact nodal bud, a shoot apex or shoot apical meristem, a root apex or root apical meristem, lateral meristem, intercalary meristem, a seedling (*e. g.*, a germinating seed or small seedling or a larger seedling with one or more true leaves), a whole seed (*e. g.*, an intact seed, or a seed with part or all of its seed coat removed or treated to make

permeable), a halved seed or other seed fragment, an embryo (*e. g.*, a mature dissected zygotic embryo, a developing embryo, a dry or rehydrated or freshly excised embryo), and callus; wherein the delivery of the at least one effector molecule includes at least one treatment selected from the group consisting of: direct application; soaking or imbibition; vacuum infiltration; application of negative or positive pressure; introduction into the vascular system; microinjection; application of ultrasound or vibration; application of hydrodynamic pressure, friction, cavitation or shear stress; vortexing; centrifugation; mechanical cell wall or cell membrane deformation or breakage; enzymatic cell wall or cell membrane breakage or permeabilization; abrasion; electroporation; and treatment with at least one chemical, enzymatic, or physical agent; and (b) regeneration of a plant from the plant cell having the genetic alteration, wherein the plant includes differentiated cells or tissues having the genetic alteration. In embodiments, delivery of the at least one effector molecule alters a target nucleotide sequence in the plant cell, resulting in a genetic alteration such as insertion of a nucleotide, deletion of a nucleotide, or replacement of a nucleotide. In embodiments, insertion of a nucleotide includes insertion of additional heterologous sequence. In embodiments, the genetic alteration results in a change (*e. g.*, increase or decrease or change in temporal or spatial specificity) in expression of the target nucleotide sequence, methylation or demethylation of the target nucleotide sequence (*e. g.*, resulting in an epigenetic change), or a phenotype, or a combination of these.

[0053] The target nucleotide sequence is one or more nucleotide sequences, including protein-coding sequence or non-coding sequence or a combination thereof. Embodiments include a plant nuclear sequence, a plant plastid sequence, a plant mitochondrial sequence, a sequence of a symbiont, pest, or pathogen of a plant, and combinations thereof. In embodiments, multiple target nucleotide sequences are altered, for example, by delivery of multiple effector molecules to the plant cell; the multiple target nucleotide sequences can be part of the same gene (*e. g.*, different locations in a single coding region or in different exons of a protein-coding gene) or different genes.

[0054] Embodiments of effector molecules include: (a) a polynucleotide selected from the group consisting of an RNA guide for an RNA-guided nuclease, a DNA encoding an RNA guide for an RNA-guided nuclease; (b) a nuclease selected from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALE-effector nuclease), Argonaute, a meganuclease or engineered meganuclease; or (c) a polynucleotide encoding one or more nucleases capable of effecting site-specific alteration of a target nucleotide sequence. Any of these nucleases can be codon-optimized, *e. g.*, plant-codon-optimized to function optimally in a plant cell. In embodiments, one or multiple effector molecules are delivered individually (*e. g.*, in separate compositions) or in combinations (*e. g.*, in a ribonucleoprotein), and in a single step or multiple steps.

[0055] Zinc finger nucleases (ZFNs) are engineered proteins including a zinc finger DNA-binding domain fused to a nucleic acid cleavage domain, *e. g.*, a nuclease. The zinc finger binding domains provide specificity and can be engineered to specifically recognize any desired target DNA sequence. For a review

of the construction and use of ZFNs in plants and other organisms, see, *e. g.*, Urnov *et al.* (2010) *Nature Rev. Genet.*, 11:636 – 646. The zinc finger DNA binding domains are derived from the DNA-binding domain of a large class of eukaryotic transcription factors called zinc finger proteins (ZFPs). The DNA-binding domain of ZFPs typically contains a tandem array of at least three zinc “fingers” each recognizing a specific triplet of DNA. A number of strategies can be used to design the binding specificity of the zinc finger binding domain. One approach, termed “modular assembly”, relies on the functional autonomy of individual zinc fingers with DNA. In this approach, a given sequence is targeted by identifying zinc fingers for each component triplet in the sequence and linking them into a multifinger peptide. Several alternative strategies for designing zinc finger DNA binding domains have also been developed. These methods are designed to accommodate the ability of zinc fingers to contact neighboring fingers as well as nucleotides bases outside their target triplet. Typically, the engineered zinc finger DNA binding domain has a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, for example, rational design and various types of selection. Rational design includes, for example, the use of databases of triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, *e. g.*, US Patents 6,453,242 and 6,534,261, both incorporated herein by reference in their entirety. Exemplary selection methods (*e. g.*, phage display and yeast two-hybrid systems) are well known and described in the literature. In addition, enhancement of binding specificity for zinc finger binding domains has been described in US Patent 6,794,136, incorporated herein by reference in its entirety. In addition, individual zinc finger domains may be linked together using any suitable linker sequences. Examples of linker sequences are publicly known, *e. g.*, see US Patents 6,479,626; 6,903,185; and 7,153,949, incorporated herein by reference in their entirety. The nucleic acid cleavage domain is non-specific and is typically a restriction endonuclease, such as FokI. This endonuclease must dimerize to cleave DNA. Thus, cleavage by FokI as part of a ZFN requires two adjacent and independent binding events, which must occur in both the correct orientation and with appropriate spacing to permit dimer formation. The requirement for two DNA binding events enables more specific targeting of long and potentially unique recognition sites. FokI variants with enhanced activities have been described; see, *e. g.*, Guo *et al.* (2010) *J. Mol. Biol.*, 400:96 - 107.

[0056] Transcription activator like effectors (TALEs) are proteins secreted by certain *Xanthomonas* species to modulate gene expression in host plants and to facilitate the colonization by and survival of the bacterium. TALEs act as transcription factors and modulate expression of resistance genes in the plants. Recent studies of TALEs have revealed the code linking the repetitive region of TALEs with their target DNA-binding sites. TALEs comprise a highly conserved and repetitive region consisting of tandem repeats of mostly 33 or 34 amino acid segments. The repeat monomers differ from each other mainly at amino acid positions 12 and 13. A strong correlation between unique pairs of amino acids at positions 12 and 13 and the corresponding nucleotide in the TALE-binding site has been found. The simple relationship between amino acid sequence and DNA recognition of the TALE binding domain allows for the design of DNA

binding domains of any desired specificity. TALEs can be linked to a non-specific DNA cleavage domain to prepare genome editing proteins, referred to as TAL-effector nucleases or TALENs. As in the case of ZFNs, a restriction endonuclease, such as FokI, can be conveniently used. For a description of the use of TALENs in plants, see Mahfouz *et al.* (2011) *Proc. Natl. Acad. Sci. USA*, 108:2623 – 2628 and Mahfouz (2011) *GM Crops*, 2:99 – 103.

[0057] Argonautes are proteins that can function as sequence-specific endonucleases by binding a polynucleotide (*e. g.*, a single-stranded DNA or single-stranded RNA) that includes sequence complementary to a target nucleotide sequence) that guides the Argonaut to the target nucleotide sequence and effects site-specific alteration of the target nucleotide sequence; see, *e. g.*, US Patent Application Publication 2015/0089681, incorporated herein by reference in its entirety.

[0058] In related embodiments, zinc finger nucleases, TALENs, and Argonautes are used in conjunction with other functional domains. For example, the nuclease activity of these nucleic acid targeting systems can be altered so that the enzyme binds to but does not cleave the DNA. Examples of functional domains include transposase domains, integrase domains, recombinase domains, resolvase domains, invertase domains, protease domains, DNA methyltransferase domains, DNA hydroxymethylase domains, DNA demethylase domains, histone acetylase domains, histone deacetylase domains, nuclease domains, repressor domains, activator domains, nuclear-localization signal domains, transcription-regulatory protein (or transcription complex recruiting) domains, cellular uptake activity associated domains, nucleic acid binding domains, antibody presentation domains, histone modifying enzymes, recruiter of histone modifying enzymes; inhibitor of histone modifying enzymes, histone methyltransferases, histone demethylases, histone kinases, histone phosphatases, histone ribosylases, histone deribosylases, histone ubiquitinases, histone deubiquitinases, histone biotinases and histone tail proteases. Non-limiting examples of functional domains include a transcriptional activation domain, a transcription repression domain, and an SHH1, SUVH2, or SUVH9 polypeptide capable of reducing expression of a target nucleotide sequence via epigenetic modification; see, *e. g.*, US Patent Application Publication 2016/0017348, incorporated herein by reference in its entirety. Genomic DNA may also be modified via base editing using a fusion between a catalytically inactive Cas9 (dCas9) is fused to a cytidine deaminase which convert cytosine (C) to uridine (U), thereby effecting a C to T substitution; see Komor *et al.* (2016) *Nature*, 533:420 – 424.

[0059] In embodiments, the plant cell capable of division and differentiation is diploid or polyploid. In embodiments, the plant cell is haploid or can be induced to become haploid; examples include but are not limited to plant cells in haploid plants and plant cells in reproductive tissues, *e. g.*, flowers, developing flowers or flower buds, ovaries, ovules, megaspores, anthers, pollen, and microspores. In embodiments where the plant cell is haploid, the method can further include the step of chromosome doubling (*e. g.*, by using a chromosome doubling agent such as colchicine) in the plant cell including the genetic alteration to produce a doubled haploid cell that is homozygous for the genetic alteration; yet other embodiments include regeneration of a doubled haploid plant from the doubled haploid cell, wherein the regenerated doubled haploid plant is homozygous for the genetic alteration. Thus, aspects of the invention are related to the

haploid cell having the genetic alteration as well as a doubled haploid cell or a doubled haploid plant that is homozygous for the genetic alteration. Another aspect of the invention is related to a hybrid plant having at least one parent plant that is a doubled haploid plant provided by the method.

[0060] A related aspect of the invention is directed to the plant having a genetic alteration, provided by the method. In embodiments, the plant is a monocot or a dicot, or is haploid, diploid, polyploid, or doubled haploid. Embodiments include plants that contain cells or tissues that do not have the genetic alteration, *e. g.*, grafted plants in which the scion or rootstock contains the genetic alteration, or chimeric plants in which some but not all cells or tissues contain the genetic alteration. In embodiments, the genetic alteration is heritable to succeeding generations; further aspects thus include seed and progeny plants of the plant having a genetic alteration, wherein the seed or progeny plants contain the genetic alteration, as well as parts of such seed or progeny plants (including plant parts used in grafting as scions or rootstocks), or products (*e. g.*, fruits or other edible plant parts, cleaned grains or seeds, edible oils, flours or starches, proteins, and other processed products) made from the seed or progeny plants. In embodiments, callus is produced from the plant cell having the genetic alteration, and plantlets and plants produced from such callus. In other embodiments, whole seedlings or plants are grown directly from the plant cell having the genetic alteration without a callus stage. Thus, additional related aspects are directed to whole seedlings and plants grown or regenerated from the plant cell having the genetic alteration, as well as the seeds of such plants. In embodiments, the grown or regenerated plant exhibits a phenotype associated with the genetic alteration. Examples of phenotypes of interest include herbicide resistance, improved tolerance of abiotic stress (*e. g.*, tolerance of temperature extremes, drought, or salt) or biotic stress (*e. g.*, resistance to bacterial or fungal pathogens), improved utilization of nutrients or water, modified lipid, carbohydrate, or protein composition, improved flavour or appearance, increased yield, altered morphology (*e. g.*, floral architecture, plant height, branching, root structure). In embodiments, the grown or regenerated plant includes in its genome two or more genetic modifications that in combination provide at least one phenotype of interest, wherein at least one genetic modification includes the genetic alteration provided by the plant cell treated by the method.

Methods for investigating reverse genetics

[0061] Another aspect of the invention is related to methods for investigating reverse genetics, for example, a method of identifying a nucleotide sequence (or alteration of a nucleotide sequence, such as a native nucleotide sequence) that is associated with a phenotype of interest. In an embodiment, the method includes the steps of altering the genome of a population of plant cells (or plant protoplasts), optionally growing or regenerating a population of calli, seedlings, plantlets, or plants from the population of plant cells, and selecting the plant cells (or grown or regenerated calli, seedlings, plantlets, or plants) exhibiting the phenotype of interest and identifying the nucleotide sequence associated with the phenotype. Embodiments of the method include culturing or growing the plant cells or protoplasts (or calli, seedlings, plantlets, or plants) under conditions that permit expression of the phenotype of interest.

[0062] In an embodiment, the method includes the steps of: (a) contacting a population of plant cells (or protoplasts) with a library of gRNAs and optionally with an RNA-guided DNA nuclease, whereby the genome of the plant cells is altered, culturing the population of plant cells under conditions that permit expression of the phenotype of interest, selecting the plant cells that exhibit the phenotype of interest, and identifying the nucleotide sequence or alteration of a nucleotide sequence, wherein the nucleotide sequence thus identified is associated with the phenotype; or (b) contacting a population of plant cells (or protoplasts) with a library of gRNAs and optionally with an RNA-guided DNA nuclease, whereby the genome of the cells is altered, regenerating a population of plants from the population of plant cells, growing the population of plants under conditions that permit expression of the phenotype of interest, selecting the plants that exhibit the phenotype of interest, and identifying the nucleotide sequence or alteration of a nucleotide sequence, wherein the nucleotide sequence thus identified is associated with the phenotype. In embodiments, the plant cells in which the genome is altered are haploid cells (*e. g.*, microspore or other gametophytic cells, or cells of a haploid plant) and the plants regenerated from these cells are haploid plants; in embodiments the method further includes the step of generating doubled-haploid cells or doubled-haploid plants from the haploid cells or plants.

[0063] In embodiments, the gRNA is provided as a polynucleotide composition including: (i) a CRISPR RNA (crRNA) that includes the gRNA, or a polynucleotide that encodes a crRNA, or a polynucleotide that is processed into a crRNA; or (ii) a single guide RNA (sgRNA) that includes the gRNA, or a polynucleotide that encodes an sgRNA, or a polynucleotide that is processed into an sgRNA. In embodiments, the plant cells contain or express the appropriate RNA-guided DNA nuclease; in other embodiments the RNA-guided DNA nuclease, or a polynucleotide encoding the RNA-guided DNA nuclease, is provided to the plant cells. In embodiments, the nuclease is selected from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TAL-effector nuclease), Argonaute, a meganuclease or engineered meganuclease. Methods and compositions useful for delivering the library of gRNAs or the RNA-guided DNA nuclease are similar to those described under the heading "Methods of altering a target nucleotide sequence in a plant cell".

Compositions and reaction mixtures

[0064] Another aspect of the invention is related to compositions and reactions mixtures useful for carrying out methods such as those described herein. In one aspect, the invention is related to a composition or a reaction mixture including: (a) at least one non-epidermal plant cell, which in embodiments is a cell in whole plant, whole seed, embryo, plant part, or plant tissue; (b) at least one effector molecule for inducing a genetic alteration in the non-epidermal plant cell, wherein the at least one effector molecule is selected from the group consisting of: (i) a polynucleotide selected from the group consisting of an RNA guide for an RNA-guided nuclease, a DNA encoding an RNA guide for an RNA-guided nuclease; (ii) a nuclease selected

from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TAL-effector nuclease), Argonaute, a meganuclease or engineered meganuclease; or (iii) a polynucleotide encoding one or more nucleases capable of effecting site-specific alteration of a target nucleotide sequence; and (c) optionally, at least one delivery agent selected from the group consisting of solvents, fluorocarbons, glycols or polyols, surfactants; primary, secondary, or tertiary amines and quaternary ammonium salts; organosilicone surfactants; lipids, lipoproteins, lipopolysaccharides; acids, bases, caustic agents; peptides, proteins, or enzymes; cell-penetrating peptides; RNase inhibitors; cationic branched or linear polymers; dendrimers; counter-ions, amines or polyamines, osmolytes, buffers, and salts; polynucleotides; transfection agents; antibiotics; non-specific DNA double-strand-break-inducing agents; antioxidants; chelating agents; particles or nanoparticles, magnetic particles or nanoparticles, abrasive or scarifying agents, needles or microneedles, matrices, and grids.

[0065] In another aspect, the invention is related to a composition or a reaction mixture including: (a) at least one non-epidermal plant cell, which in embodiments is a cell in whole plant, whole seed, embryo, plant part, or plant tissue; (b) at least one guide RNA (gRNA) having a nucleotide sequence designed to alter a target nucleotide sequence in the non-epidermal plant cell, wherein the gRNA is provided as a polynucleotide composition including: (i) a CRISPR RNA (crRNA) that includes the gRNA, or a polynucleotide that encodes a crRNA, or a polynucleotide that is processed into a crRNA; or (ii) a single guide RNA (sgRNA) that includes the gRNA, or a polynucleotide that encodes an sgRNA, or a polynucleotide that is processed into an sgRNA; (c) optionally, at least one nuclease, or at least one polynucleotide that encodes the nuclease, wherein the nuclease is selected from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TAL-effector nuclease), Argonaute, a meganuclease or engineered meganuclease; and (d) optionally, at least one delivery agent selected from the group consisting of solvents, fluorocarbons, glycols or polyols, surfactants; primary, secondary, or tertiary amines and quaternary ammonium salts; organosilicone surfactants; lipids, lipoproteins, lipopolysaccharides; acids, bases, caustic agents; peptides, proteins, or enzymes; cell-penetrating peptides; RNase inhibitors; cationic branched or linear polymers; dendrimers; counter-ions, amines or polyamines, osmolytes, buffers, and salts; polynucleotides; transfection agents; antibiotics; non-specific DNA double-strand-break-inducing agents; antioxidants; chelating agents; particles or nanoparticles, magnetic particles or nanoparticles, abrasive or scarifying agents, needles or microneedles, matrices, and grids. In embodiments, the gRNA is a single guide RNA (sgRNA) that includes the gRNA, wherein the composition further includes an RNA-guided nuclease, and wherein the sgRNA and RNA guided-nuclease are provided as a ribonucleoprotein (RNP) complex. In embodiments, the at least one plant cell or plant protoplast is a population of plant cells or plant protoplasts, the at least one gRNA is two or

more sgRNAs, wherein the composition further includes an RNA-guided nuclease, and wherein the two or more sgRNAs are each provided as a ribonucleoprotein (RNP) complex with the RNA guided-nuclease.

[0066] In embodiments of these compositions and reaction mixtures, the at least one non-epidermal plant cell is a plant cell located in plant tissue, a plant part, or an intact plant or seed, or is a plant cell in callus. In embodiments, the at least one non-epidermal plant cell is obtained from a monocot or a dicot. In various embodiments, the at least one non-epidermal plant cell is haploid, diploid, or polyploid.

[0067] The foregoing description and the examples presented in this disclosure describe the subject matter of this invention, which includes the following: (I) a method of delivering a guide RNA (gRNA) to a non-epidermal plant cell, wherein the non-epidermal plant cell is in a plant or part of a plant, wherein the gRNA has a nucleotide sequence designed to alter a target nucleotide sequence in the non-epidermal plant cell, and wherein the gRNA is provided as a polynucleotide composition including: (i) a CRISPR RNA (crRNA) that includes the gRNA, or a polynucleotide that encodes a crRNA, or a polynucleotide that is processed into a crRNA; or (ii) a single guide RNA (sgRNA) that includes the gRNA, or a polynucleotide that encodes an sgRNA, or a polynucleotide that is processed into an sgRNA; wherein the delivery of the polynucleotide composition includes at least one treatment selected from the group consisting of: direct application; soaking or imbibition; vacuum infiltration; application of negative or positive pressure; introduction into the vascular system; microinjection; application of ultrasound or vibration; application of hydrodynamic pressure, friction, cavitation or shear stress; vortexing; centrifugation; mechanical cell wall or cell membrane deformation or breakage; enzymatic cell wall or cell membrane breakage or permeabilization; abrasion; electroporation; and treatment with at least one chemical, enzymatic, or physical agent; whereby the gRNA is delivered to the non-epidermal plant cell; wherein the method is optionally further characterized by one or more of the following: (1) wherein the plant or part of a plant is selected from the group consisting of a plant tissue, a whole plant, an intact nodal bud, a shoot apex or shoot apical meristem, a root apex or root apical meristem, lateral meristem, intercalary meristem, a seedling, a whole seed, a halved seed or other seed fragment, an embryo, and callus; (2) wherein the plant is a dicot or a monocot; (3) wherein delivery of the gRNA results in alteration of the target nucleotide sequence in the non-epidermal plant cell; (4) wherein: (a) the polynucleotide composition optionally includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease; or (b) the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; or (c) the non-epidermal plant cell includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; (5) wherein (a) the polynucleotide composition further includes a chemical agent or a physical agent or a combination of both chemical and physical agents, or (b) the method further includes the step of treating the plant cell with a chemical agent or a physical agent or a combination of both chemical and physical agents; wherein the chemical agent is at least one selected from the group consisting of solvents, fluorocarbons, glycols or polyols, surfactants; primary, secondary, or

tertiary amines and quaternary ammonium salts; saponins; organosilicone surfactants; lipids, lipoproteins, lipopolysaccharides; acids, bases, caustic agents; peptides, proteins, or enzymes; cell-penetrating peptides; RNase inhibitors; cationic branched or linear polymers; dendrimers; counter-ions, amines or polyamines, osmolytes, buffers, and salts; polynucleotides; transfection agents; antibiotics; non-specific DNA double-strand-break-inducing agents; antioxidants; and chelating agents; and wherein the physical agent is at least one selected from the group consisting of particles or nanoparticles, magnetic particles or nanoparticles, abrasive or scarifying agents, needles or microneedles, matrices, and grids; (6) wherein the crRNA, the polynucleotide that encodes a crRNA, the polynucleotide that is processed into a crRNA, the sgRNA, the polynucleotide that encodes an sgRNA, or the polynucleotide that is processed into an sgRNA further includes one or more additional nucleotide sequences selected from the group consisting of an aptamer or riboswitch sequence, a nucleotide sequence that provides secondary structure, a nucleotide sequence that provides a sequence-specific site for an enzyme, T-DNA sequence, a DNA nuclear-targeting sequence, a regulatory sequence, and a transcript-stabilizing sequence; (7) wherein the crRNA, the polynucleotide that encodes a crRNA, the polynucleotide that is processed into a crRNA, the sgRNA, the polynucleotide that encodes an sgRNA, or the polynucleotide that is processed into an sgRNA includes: (a) double-stranded RNA, (b) single-stranded RNA; (c) chemically modified RNA; (d) a combination of (a) – (c); (8) wherein the polynucleotide composition includes a liquid, a solution, a suspension, an emulsion, a reverse emulsion, a colloid, a dispersion, a gel, liposomes, micelles, an injectable material, an aerosol, a solid, a powder, a particulate, a nanoparticle, or a combination thereof; (9) wherein the polynucleotide composition is provided at a location in the plant or plant part other than the non-epidermal plant cell; (10) wherein: (a) the polynucleotide composition optionally includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease; or (b) the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; or (c) the non-epidermal plant cell includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; and wherein the RNA-guided nuclease is selected from the group consisting of an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, and a codon-optimized nuclease; (11) wherein: (a) the polynucleotide composition optionally includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease; or (b) the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; or (c) the non-epidermal plant cell includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; and wherein the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease is provided: (a) as a ribonucleoprotein complex including the crRNA and the RNA-guided nuclease; (b) as a complex including the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease and at least one peptide selected from the group consisting of a cell-penetrating peptide, viral movement protein, or transfecting peptide; (c) as a fusion protein including the RNA-guided nuclease and at least one peptide selected from the group consisting of a cell-penetrating peptide, viral movement

protein, or transfecting peptide; (d) on a carrier molecule or a particulate; (e) in a liposome, micelle, protoplast or protoplast fragment; or (f) using a combination of any of (a) – (e); (12) wherein: (a) the polynucleotide composition optionally includes an RNA-guided nuclease, or a polynucleotide that encodes the RNA-guided nuclease; or (b) the method further includes the step of providing to the non-epidermal plant cell an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; or (c) the non-epidermal plant cell includes an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease; and wherein the RNA-guided nuclease is provided: (a) by contacting the non-epidermal plant cell with the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease; (b) by transporting the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease into the non-epidermal plant cell using a chemical, enzymatic, or physical agent; (c) by bacterially mediated transfection with a polynucleotide encoding the RNA-guided nuclease; or (d) by transcription of a polynucleotide that encodes the RNA-guided nuclease; and (13) wherein the non-epidermal plant cell is: (a) diploid or (b) haploid.

[0068] The subject matter of this invention further includes: (II) A non-epidermal plant cell including an altered target nucleotide sequence, provided by at least one of the methods described above under (I), wherein the method is optionally further characterized by further including growth or regeneration of a plant from the non-epidermal plant cell including an altered target nucleotide sequence, wherein the plant includes cells having the altered target nucleotide sequence. The subject matter of this invention therefore further includes: (III) A regenerated plant provided by at least one of the methods described above under (I), or seed or plant parts of the regenerated plant, wherein the method further includes growth or regeneration of a plant from the non-epidermal plant cell including an altered target nucleotide sequence, wherein the plant includes cells having the altered target nucleotide sequence; and wherein the regenerated plant is optionally further characterized by one or more of the following: (1) wherein the regenerated plant exhibits a phenotype associated with the altered target nucleotide sequence; and (2) wherein the regenerated plant includes in its genome two or more genetic modifications that in combination provide at least one phenotype of interest, wherein at least one genetic modification includes the altered target nucleotide sequence in the non-epidermal plant cell.

[0069] The subject matter of this invention further includes: (IV) At least one of the methods described above under (I), wherein the method further includes the step of chromosome doubling in the non-epidermal plant cell including the altered target nucleotide sequence to produce a doubled haploid cell that is homozygous for the altered target nucleotide sequence; and wherein the method optionally further includes the step of regeneration of a doubled haploid plant from the doubled haploid cell, wherein the regenerated doubled haploid plant includes cells that are homozygous for the altered target nucleotide sequence. The subject matter of this invention therefore further includes: (V) A doubled haploid cell that is homozygous for the altered target nucleotide sequence, provided by at least one of the methods described above under (I), wherein the method further includes the step of chromosome doubling in the non-epidermal plant cell including the altered target nucleotide sequence to produce a doubled haploid cell that is homozygous for the

altered target nucleotide sequence. The subject matter of this invention therefore further includes: (VI) A regenerated doubled haploid plant, or seed or plant parts of the regenerated doubled haploid plant, wherein the regenerated doubled haploid plant is provided by at least one of the methods described above under (I), wherein the method further includes the steps of (a) chromosome doubling in the non-epidermal plant cell including the altered target nucleotide sequence to produce a doubled haploid cell that is homozygous for the altered target nucleotide sequence, and (b) regeneration of a doubled haploid plant from the doubled haploid cell, wherein the regenerated doubled haploid plant includes cells that are homozygous for the altered target nucleotide sequence. The subject matter of this invention also further includes: (VII) A hybrid plant having at least one parent plant that is a regenerated doubled haploid plant provided according to (VI) above.

[0070] The subject matter of this invention further includes: (VIII) A method of providing a plant having a genetic alteration, including: (a) delivery of an effector molecule to a plant cell capable of division and differentiation, resulting in a genetic alteration of the plant cell; wherein the plant cell is a cell in a plant or part of a plant selected from the group consisting of a plant tissue, a whole plant, an intact nodal bud, a shoot apex or shoot apical meristem, a root apex or root apical meristem, lateral meristem, intercalary meristem, a seedling, a whole seed, a halved seed or other seed fragment, an embryo, and callus; wherein the plant is a monocot or a dicot; wherein the effector molecule is at least one selected from the group consisting of: (i) a polynucleotide selected from the group consisting of an RNA guide for an RNA-guided nuclease, a DNA encoding an RNA guide for an RNA-guided nuclease; (ii) a nuclease selected from the group consisting of an RNA-guided nuclease, an RNA-guided DNA endonuclease, a type II Cas nuclease, a Cas9, a type V Cas nuclease, a Cpf1, a CasY, a CasX, a C2c1, a C2c3, an engineered nuclease, a codon-optimized nuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a meganuclease or engineered meganuclease; or (iii) a polynucleotide encoding one or more nucleases capable of effecting site-specific alteration of a target nucleotide sequence; wherein delivery includes at least one treatment selected from the group consisting of at least one treatment selected from the group consisting of: direct application; soaking or imbibition; vacuum infiltration; application of negative or positive pressure; introduction into the vascular system; microinjection; application of ultrasound or vibration; application of hydrodynamic pressure, friction, cavitation or shear stress; vortexing; centrifugation; mechanical cell wall or cell membrane deformation or breakage; enzymatic cell wall or cell membrane breakage or permeabilization; abrasion; electroporation; and treatment with at least one chemical, enzymatic, or physical agent; and (b) regeneration of a plant from the plant cell, wherein the plant includes differentiated cells or tissues having the genetic alteration; wherein the method is optionally further characterized by one or more of the following: (1) wherein the genetic alteration is at least one sequence alteration selected from the group consisting of insertion of a nucleotide, deletion of a nucleotide, or replacement of a nucleotide; (2) wherein the genetic alteration is heritable to succeeding generations; and (3) wherein the plant cell is a cell in a seedling, a whole seed, a seed fragment, a mature dissected zygotic embryo, or a developing embryo, and wherein the regeneration of a plant from the plant cell includes growing the plant from the seedling, whole seed, seed fragment, mature dissected zygotic embryo, or

developing embryo. The subject matter of this invention also further includes: (IX) A plant having a genetic alteration produced by any of the methods described above under (VIII), as well as seed of such a plant, wherein the seed contains the genetic alteration.

[0071] The subject matter of this invention further includes: (X) A method of identifying a nucleotide sequence associated with a phenotype of interest, including: (a) contacting a population of plant cells/protoplasts with a library of gRNAs or a library of polynucleotides encoding gRNAs and optionally with an RNA-guided DNA nuclease, whereby the genome of the cells/protoplasts is altered, culturing the population of plant cells or plant protoplasts under conditions that permit expression of the phenotype of interest, selecting the plant cells or plant protoplasts that exhibit the phenotype of interest, and identifying the nucleotide sequence or alteration of a nucleotide sequence, wherein the nucleotide sequence thus identified is associated with the phenotype; or (b) contacting a population of plant cells/protoplasts with a library of gRNAs or a library of polynucleotides encoding gRNAs and optionally with an RNA-guided DNA nuclease, whereby the genome of the cells/protoplasts is altered, regenerating a population of plants from the population of plant cells or plant protoplasts, growing the population of plants under conditions that permit expression of the phenotype of interest, selecting the plants that exhibit the phenotype of interest, and identifying the nucleotide sequence or alteration of a nucleotide sequence, wherein the nucleotide sequence thus identified is associated with the phenotype.

[0072] The above-described subject matter is further illustrated by the non-limiting embodiments described throughout the specification and in the Examples that follow.

EXAMPLES

Example 1

[0073] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example describes delivery of an RNA guide for an RNA-guided nuclease (*i. e.*, an sgRNA) and the corresponding RNA-guided nuclease (*i. e.*, Cas9) to a non-epidermal plant cell (*i. e.*, a cell in a soybean embryo), resulting in editing of an endogenous plant gene (*i. e.*, phytoene desaturase, PDS) in germline cells of excised soybean embryos. This example demonstrates delivery of polynucleotides encoding effector molecules (sgRNAs, nucleases) through multiple barriers (*e. g.*, multiple cell layers, seed coat, cell walls, plasma membrane) directly into soybean germline cells, resulting in a heritable alteration of the target nucleotide sequence, PDS. The methods described do not employ the common techniques of bacterially mediated transformation (*e. g.*, by *Agrobacterium* sp.) or biolistics.

[0074] Plasmids were designed for delivery of Cas9 (Csn1) endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system and a single guide RNA (sgRNA) targeting the endogenous phytoene desaturase (PDS) in soybean, *Glycine max*. The sequences of these plasmids and specific elements contained therein are described in **Tables 1** and **2** below.

Table 1: sgRNA vector (SEQ ID NO:1), 3079 base pairs DNA

Nucleotide position in SEQ ID NO:1	Description	Comment
1 – 3079	Intact plasmid	SEQ ID NO:1
379 – 395	M13 forward primer for sequencing	
412 – 717	<i>Glycine max</i> U6 promoter	
717 – 736	<i>Glycine max</i> phytoene desaturase targeting sequence (gRNA)	SEQ ID NO:2
737 – 812	guide RNA scaffold sequence for <i>S. pyogenes</i> CRISPR/Cas9 system	SEQ ID NO:3
856 – 874	M13 reverse primer for sequencing	complement
882 – 898	lac repressor encoded by lacI	
906 – 936	lac promoter for the <i>E. coli</i> lac operon	complement
951 – 972	<i>E. coli</i> catabolite activator protein (CAP) binding site	
1260 – 1848	high-copy-number ColE1/pMB1/pBR322/pUC origin of replication (left direction)	complement
2019 – 2879	CDS for <i>bla</i> , <i>beta</i> -lactamase, AmpR	complement; ampicillin selection
2880 – 2984	<i>bla</i> promoter	complement

[0075] The sgRNA vector having the sequence of **SEQ ID NO:1** contains nucleotides at positions 717 – 812 encoding a single guide RNA having the sequence of **SEQ ID NO:4**, which includes both a targeting sequence (gRNA) (**SEQ ID NO:2**) and a guide RNA scaffold (**SEQ ID NO:3**); transcription of the sgRNA is driven by a *Glycine max* U6 promoter at nucleotide positions 412 – 717. The sgRNA vector also includes lac operon and ampicillin resistance sequences for convenient selection of the plasmid in bacterial cultures.

Table 2: endonuclease vector (SEQ ID NO:5), 8569 base pairs DNA

Nucleotide position in SEQ ID NO:4	Description	Comment
1 – 8569	Intact plasmid	SEQ ID NO:5
379 – 395	M13 forward primer for sequencing	
419 – 1908	<i>Glycine max</i> UbiL promoter	
1917 – 6020	Cas9 (Csn1) endonuclease from the <i>Streptococcus pyogenes</i> type II CRISPR/Cas system	SEQ ID NO:6 (encodes protein with sequence of SEQ ID NO:7)
6033 – 6053	nuclear localization signal of SV40 large T antigen	SEQ ID NO:8 (encodes peptide with sequence of SEQ ID NO:9)
6065 – 6317	nopaline synthase (NOS) terminator and poly(A) signal	
6348 – 6364	M13 reverse primer for sequencing	complement
6372 – 6388	lac repressor encoded by lacI	
6396 – 6426	lac promoter for the <i>E. coli</i> lac operon	complement
6441 – 6462	<i>E. coli</i> catabolite activator protein (CAP) binding site	
6750 – 7338	high-copy-number ColE1/pMB1/pBR322/pUC origin of replication (left direction)	complement
7509 – 8369	CDS for <i>bla</i> , <i>beta</i> -lactamase, AmpR	complement; ampicillin selection
8370 – 8474	<i>bla</i> promoter	complement

[0076] The endonuclease vector having the sequence of **SEQ ID NO:5** contains nucleotides at positions 1917 – 6020 having the sequence of **SEQ ID NO:6** and encoding the Cas9 nuclease from *Streptococcus pyogenes* that has the amino acid sequence of **SEQ ID NO:7**, and nucleotides at positions 6033 – 6053 having the sequence of **SEQ ID NO:8** and encoding the nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen that has the amino acid sequence of **SEQ ID NO:9**. Transcription of the Cas9 nuclease and adjacent SV40 nuclear localization signal is driven by a *Glycine max* UbiL promoter at nucleotide positions 419 – 1908; the resulting transcript including nucleotides at positions 1917 – 6053 having the sequence of **SEQ ID NO:10** encodes a fusion protein having the sequence of **SEQ ID NO:11** wherein the Cas9 nuclease is linked through a 4-residue peptide linker to the SV40 nuclear localization signal. The endonuclease vector also includes lac operon and ampicillin resistance sequences for convenient selection of the plasmid in bacterial cultures.

[0077] Similar vectors for expression of nucleases and sgRNAs are also described, *e. g.*, in Fauser *et al.* (2014) *Plant J.*, 79:348 – 359; and described at [www\[dot\]addgene\[dot\]org/crispr](http://www.addgene.org/crispr). It will be apparent to one skilled in the art that analogous plasmids are easily designed to encode other guide polynucleotide or nuclease sequences, optionally including different elements (*e. g.*, different promoters, terminators, selectable or detectable markers, a cell-penetrating peptide, a nuclear localization signal, a chloroplast transit peptide, or a mitochondrial targeting peptide, etc.), and used in a similar manner. Embodiments of nuclease fusion proteins include fusions (with or without an optional peptide linking sequence) between the Cas9 nuclease from *Streptococcus pyogenes* that has the amino acid sequence of **SEQ ID NO:7** and at least one of the following peptide sequences: (a) GRKKRRQRRRPPQ (“HIV-1 Tat (48-60)”, **SEQ ID NO:12**), (b) GRKKRRQRRRPPQ (“TAT”, **SEQ ID NO:13**), (c) YGRKKRRQRRR (“TAT (47-57)”, **SEQ ID NO:14**), (d) KLALKLALKALKALKLA (“MAP (KLAL)”, **SEQ ID NO:15**), (e) RQIRIWFQNRRMRWRR (“Penetratin-Arg”, **SEQ ID NO:16**), (f) CSIPPEVKFNKPFVYLI (“antitrypsin (358-374)”, **SEQ ID NO:17**), (g) RRRQRRKKRGGDIMGEWGNEIFGAIAGFLG (“TAT-HA2 Fusion Peptide”, **SEQ ID NO:18**), (h) FVQWFSKFLGRIL-NH2 (“Temporin L, amide”, **SEQ ID NO:19**), (i) LLILRRRIRKQAHAAHSK (“pVEC (Cadherin-5)”, **SEQ ID NO:20**), (j) LGTYTQDFNKFHTFPQTAIGVGAP (“Calcitonin”, **SEQ ID NO:21**), (k) GAEEAAARVYDLGLRRLRQRRRLRRERVRA (“Neurturin”, **SEQ ID NO:22**), (l) MGLGLHLLVLAALQGAWSQPKKKRKV (“Human P1”, **SEQ ID NO:23**), (m) RQIKIWFQNRRMKWKKGG (“Penetratin”, **SEQ ID NO:24**), poly-arginine peptides including (n) RRRRRRRR (“octo-arginine”, **SEQ ID NO:25**) and (o) RRRRRRRRR (“nono-arginine”, **SEQ ID NO:26**), and (p) KKLFFKILKYLKKLFFKILKYLKKKKKKKK (“(BP100x2)-K8”, **SEQ ID NO:27**); these nuclease fusion proteins are specifically claimed herein. In other embodiments, such vectors are used to produce a guide RNA (such as one or more crRNAs or sgRNAs) or the nuclease protein; guide RNAs and nucleases can be combined to produce a specific ribonucleoprotein complex for delivery to the plant cell; in an example, a ribonucleoprotein including the sgRNA having the sequence of **SEQ ID NO:4** and the Cas9-NLS fusion protein having the sequence of **SEQ ID NO:11** is produced for delivery to the plant cell.

Related aspects of the invention thus encompass ribonucleoprotein compositions containing the ribonucleoprotein including the sgRNA having the sequence of **SEQ ID NO:4** and a Cas9 fusion protein such as the Cas9-NLS fusion protein having the sequence of **SEQ ID NO:11**, and polynucleotide compositions containing one or more polynucleotides including the sequences of **SEQ ID NOs: 4 or 10**. The above sgRNA and nuclease vectors are delivered to plant cells using compositions and methods described in the specification.

[0078] In a first series of experiments, the sgRNA and nuclease vectors were delivered to non-epidermal plant cells in soybean embryos using combinations of delivery agents and electroporation. Mature, dry soybean seeds (cv. Williams 82) were surface-sterilized as follows. Dry soybean seeds were held for 4 hours in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid were freshly added. Seeds remained desiccated after this sterilization treatment. The sterilized seeds were split into 2 halves by manual application of a razor blade and the embryos manually separated from the cotyledons. Each test or control treatment was carried out on 20 excised embryos.

[0079] Experiment 1: A delivery solution containing the sgRNA and nuclease vectors (100 nanograms per microliter of each plasmid) in 0.01% CTAB (cetyltrimethylammonium bromide, a quaternary ammonium surfactant) in sterile-filtered milliQ water was prepared. Each solution was chilled to 4 degrees Celsius and 500 microliters were added directly to the embryos, which were then immediately placed on ice in a vacuum chamber and subjected to a negative pressure (2×10^{-3} millibar) treatment for 15 minutes. Following the chilling/negative pressure treatments, the embryos were treated with electric current using a BTX-Harvard ECM-830 electroporation device set with the following parameters: 50V, 25 millisecond pulse length, 75 millisecond pulse interval for 99 pulses.

[0080] Experiment 2: conditions identical to Experiment 1, except that the initial contacting with delivery solution and negative pressure treatments were carried out at room temperature.

[0081] Additional experiments are performed as follows:

[0082] Experiment 3: conditions identical to Experiment 1, except that the delivery solution is prepared without CTAB but includes 0.1% Silwet L-77TM (CAS Number 27306-78-1, available from Momentive Performance Materials, Albany, N.Y.). Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0083] Experiment 4: conditions identical to Experiment 3, except that several delivery solutions are prepared, where each further includes 20 micrograms/milliliter of one single-walled carbon nanotube preparation selected from those with catalogue numbers 704113, 750530, 724777, and 805033, all obtainable from Sigma-Aldrich, St. Louis, MO. Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0084] Experiment 5: conditions identical to Experiment 3, except that the delivery solution further includes 20 micrograms/milliliter of triethoxypylpropylaminosilane-functionalized silica nanoparticles

(catalogue number 791334, Sigma-Aldrich, St. Louis, MO). Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0085] Experiment 6: conditions identical to Experiment 3, except that the delivery solution further includes 9 micrograms/milliliter branched polyethylenimine, molecular weight ~25,000 (CAS Number 9002-98-6, catalogue number 408727, Sigma-Aldrich, St. Louis, MO) or 9 micrograms/milliliter branched polyethylenimine, molecular weight ~800 (CAS Number 25987-06-8, catalogue number 408719, Sigma-Aldrich, St. Louis, MO). Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0086] Experiment 7: conditions identical to Experiment 3, except that the delivery solution further includes 20% v/v dimethylsulfoxide (DMSO, catalogue number D4540, Sigma-Aldrich, St. Louis, MO). Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0087] Experiment 8: conditions identical to Experiment 3, except that the delivery solution further contains 50 micromolar nono-arginine (RRRRRRRRR, **SEQ ID NO:26**). Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0088] Experiment 9: conditions identical to Experiment 3, except that following the vacuum treatment, the embryos and treatment solutions are transferred to microcentrifuge tubes and centrifuged 2, 5, 10, or 20 minutes at 4000x g. Half (10 of 20) of the embryos receiving each treatment undergo electroporation, and the other half of the embryos do not.

[0089] Experiment 10: conditions identical to Experiment 3, except that following the vacuum treatment, the embryos and treatment solutions are transferred to microcentrifuge tubes and centrifuged 2, 5, 10, or 20 minutes at 4000x g.

[0090] Experiment 11: conditions identical to Experiment 4, except that following the vacuum treatment, the embryos and treatment solutions are transferred to microcentrifuge tubes and centrifuged 2, 5, 10, or 20 minutes at 4000x g.

[0091] Experiment 12: conditions identical to Experiment 5, except that following the vacuum treatment, the embryos and treatment solutions are transferred to microcentrifuge tubes and centrifuged 2, 5, 10, or 20 minutes at 4000x g.

[0092] After the delivery treatment, each treatment group of embryos is washed 5 times with sterile water, transferred to a petri dish containing ½ MS solid medium (2.165 g Murashige and Skoog medium salts, catalogue number MSP0501, Caisson Laboratories, Smithfield, UT), 10 grams sucrose, and 8 grams Bacto agar, made up to 1.00 liter in distilled water), and placed in a tissue culture incubator set to 25 degrees Celsius. After the embryos have elongated, developed roots and true leaves have emerged, the seedlings are transferred to soil and grown out. Modification of all endogenous PDS alleles results in a plant unable to produce chlorophyll and having a visible bleached phenotype. Modification of a fraction of all endogenous PDS alleles results in plants still able to produce chlorophyll; plants that are heterozygous for an altered PDS

gene will are grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

Example 2

[0093] This example illustrates a method of delivering polynucleotides encoding effector molecules (sgRNAs, nucleases) to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example describes delivery of an RNA guide for an RNA-guided nuclease (*i. e.*, an sgRNA) and the corresponding RNA-guided nuclease (*i. e.*, Cas9) to a non-epidermal plant cell (*i. e.*, a cell in a soybean embryo), resulting in editing of an endogenous plant gene (*i. e.*, phytoene desaturase, PDS) in germline cells of excised soybean embryos. The polynucleotides are delivered using combinations of chemical agents such as cationic polymers, and physical treatments such as use of negative pressure.

[0094] The sgRNA vector (**SEQ ID NO:1**) and nuclease vector (**SEQ ID NO:5**) described in Example 1 are used in a series of experiments. Mature, dry soybean seeds (cv. Williams 82) are surface-sterilized as follows. Dry soybean seeds are held for 4 hours in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid are freshly added. Seeds remained desiccated after this sterilization treatment. The sterilized seeds are split into 2 halves by manual application of a razor blade and the embryos manually separated from the cotyledons. Each test and control treatment experiment is carried out on 20 excised embryos, with treatments carried out in 96-well plates (two embryos per well).

[0095] Solutions are prepared with branched polyethylenimine ("PEI", CAS Number 9002-98-6) in sterile-filtered milliQ water. Three molecular weights of PEI are used: molecular weight ~25,000 (catalogue number 408727), ~5,000 (catalogue number 764582), and ~2,500 (catalogue number 764604) (all from Sigma Aldrich, St. Louis, MO). Four concentrations of PEI are used: 1, 5, 25, and 125 micrograms/milliliter final concentrations. The sgRNA and nuclease vectors (final concentrations of 5 micrograms/microliter of each plasmid) are added to the PEI solutions, and the mixtures incubated for 1 hour at room temperature to allow PEI/DNA complexes to form. Each solution is then chilled to 4 degrees Celsius and 500 microliters is added directly to the embryos, which are then immediately placed on ice and vacuum infiltrated (2×10^{-3} millibar) for 2 hours with shaking at 100 rpm.

[0096] Following vacuum infiltration, the embryos are rinsed with 5% sodium hypochlorite solution, washed 5 times with sterile water, transferred to a petri dish containing ½xMS medium (2.165 g Murashige and Skoog medium salts, catalogue number MSP0501, Caisson Laboratories, Smithfield, UT), 10 grams sucrose, and 8 grams Bacto agar, made up to 1.00 liter in distilled water), and placed in a tissue culture incubator set to 25 degrees Celsius. After the embryos have elongated, developed roots and true leaves have emerged, the seedlings are transferred to soil and grown out. Modification of all endogenous PDS alleles results in a plant unable to produce chlorophyll and having a visible bleached phenotype. Modification of a fraction of all endogenous PDS alleles results in plants still able to produce chlorophyll;

plants that are heterozygous for an altered PDS gene will be grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

Example 3

[0097] This example illustrates a method of delivering polynucleotides encoding effector molecules (sgRNAs, nucleases) to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example describes delivery of a single vector encoding both an RNA guide for an RNA-guided nuclease (*i. e.*, an sgRNA) and the corresponding RNA-guided nuclease (*i. e.*, Cas9) to a non-epidermal plant cell (*i. e.*, a cell in a soybean embryo), resulting in editing of the endogenous phytoene desaturase (PDS) in germline cells of excised soybean embryos. The polynucleotides are delivered using combinations of chemical agents such as a cell-penetrating peptide or a cationic polymer, and physical treatments such as use of negative pressure.

[0098] A plasmid ("pCas9TPC-GmPDS") having the nucleotide sequence of **SEQ ID NO:28** was designed for delivery of Cas9 (Csn1) endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system and a single guide RNA (sgRNA) targeting the endogenous phytoene desaturase (PDS) in soybean, *Glycine max*. The sequences of this plasmid and specific elements contained therein are described in **Table 3** below.

Table 3: pCas9TPC-GmPDS vector (SEQ ID NO:28), 14548 base pairs DNA

Nucleotide position in SEQ ID NO:28	Description	Comment
1 – 14548	Intact plasmid	SEQ ID NO:28
1187 – 1816	pVS1 StaA	stability protein from the <i>Pseudomonas</i> plasmid pVS1
2250 – 3317	pVS1 RepA	replication protein from the <i>Pseudomonas</i> plasmid pVS1
3383 – 3577	pVS1 oriV	origin of replication for the <i>Pseudomonas</i> plasmid pVS1
3921 - 4061	basis of mobility region from pBR322	
4247 – 4835	high-copy-number ColE1/pMB1/pBR322/pUC origin of replication (left direction)	complement
5079 – 5870	aminoglycoside adenyltransferase (aadA), confers resistance to spectinomycin and streptomycin	complement
6398 – 6422	left border repeat from nopaline C58 T-DNA	
6599 – 6620	<i>E. coli</i> catabolite activator protein (CAP) binding site	
6635 – 6665	lac promoter for the <i>E. coli</i> lac operon	
6673 – 6689	lac repressor encoded by lacI	
6697 – 6713	M13 reverse primer for sequencing	
6728 – 7699	PcUbi4-2 promoter	
7714 – 11817	Cas9 (Csn1) endonuclease from the <i>Streptococcus pyogenes</i> type II CRISPR/Cas system	SEQ ID NO:6 (encodes protein with sequence of SEQ ID NO:7)

11830 – 11850	nuclear localization signal of SV40 large T antigen	SEQ ID NO:8 (encodes peptide with sequence of SEQ ID NO:9)
11868 – 12336	Pea3A terminator	
12349 – 12736	AtU6-26 promoter	
12737 – 12756	<i>Glycine max</i> phytoene desaturase targeting sequence (gRNA)	SEQ ID NO:2
12757 – 12832	guide RNA scaffold sequence for <i>S. pyogenes</i> CRISPR/Cas9 system	SEQ ID NO:3
12844 – 12868	attB2; recombination site for Gateway® BP reaction	complement
13549 – 14100	<i>Streptomyces hygroscopicus</i> bar or pat, encodes phosphinothricin acetyltransferase, confers resistance to bialaphos or phosphinothricin	
14199 – 14215	M13 forward primer, for sequencing	complement
14411 – 14435	right border repeat from nopaline C58 T-DNA	

[0099] The pCas9TPC-GmPDS vector having the sequence of **SEQ ID NO:28** contains nucleotides at positions 12737 – 12832 encoding a single guide RNA having the sequence of **SEQ ID NO:4**, which includes both a targeting sequence (gRNA) (**SEQ ID NO:2**) and a guide RNA scaffold (**SEQ ID NO:3**); transcription of the single guide RNA is driven by a AtU6-26 promoter at nucleotide positions 12349 – 12736. This vector further contains nucleotides at positions 7714 – 11817 having the sequence of **SEQ ID NO:6** and encoding the Cas9 nuclease from *Streptococcus pyogenes* that has the amino acid sequence of **SEQ ID NO:7**, and nucleotides at positions 11830 – 11850 having the sequence of **SEQ ID NO:8** and encoding the nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen that has the amino acid sequence of **SEQ ID NO:9**. Transcription of the Cas9 nuclease and adjacent SV40 nuclear localization signal is driven by a PcUbi4-2 promoter at nucleotide positions 6728 – 7699; the resulting transcript including nucleotides at positions 7714 – 11850 having the sequence of **SEQ ID NO:10** encodes a fusion protein having the sequence of **SEQ ID NO:11** wherein the Cas9 nuclease is linked through a 4-residue peptide linker to the SV40 nuclear localization signal. The pCas9TPC-GmPDS vector also includes lac operon, aminoglycoside adenyltransferase, and phosphinothricin acetyltransferase sequences for convenient selection of the plasmid in bacterial or plant cultures.

[00100] In a series of experiments, the pCas9TPC-GmPDS vector (**SEQ ID NO:28**) was delivered to non-epidermal plant cells in soybean embryos using combinations of delivery agents and application of negative pressure. Mature, dry soybean seeds (cv. Williams 82) were surface-sterilized as follows. Dry soybean seeds were held for 4 hours in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid were freshly added. Seeds remained desiccated after this sterilization treatment. The sterilized seeds were split into 2 halves by manual application of a razor blade and the embryos manually separated from the cotyledons. Each test or control treatment experiment was carried out on 10 dry excised embryos. The excised embryos were transferred to 1.5-microliter microcentrifuge tubes.

[00101] Treatments included use of delivery agents including a cell-penetrating peptide (“CPP”) having the amino acid sequence KKLFFKKILKYLKKLFFKKILKYLKKKKKKKK (“(BP100x2)-K8”, **SEQ ID NO:27**), and a cationic polymer, branched polyethylenimine (“PEI”), molecular weight ~25,000 (CAS Number 9002-98-6, catalogue number 408727, Sigma-Aldrich, St. Louis, MO). Stock solutions of the pCas9TPC-GmPDS vector (**SEQ ID NO:28**) (500 nanograms/microliter), CPP (10 micrograms/microliter), and PEI (10 micrograms/microliter) were prepared. Treatment solutions were prepared according to **Table 4** and incubated 1 hour at room temperature to allow the plasmid to form complexes with the CPP or PEI delivery agents. Control treatment solutions included sterile milliQ water (no plasmid, no delivery agents) as well as a solution of the plasmid in milliQ water with no delivery agents.

Table 4

Treatment	Plasmid (SEQ ID NO:28), micrograms/milliliter	Cell-penetrating peptide (SEQ ID NO:27), micrograms/milliliter	Branched PEI, micrograms/milliliter
CPP-1	20	1	0
CPP-2	20	10	0
CPP-3	20	500	0
CPP-4	1	500	0
PEI-1	5	0	50
PEI-2	5	0	75
PEI-3	5	0	100
PEI-4	5	0	250

[00102] Each tube received 1000 microliters of the prepared treatment solutions and placed on ice. The tubes were vacuum infiltrated (2×10^{-3} millibar) for 2 hours in a desiccator submerged in ice. Following vacuum infiltration, the embryos were rinsed with 5% sodium hypochlorite solution, washed 5 times with sterile water, transferred to a petri dish containing ½xMS medium (2.165 g Murashige and Skoog medium salts, catalogue number MSP0501, Caisson Laboratories, Smithfield, UT), 10 grams sucrose, and 8 grams Bacto agar, made up to 1.00 liter in distilled water), and placed in a tissue culture incubator set to 25 degrees Celsius. After the embryos have elongated, developed roots and true leaves have emerged, the seedlings are transferred to soil and grown out to seed. Modification of all endogenous PDS alleles results in a plant unable to produce chlorophyll and having a visible bleached phenotype. Modification of a fraction of all endogenous PDS alleles results in plants still able to produce chlorophyll; plants that are heterozygous for an altered PDS gene will be grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

Example 4

[00103] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example illustrates a method of delivering a polynucleotide composition including a guide RNA (gRNA) to a non-epidermal plant cell in a seed of a plant and editing of an endogenous plant gene (phytoene desaturase, PDS) in germline cells of *Nicotiana benthamiana* seeds. This example demonstrates delivery of polynucleotides

encoding effector molecules (sgRNAs, nucleases) through multiple barriers (*e. g.*, multiple cell layers, seed coat, cell walls, plasma membrane) directly into *Nicotiana benthamiana* germline cells, resulting in a heritable alteration of the target nucleotide sequence, PDS. The methods described do not employ the common techniques of bacterially mediated transformation (*e. g.*, by *Agrobacterium* sp.) or biolistics.

[00104] A plasmid (“pCas9TPC-NbPDS”) having the nucleotide sequence of **SEQ ID NO:29** was designed for delivery of Cas9 (Csn1) endonuclease from the *Streptococcus pyogenes* Type II CRISPR/Cas system and a single guide RNA (sgRNA) targeting the endogenous phytoene desaturase (PDS) in *Nicotiana benthamiana*; see Nekrasov *et al.* (2013) *Nature Biotechnol.*, 31:691 – 693. The sequences of this plasmid and specific elements contained therein are described in **Table 5** below.

Table 5: pCas9TPC-NbPDS vector (SEQ ID NO:29), 14548 base pairs DNA

Nucleotide position in SEQ ID NO:29	Description	Comment
1 – 14548	Intact plasmid	SEQ ID NO:29
1187 – 1816	pVS1 StaA	stability protein from the <i>Pseudomonas</i> plasmid pVS1
2250 – 3317	pVS1 RepA	replication protein from the <i>Pseudomonas</i> plasmid pVS1
3383 – 3577	pVS1 oriV	origin of replication for the <i>Pseudomonas</i> plasmid pVS1
3921 - 4061	basis of mobility region from pBR322	
4247 – 4835	high-copy-number ColE1/pMB1/pBR322/pUC origin of replication (left direction)	Complement
5079 – 5870	aminoglycoside adenylyltransferase (aadA), confers resistance to spectinomycin and streptomycin	Complement
6398 – 6422	left border repeat from nopaline C58 T-DNA	
6599 – 6620	<i>E. coli</i> catabolite activator protein (CAP) binding site	
6635 – 6665	lac promoter for the <i>E. coli</i> lac operon	
6673 – 6689	lac repressor encoded by lacI	
6697 – 6713	M13 reverse primer for sequencing	
6728 – 7699	PcUbi4-2 promoter	
7714 – 11817	Cas9 (Csn1) endonuclease from the <i>Streptococcus pyogenes</i> type II CRISPR/Cas system	SEQ ID NO:6 (encodes protein with sequence of SEQ ID NO:7)
11830 – 11850	nuclear localization signal of SV40 large T antigen	SEQ ID NO:8 (encodes peptide with sequence of SEQ ID NO:9)
11868 – 12336	Pea3A terminator	
12349 – 12736	AtU6-26 promoter	
12737 – 12756	<i>Nicotiana benthamiana</i> phytoene desaturase targeting sequence	SEQ ID NO:30
12757 – 12832	guide RNA scaffold sequence for <i>S. pyogenes</i> CRISPR/Cas9 system	SEQ ID NO:3
12844 – 12868	attB2; recombination site for Gateway® BP reaction	Complement
13549 – 14100	<i>Streptomyces hygrosopicus</i> bar or pat, encodes phosphinothricin acetyltransferase, confers resistance to bialaphos or phosphinothricin	

14199 – 14215	M13 forward primer, for sequencing	Complement
14411 – 14435	right border repeat from nopaline C58 T-DNA	

[00105] The pCas9TPC-NbPDS vector having the sequence of **SEQ ID NO:29** contains nucleotides at positions 12737 – 12832 encoding a single guide RNA having the sequence of **SEQ ID NO:31**, which includes both a targeting sequence (gRNA) (**SEQ ID NO:30**) and a guide RNA scaffold (**SEQ ID NO:3**); transcription of the single guide RNA is driven by a AtU6-26 promoter at nucleotide positions 12349 – 12736. This vector further contains nucleotides at positions 7714 – 11817 having the sequence of **SEQ ID NO:6** and encoding the Cas9 nuclease from *Streptococcus pyogenes* that has the amino acid sequence of **SEQ ID NO:7**, and nucleotides at positions 11830 – 11850 having the sequence of **SEQ ID NO:8** and encoding the nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen that has the amino acid sequence of **SEQ ID NO:9**. Transcription of the Cas9 nuclease and adjacent SV40 nuclear localization signal is driven by a PcUbi4-2 promoter at nucleotide positions 6728 – 7699; the resulting transcript including nucleotides at positions 7714 – 11850 having the sequence of **SEQ ID NO:10** encodes a fusion protein having the sequence of **SEQ ID NO:11** wherein the Cas9 nuclease is linked through a 4-residue peptide linker to the SV40 nuclear localization signal. The pCas9TPC-NbPDS vector also includes lac operon, aminoglycoside adenyltransferase, and phosphinothricin acetyltransferase sequences for convenient selection of the plasmid in bacterial or plant cultures.

[00106] In a series of experiments, the pCas9TPC-NbPDS (**SEQ ID NO:29**) was delivered to non-epidermal plant cells in *Nicotiana benthamiana* intact seeds using combinations of delivery agents and physical techniques. Mature, dry *N. benthamiana* seeds were surface-sterilized as follows. Dry *N. benthamiana* seeds were held for 3 hours in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid were freshly added. Seeds remained desiccated after this sterilization treatment. Each test or control treatment experiment was carried out on 10 sterilized seeds. The following treatments were performed:

[00107] Treatment 1 (vacuum control): Seeds were pre-incubated in 400 microliters Tris-EDTA (TE) buffer on ice for 3 hours under vacuum, followed by an overnight incubation at 4 degrees Celsius and then a recovery period of 7 hours at room temperature. The TE buffer was removed by aspiration and the seeds resuspended in 400 microliters of 50% glycerol in milliQ water. Seeds were plated on ½ MS solid media (see Example 1) and germination scored.

[00108] Treatment 2 (vacuum/electroporation control): identical to treatment 1, except that after removal of the TE buffer, the seeds were resuspended in 400 or 800 microliters of 50% glycerol in milliQ water, transferred respectively to a 2 or 4 millimeter gap cuvette, and subjected to electroporation using a BTX-Harvard ECM-830 electroporation device set with the following parameters: 100V, 25 millisecond pulse length, 75 millisecond pulse interval for 99 pulses, followed by 400V, 99 millisecond pulse length, 297 millisecond pulse interval for 5 pulses. The seeds were then plated on ½ MS solid media (see Example 1) and germination scored.

[00109] Treatment 3 (vacuum/DNA): identical to treatment 1, except that seeds were incubated in a solution of 1 microgram of the pCas9TPC-NbPDS (**SEQ ID NO:29**) DNA in 200 microliters TE buffer.

[00110] Treatment 4: (vacuum/electroporation/DNA): identical to treatment 2, except that seeds were incubated in a solution of 1 microgram of the pCas9TPC-NbPDS (**SEQ ID NO:29**) DNA in 200 microliters TE buffer, and the electroporation solution further includes 5 nanograms/microliter of the pCas9TPC-NbPDS (**SEQ ID NO:29**) DNA.

[00111] Modification of all endogenous PDS alleles results in a plant unable to produce chlorophyll and having a visible bleached phenotype. Modification of a fraction of all endogenous PDS alleles results in plants still able to produce chlorophyll; plants that are heterozygous for an altered PDS gene will be grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

Example 5

[00112] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example illustrates a method of delivering a polynucleotide composition including a guide RNA (gRNA) to a non-epidermal plant cell in a seed of a plant and editing of an endogenous plant gene (alcohol dehydrogenase, ADH1, NCBI locus tag ZEAMMB73_889219, with the sequence of **SEQ ID NO:32**) in germline cells of maize (*Zea mays*) seeds. This example demonstrates delivery of effector molecules including polynucleotides (multiple crRNA:tracrRNA combinations) and an RNA-guided nuclease in the form of a ribonucleoprotein complex, through multiple barriers (*e. g.*, multiple cell layers, seed coat, cell walls, plasma membrane) directly into *Zea mays* germline cells, resulting in a selectable, heritable alteration of the target nucleotide sequence, ADH1, thus conferring resistance to allyl alcohol. The methods described do not employ the common techniques of bacterially mediated transformation (*e. g.*, by *Agrobacterium* sp.) or biolistics.

[00113] Three individual crRNAs (Alt-R™) including the guide RNA (gRNA) sequences GGCAAGCCACTGTCGATCG (**SEQ ID NO:33**), GGCCTCCCAGAAGTAGACGT (**SEQ ID NO:34**), and ACGCGCACCTCCATGGCCTG (**SEQ ID NO:35**) were synthesized by IDT (Coralville, IA). Individual crRNA:tracrRNA duplex solutions are prepared by combining equimolar amounts of a single crRNA with a tracrRNA synthesized by IDT (Coralville, IA) to a final concentration of 100 micromolar; each crRNA/tracrRNA mixture is heated to 95 degrees Celsius for 5 minutes and then allowed to cool to room temperature to form the crRNA:tracrRNA duplex solutions. Ribonucleoprotein (RNP) solutions are prepared by combining equimolar amounts of each crRNA:tracrRNA duplex and a purified Cas9 fusion protein having a nuclear localization signal (NLS) on either terminus (sNLS-spCas9-sNLS, purchased from Aldevron, Fargo, ND) to a final concentration of 100 micromolar and incubating the mixtures at room temperature for 5 minutes to allow the ribonucleoprotein complexes to form.

[00114] Mature, dry kernels (seeds) of B73 maize are surface-sterilized as follows. Dry maize kernels are held for 4 hours in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid are freshly added. The kernels remain desiccated after this sterilization treatment. Embryos are manually separated from the cotyledons and endosperm using a scalpel blade. Each test or control treatment experiment is carried out on 10 dry excised embryos. The excised embryos are transferred to 1.5-microliter microcentrifuge tubes.

[00115] To each microfuge tube is added 180 microliters of maize washing solution (0.6 molar D-mannitol, 4 millimolar 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.7, 20 millimolar KCl) and 20 microliters of the RNP solution. The microcentrifuge tubes are tapped gently to mix and immediately placed on ice and vacuum infiltrated (2×10^{-3} millibar) for 2 hours with shaking at 100 rpm. Following vacuum infiltration, the embryos are rinsed with 5% sodium hypochlorite solution, washed 5 times with sterile water, transferred to a petri dish containing $\frac{1}{2}$ xMS medium (2.165 g Murashige and Skoog medium salts, catalogue number MSP0501, Caisson Laboratories, Smithfield, UT), 10 grams sucrose, and 8 grams Bacto agar, made up to 1.00 liter in distilled water), and placed in a tissue culture incubator set to 25 degrees Celsius. After the embryos have elongated, developed roots and true leaves have emerged, the seedlings are treated with 5 or 20 micromolar allyl alcohol for 24 hours. The seedlings are then washed 5 times with sterile water, transferred to soil and grown out. Modification of the endogenous ADH1 results in a plant having an observable phenotype, *i. e.*, resistance to allyl alcohol-induced toxicity. Surviving maize seedlings are grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

Example 6

[00116] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example illustrates a method of delivering a polynucleotide composition including at least one crRNA or gRNA or sgRNA to a non-epidermal plant cell in a seed of a plant, resulting in editing of at least one endogenous plant gene. This example demonstrates direct delivery by microinjection of effector molecules (*e. g.*, at least one crRNA or sgRNA or a polynucleotide encoding at least one crRNA or sgRNA or an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease) directly into maize (*Zea mays*) zygotes; the embryos are isolated and allowed to shoot, and the resulting maize plants containing the desired genomic edit or alteration of the target nucleotide sequence are subsequently identified. The methods described do not employ the common techniques of bacterially mediated transformation (*e. g.*, by *Agrobacterium* sp.) or biolistics.

[00117] A non-limiting example of a microinjection protocol utilized maize B73 fertilized cobs (ears) (collected 1 day after pollination). All steps of this protocol were performed under a laminar flow hood. Husks and silks were removed from the cobs. The cobs were transversely cut into approximately 3-centimeter segments with the top and bottom two centimeters of each cob discarded. The segments were

surface-sterilized for 10 minutes in 70% ethanol followed by three washes in distilled, autoclaved water of one minute each.

[00118] Ethanol-sterilized fifty-milliliter tube caps were used as specimen mounting blocks, to which two pairs of ovaries cut from the prepared cob slices were glued with a thin layer of fast-facing adhesive (*e. g.*, Loctite Control Gel Premium Super Glue); one pair of ovaries was mounted facing the other pair's basal ends. The mounted ovaries were attached to a modified specimen tray of a Vibratome (PELCO easiSlicer™, Ted Pella, Inc.) with the stylar ends facing the blade. Ovaries were sectioned at 220 micrometers from the ovarian surface. Sections that contained embryo sacs were collected for microinjection on MMIM (modified maize induction medium). To prepare MMIM, 2.2 g Murashige and Skoog (MS) medium, 50 g sucrose, 10 g mannitol, and 2.5 g Phytigel were dissolved in 500 milliliters water and pH adjusted to 5.8; after autoclaving, indole acetic acid or 1-naphthaleneacetic acid (0.1 milligrams/liter final concentration), 6-benzylaminopurine (0.5 milligrams/liter final concentration), and vitamins (1x final concentration) are added.

[00119] The target gene selected for editing was the maize (*Zea mays*) alcohol dehydrogenase ADH1 (see [www\[dot\]maizegdb\[dot\]org/gene_center/gene/GRMZM2G442658](http://www.maizegdb.org/gene_center/gene/GRMZM2G442658)) with the partial genomic sequence:

GAACAGTGCCGAGTGGCGCTGATCTTGATGCTATCCTGCAATCGTGGTGAACCTATTTCTTTATATCC
TTTACTCCCATGAAAAGGCTAGTAATCTTCTCGATGTAACATCGTCCAGCACTGCTATTACCGTGTGGTC
CATCCGACAGTCTGGCTGAACACATCATACGATCTATGGAGCAAAAATCTATCTTCCCTGTTCTTTAATGA
AGGACGTCATTTTCATTAGTATGATCTAGGAATGTTGCAACTTGCAAGGAGGCGTTTCTTTCTTTGAATTT
AACTAACTCGTTGAGTGGCCCTGTTTCTCGGACGTAAGGCCTTTGCTGCTCCACACATGTCCATTCTGAATT
TTACCGTGTTTAGCAAGGGCGAAAAGTTTGCATCTTGATGATTTAGCTTGACTATGCGATTGCTTTCTCTG
GACCCGTGCAGCTGCGGTGGCATGGGAGGCCGGCAAGCCACTGTCGATCGAGGAGGTGGAGGTA
GCGCCTCCGAGGCCATGGAGGTGCGCGTCAAGATCCTCTTACCTCGCTCTGCCACACCGACGTC
TACTTCTGGGAGGCCAAGGTATCTAATCAGCCATCCCATTTGTGATCTTTGTGAGTAGATATGATACAAC
AACTCGCGTTGACTTGCGCCTTCTGGCGGCTTATCTGTCTTAGGGGCAGACTCCCGTGTTCCTCGGAT
CTTTGGCCACGAGGCTGGAGGGTA (SEQ ID NO:36); the first exon (SEQ ID NO:37), located at
nucleotide positions 409 – 571 of SEQ ID NO:36 is indicated by bold, underlined text and guide RNA
(crRNA) sequences were designed to edit this exon.

[00120] A ribonucleoprotein (RNP) was prepared with Cas9 nuclease (Aldevron, Fargo, ND) and a guide RNA complex of a crRNA (ZmADH1-B) having the sequence
GGCCUCCAGAAGUAGACGUGUUUAGAGCUAUGCU (SEQ ID NO:38) and a tracrRNA (both
purchased from Integrated DNA Technologies, Coralville, IA). A guide RNA (gRNA) complex was
prepared as follows: 30 microliters of 100 micromolar crRNA were mixed with 30 microliters of 100
micromolar tracrRNA, heated at 95 degrees Celsius for 5 minutes, and then cooled to room temperature. To
the cooled gRNA solution, 100 micrograms Cas9 nuclease (Aldevron, Fargo, ND) was added and the
mixture incubated 5 minutes at room temperature to allow the ribonucleoprotein (RNP) complex to form. A
microinjection mixture containing the RNP complex was prepared by taking a volume (*e. g.*, 30 microliters)

of the RNP solution and adding sufficient 10x Cas9 reaction buffer (20 millimolar HEPES, 1 molar NaCl, 50 millimolar MgCl₂, 1 millimolar EDTA) to yield a 1x buffer concentration in the final mixture. The microinjection mixture was centrifuged through a Millipore filter (UFC30VV25) at 13,000 rpm for 10 minutes at room temperature.

[00121] For microinjection of the maize zygotes, 2.5 microliters of the filtered injection mix were loaded into a borosilicate needle (catalogue number G100F-4, Warner Instruments, Hamden, CT), previously pulled with a P1000 micropipette puller (Sutter Instrument, Novato, CA) with the following settings: Heat: Ramp-20; Pull: 140; Velocity: 70; Delay: 200; Pressure: 510; Ramp: 499. The needle was opened with a micropipette beveller (BV-10, Sutter Instrument, Novato, CA) with an angle of 35 degrees. The egg apparatus was visualized with basal illumination with a fluorescence stereoscope (model SMZ18, Nikon, Tokyo, Japan). The injection mix was injected into the egg apparatus using a FemtoJet 4i with a PatchMan micromanipulator (both from Eppendorf, Hauppauge, NY). Embryo sacs were recovered in MMIM medium. The embryos were kept in the dark at 26 degrees Celsius until shoots formed, and then kept in light at 26 degrees Celsius. Shoots thus produced are optionally grown under culture conditions including exposure to low concentrations (*e. g.*, 5 or 20 micromolar) of allyl alcohol (which is converted by a functional ADH1 to acrolein, which is toxic to the cell), thus permitting selection by expression of the predicted phenotype, *i. e.*, decreased allyl alcohol susceptibility in shoots or plants wherein one or both copies of the endogenous ADH1 gene has been disrupted. Surviving maize seedlings are grown out to seed and the efficiency of heritable genome modification is determined by molecular analysis of the progeny seeds.

[00122] One of skill in the art would recognize that there are alternative reagents and compositions including such reagents that are useful for introducing alterations or edits into the genome (*e. g.*, use of CRISPR nucleases other than Cas9, such as CasX, CasY, and Cpf1, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TAL-effector nucleases or TALENs), Argonaute proteins, or a meganuclease or engineered meganuclease) and thus similar embodiments of the microinjection technique described herein include use of any of these reagents. Similarly, the microinjection technique described herein is generally applicable to any plant cell of sufficient size to permit microinjection (*e. g.*, germline cells or cells that develop into germline cells, egg cells, zygote cells, embryo cells, meristematic cells), and of any plant species (*e. g.*, alfalfa (*Medicago sativa*), almonds (*Prunus dulcis*), apples (*Malus x domestica*), apricots (*Prunus armeniaca*, *P. brigantiae*, *P. mandshurica*, *P. mume*, *P. sibirica*), asparagus (*Asparagus officinalis*), bananas (*Musa* spp.), barley (*Hordeum vulgare*), beans (*Phaseolus* spp.), blueberries and cranberries (*Vaccinium* spp.), cacao (*Theobroma cacao*), canola and rapeseed or oilseed rape, (*Brassica napus*), carnation (*Dianthus caryophyllus*), carrots (*Daucus carota sativus*), cassava (*Manihot esculentum*), cherry (*Prunus avium*), chickpea (*Cicer arietinum*), chicory (*Cichorium intybus*), chili peppers and other capsicum peppers (*Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, *C. baccatum*), chrysanthemums (*Chrysanthemum* spp.), coconut (*Cocos nucifera*), coffee (*Coffea* spp. including *Coffea arabica* and *Coffea canephora*), cotton (*Gossypium hirsutum* L.), cowpea (*Vigna unguiculata*), cucumber

(*Cucumis sativus*), currants and gooseberries (*Ribes* spp.), eggplant or aubergine (*Solanum melongena*), eucalyptus (*Eucalyptus* spp.), flax (*Linum usitatissimum* L.), geraniums (*Pelargonium* spp.), grapefruit (*Citrus x paradisi*), grapes (*Vitis* spp.) including wine grapes (*Vitis vinifera*), guava (*Psidium guajava*), irises (*Iris* spp.), lemon (*Citrus limon*), lettuce (*Lactuca sativa*), limes (*Citrus* spp.), maize (*Zea mays* L.), mango (*Mangifera indica*), mangosteen (*Garcinia mangostana*), melon (*Cucumis melo*), millets (*Setaria* spp., *Echinochloa* spp., *Eleusine* spp., *Panicum* spp., *Pennisetum* spp.), oats (*Avena sativa*), oil palm (*Ellis quineensis*), olive (*Olea europaea*), onion (*Allium cepa*), orange (*Citrus sinensis*), papaya (*Carica papaya*), peaches and nectarines (*Prunus persica*), pear (*Pyrus* spp.), pea (*Pisa sativum*), peanut (*Arachis hypogaea*), peonies (*Paeonia* spp.), petunias (*Petunia* spp.), pineapple (*Ananas comosus*), plantains (*Musa* spp.), plum (*Prunus domestica*), poinsettia (*Euphorbia pulcherrima*), Polish canola (*Brassica rapa*), poplar (*Populus* spp.), potato (*Solanum tuberosum*), pumpkin (*Cucurbita pepo*), rice (*Oryza sativa* L.), roses (*Rosa* spp.), rubber (*Hevea brasiliensis*), rye (*Secale cereale*), safflower (*Carthamus tinctorius* L.), sesame seed (*Sesame indium*), sorghum (*Sorghum bicolor*), soybean (*Glycine max* L.), squash (*Cucurbita pepo*), strawberries (*Fragaria* spp., *Fragaria x ananassa*), sugar beet (*Beta vulgaris*), sugarcane (*Saccharum* spp.), sunflower (*Helianthus annuus*), sweet potato (*Ipomoea batatas*), tangerine (*Citrus tangerina*), tea (*Camellia sinensis*), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum*), tulips (*Tulipa* spp.), turnip (*Brassica rapa rapa*), walnuts (*Juglans* spp. L.), watermelon (*Citrulus lanatus*), wheat (*Triticum aestivum*), and yams (*Discorea* spp.). Non-limiting embodiments include microinjection delivery of DNA or RNP editing reagents to egg cells, zygote cells, embryo cells, and meristematic cells of maize, rice, wheat, barley, rye, millet, sorghum, soybean, cotton, brassicas (including oilseed brassicas and sugar beet), solanaceous plants (including tomato, pepper, potato, and eggplant), strawberry, banana, plantain, citrus fruits, coffee, cacao, and sugarcane.

Example 7

[00123] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. More specifically, this non-limiting example illustrates a method of delivering a polynucleotide composition including at least one crRNA or gRNA or sgRNA to non-epidermal plant cells, resulting in editing of at least one endogenous plant gene. This example demonstrates direct delivery of effector molecules (*e. g.*, at least one crRNA or sgRNA or a polynucleotide encoding at least one crRNA or sgRNA or an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease) by gold microparticle bombardment directly into germline cells of excised soybean (*Glycine max*) embryos.

[00124] The target genes selected for editing were two endogenous soybean (*Glycine max*) phytoene desaturase (PDS) genes, Glyma.11g239000 and Glyma.18g003900 (see, *e. g.*, [www\[dot\]soybase\[dot\]org/sbt/search/search_results.php?category=FeatureName&version=Glyma2\[dot\]0&search_term=Glyma\[dot\]18g003900](http://www[dot]soybase[dot]org/sbt/search/search_results.php?category=FeatureName&version=Glyma2[dot]0&search_term=Glyma[dot]18g003900)). The first PDS gene, Glyma.11g239000 has the genomic sequence of **SEQ ID NO:39**. The second PDS gene, Glyma.18g003900, has the genomic sequence of **SEQ ID NO:40**.

[00125] Four guide RNA (gRNA) sequences were designed to cleave both soybean PDS genes, Glyma.11g253000 (SEQ ID NO:39) and Glyma18g003900 (SEQ ID NO:40): GmPDS gRNA.1 GAAGCAAGAGACGUUCUAGG (SEQ ID NO:41), GmPDS gRNA.2 GGUUGCUGCAUGGAAAGACA (SEQ ID NO:42), GmPDS gRNA.3 CCAUAUGUUGAGGCUCAAGA (SEQ ID NO:43), and GmPDS gRNA.4 GAUCAUAUUCAGUCCUUGGG (SEQ ID NO:44). These were provided as single guide RNAs (sgRNAs), chimeric RNAs, each of which included one of the gRNA sequences and a guide RNA scaffold sequence (SEQ ID NO:3). All four gRNA sequences had been previously validated by an *in vitro* Cas9 assay and were shown to be capable of cleaving both soybean phytoene desaturase genes. The first PDS gene, Glyma.11g239000 (SEQ ID NO:39) is cleaved between nucleotide positions 2379 – 2340, 2653 – 2654, 3931 – 3932, and 4795 – 4796. The second PDS gene, Glyma.18g003900, (SEQ ID NO:40) is cleaved between nucleotide positions 2217 – 2218, 2490 – 2491, 5370 – 5371, and 6130 – 6131.

[00126] In a first series of experiments, the sgRNA and nuclease vectors were delivered by gold microparticle bombardment to non-epidermal cells in soybean embryonic axes. Mature, dry soybean seeds (cv. Williams 82) were surface-sterilized by holding overnight in an enclosed chamber holding a beaker containing 100 milliliters 5% sodium hypochlorite solution to which 4 milliliters hydrochloric acid were freshly added. The sterilized seeds were imbibed in sterile water for 2 – 20 hours. Seeds were divided by inserting a razor blade into the hilum leaving the embryonic axes intact. The pericarp was removed and the tip of the radicle excised. The leaf primordia and a thin layer of the shoot apical meristems were excised with a scalpel with the aid of a dissecting microscope. Prepared explants were placed on pre-bombardment medium ("Recipe X" with the addition of 2 milligrams/liter 6-benzylaminopurine) for 2 – 3 days in the dark at 26 (plus or minus 2) degrees Celsius. In an alternative protocol, explants were placed on osmoticum medium ("Recipe X" modified by the addition of 36.8 grams/liter sorbitol and 36.8 grams/liter mannitol) for four hours prior to bombardment. To make a 1-liter quantity of "Recipe X" medium, mix 4.43g MS salts with B5 vitamins, 10 milliliter 0.2 molar MES hydrate stock solution, 100 milligrams *myo*-inositol, 30 grams sucrose, 8 grams Oxoid agar (Remel, Inc. Lenexa, KS) and bring volume to 1 liter with water. Adjust pH to 5.8 before adding agar and autoclaving. Add 6-benzylaminopurine (BA) after cooling to about 50 degrees Celsius.

[00127] Gold microparticles were prepared as follows. In the following non-limiting experiments, 1.0 micrometer gold microparticles were used (Bio-Rad, Hercules, CA). In other protocols, gold microparticles of other sizes (*e. g.*, 0.6 or 1.6 micrometer) are also useful gold. Approximately 15- 20 milligrams of gold microparticles were transferred to sterile 1.5 milliliter microcentrifuge tubes. Cold absolute ethanol (500 microliters) was added to each tube, and the tubes were placed in the ultrasonication water bath for 15 seconds. Gold microparticles were allowed to settle ~ 10 – 30 minutes followed by pelleting by centrifugation for 1 minute at 3000 rpm. The supernatant was removed and the pellet was carefully rinsed with 1 milliliter ice-cold sterile water. The tubes were tapped gently to disturb the pellets, which were then allowed to settle again. The rinse step was repeated two more times. After the third rinse, the microparticles were pelleted 15 seconds at 5000 rpm, and the final supernatant removed. The pellet was

resuspended in 500 microliters sterile water to form a "1X" concentration, placed in the ultrasonication water bath for 15 seconds, and immediately after was vortexed. Aliquots of 50 microliters were transferred to 1.5-milliliter microcentrifuge tubes, with the original preparation continually vortexed during the transfers. The 1X aliquots were stored at -20 degrees Celsius.

[00128] Prior to precipitation of DNA on gold microparticles, soy explants are embedded in pre-bombardment medium with the shoot apical meristem arranged parallel with the medium's surface and directly facing the trajectory of the DNA coated microparticles. Approximately, 20 – 40 explants were placed in the center of the plate, corresponding to the ~ 3.5-centimeter diameter circle of the tissue platform (Bio-Rad, Hercules, CA). A tube of 1X prepared gold was used for bombardment of three media plates of soy explants. Prepared 1X tubes were thawed on ice, placed in the ultrasonication water bath for 15 seconds, and then centrifuged at 2000 rpm for 2 minutes. The supernatant was removed and the gold microparticles were resuspended in either 25 microliters DNA (1 microgram/microliter) solution or 25 microliters sterile water as a control. The following was added in order, vortexing between each addition: 220 microliters sterile water, 250 microliters 2.5 molar calcium chloride, and 50 microliters 0.1 molar spermidine. The tubes were placed on ice for 5 minutes, vortexed for ~2 minutes at room temperature, and then centrifuged at 500 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 600 microliters absolute ethanol. The tubes were centrifuged for 1 minute at 14K rpm. The supernatant was removed and the pellet was resuspended in 36 microliters absolute ethanol. (To conserve the amount of gold used, the pellet can be resuspended in about 90 microliters absolute ethanol, and about 10 microliters or about 444 nanograms gold used for each shot for 9 shots.) DNA-coated gold (11 microliters) was placed in the center of autoclaved macrocarriers (Bio-Rad, Hercules, CA) and allowed to dry for approximately 5 -10 minutes. The PDS-1000/He biolistic® particle delivery system (Bio-Rad, Hercules, CA) was assembled. The rupture discs (1,100 psi rupture discs, Bio-Rad, Hercules, CA; 900 or 650 psi rupture discs can also be used) were dipped in 70% ethanol to sterilize, placed in the retaining cap, and tightened with the manufacturer's supplied wrench. The autoclaved stopping screen was placed in the macrocarrier assembly followed by the DNA-coated gold macrocarrier. The system was assembled as directed in the manual. The distance used from stopping screen to soy explants was 6 centimeters. The gun was fired when the vacuum in the chamber reached 27 – 28 inches of Hg.

[00129] After bombardment, explants were transferred to Recipe X medium containing 0.5 milligrams/liter 6-benzylaminopurine. Plates with bombarded explants were placed in the dark for 2 – 4 days at 26 (plus or minus 2) degrees Celsius, then moved to a 16-hour light (75 micromoles)/8-hour dark light regime at 26 (plus or minus 2) degrees Celsius for several days to weeks depending on assay performed. For non-destructive assays, soybean shoots were sampled and explants moved to fresh Recipe X medium containing 0.5 milligrams/liter 6-benzylaminopurine. When shoots reached about 2 – 3 centimeters in length, explants were transferred to shoot elongation media ("Recipe Y"). To make 1 liter of "Recipe Y" medium, mix 4.43 grams MS salts with B5 vitamins, 0.59 grams MES hydrate, and 30 grams sucrose in 1 liter water, adjust pH to 5.7, and add 3 grams Phytagel. Autoclave 35 minutes on liquid cycle and cool to

50 degrees Celsius. In a laminar flow hood, add to 1 liter of cooled medium 0.5 milligrams gibberellic acid (as a premade stock, G362, PhytoTechnologies Laboratories, Shawnee Mission, KS), 500 microliters 50 milligrams/milliliter asparagine stock solution, 5 milligrams glutamine, 400 microliters indole acetic acid (as a 1 milligram/milliliter stock), and 1 milligram trans-zeatin riboside. Pour 100 milliliters per phytatray and allow to cool; store at room temperature. After approximately two weeks of shoot elongation, shoots were of sufficient size to transfer to Jiffy peat pellets, and were later transplanted to soilless mix in pots for maturation. Modification of the endogenous PDS gene(s) results in a plant having an observable bleached phenotype.

[00130] In another series of experiments, ribonucleoprotein (RNP) including a Cas9 nuclease and a guide RNA (gRNA) complex (crRNA-tracrRNA complex) was used for delivery of soybean phytoene desaturase guide RNAs to shoot apical meristem cells via gold microparticle bombardment. The RNP was prepared using procedures similar to those described in Example 6, but using 6 microliters of 100 micromolar crRNA (containing the soybean PDS guide RNA sequences described above) annealed with 6 microliters of 100 micromolar tracrRNA, and complexed with 20 micrograms Cas9 nuclease. The RNP preparation was added to a tube of 1X gold microparticles in 50 microliters water, mixed gently, and used at a rate of 14 microliters RNP-coated gold per macrocarrier. Sixty microliters 2.5 molar calcium chloride and 20 microliters 0.1 molar spermidine were optionally added, with vortexing, to this preparation. (To conserve the amount of gold used, one tube of ~1.5 mg of gold coated with 5 micrograms Cas9 complexed with 2.5 micrograms crRNA-tracrRNA complex is sufficient for 9 shots.) The samples were dried in Petri dishes with Drierite desiccant (W. A. Hammond DRIERITE Co., LTD, Xenia, OH) for 1 – 2 hours. The rest of the bombardment procedure was similar to that described above for the DNA-coated gold microparticles.

[00131] The shoot apical meristems of 48 soybean embryonic axes were sampled 5 days after bombardment by RNPs containing GmPDS g.RNA4 (**SEQ ID NO:44**). PCR amplification flanking the guide region was performed and six products were pooled together prior to Monarch PCR purification. The GmPDS gRNA.4 sequence contains a StyI restriction site which allows for enrichment of edited sequences; StyI restriction digest of the wild-type (unedited) sequence enriches the sample for edited sequences. After PCR purification, the products were digested for 4 hours with StyI at 37 degrees Celsius. The reactions were loaded on 2% E-gels (Invitrogen, Carlsbad, CA) and the uncut ~280 base-pair product was excised and purified using the Monarch Gel Extraction kit (New England Biolabs). The eluted product was submitted for Sanger sequencing and analyzed for editing. From the six pools representing 48 bombarded soybean axes, one pool showed evidence of editing at the correct location on the genome predicted to be edited by the GmPDS g.RNA4 (**SEQ ID NO:44**) guide sequence.

[00132] Addition bombardment experiments using GmPDS gRNA.1 (**SEQ ID NO:41**) delivered as DNA-coated or RNP-coated gold microparticles, or GmPDS gRNA.1 (**SEQ ID NO:41**) and GmPDS gRNA.2 (**SEQ ID NO:42**) delivered as RNP-coated gold microparticles, were evaluated for editing of the endogenous soybean PDS genes by various molecular assays, including, *e. g.*, T7E1 assay, fragment

analyzer assay, Sanger sequencing, and enrichment of edited amplicons by restriction digest and NGS amplicon sequencing.

[00133] One of skill in the art would recognize that there are alternative reagents and compositions (e. g., DNA encoding a nuclease or RNPs including a nuclease) including such reagents that are useful for introducing alterations or edits into the genome (e. g., use of CRISPR nucleases other than Cas9, such as CasX, CasY, and Cpf1, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALE effector nucleases or TALENs), Argonaute proteins, or a meganuclease or engineered meganuclease) and thus similar embodiments of the bombardment technique described herein include use of any of these reagents or compositions. Similarly, the bombardment technique described herein is generally applicable to any plant part, plant tissue, or whole plant, seed, seedling, or embryo (e. g., excised embryos, callus, leaf or other plant part, meristematic tissue), and of any plant species (e. g., alfalfa (*Medicago sativa*), almonds (*Prunus dulcis*), apples (*Malus x domestica*), apricots (*Prunus armeniaca*, *P. brigantiae*, *P. mandshurica*, *P. mume*, *P. sibirica*), asparagus (*Asparagus officinalis*), bananas (*Musa* spp.), barley (*Hordeum vulgare*), beans (*Phaseolus* spp.), blueberries and cranberries (*Vaccinium* spp.), cacao (*Theobroma cacao*), canola and rapeseed or oilseed rape, (*Brassica napus*), carnation (*Dianthus caryophyllus*), carrots (*Daucus carota sativus*), cassava (*Manihot esculentum*), cherry (*Prunus avium*), chickpea (*Cicer arietinum*), chicory (*Cichorium intybus*), chili peppers and other capsicum peppers (*Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, *C. baccatum*), chrysanthemums (*Chrysanthemum* spp.), coconut (*Cocos nucifera*), coffee (*Coffea* spp. including *Coffea arabica* and *Coffea canephora*), cotton (*Gossypium hirsutum* L.), cowpea (*Vigna unguiculata*), cucumber (*Cucumis sativus*), currants and gooseberries (*Ribes* spp.), eggplant or aubergine (*Solanum melongena*), eucalyptus (*Eucalyptus* spp.), flax (*Linum usitatissimum* L.), geraniums (*Pelargonium* spp.), grapefruit (*Citrus x paradisi*), grapes (*Vitis* spp.) including wine grapes (*Vitis vinifera*), guava (*Psidium guajava*), irises (*Iris* spp.), lemon (*Citrus limon*), lettuce (*Lactuca sativa*), limes (*Citrus* spp.), maize (*Zea mays* L.), mango (*Mangifera indica*), mangosteen (*Garcinia mangostana*), melon (*Cucumis melo*), millets (*Setaria* spp, *Echinochloa* spp, *Eleusine* spp, *Panicum* spp., *Pennisetum* spp.), oats (*Avena sativa*), oil palm (*Ellis quineensis*), olive (*Olea europaea*), onion (*Allium cepa*), orange (*Citrus sinensis*), papaya (*Carica papaya*), peaches and nectarines (*Prunus persica*), pear (*Pyrus* spp.), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), peonies (*Paeonia* spp.), petunias (*Petunia* spp.), pineapple (*Ananas comosus*), plantains (*Musa* spp.), plum (*Prunus domestica*), poinsettia (*Euphorbia pulcherrima*), Polish canola (*Brassica rapa*), poplar (*Populus* spp.), potato (*Solanum tuberosum*), pumpkin (*Cucurbita pepo*), rice (*Oryza sativa* L.), roses (*Rosa* spp.), rubber (*Hevea brasiliensis*), rye (*Secale cereale*), safflower (*Carthamus tinctorius* L.), sesame seed (*Sesame indicum*), sorghum (*Sorghum bicolor*), soybean (*Glycine max* L.), squash (*Cucurbita pepo*), strawberries (*Fragaria* spp., *Fragaria x ananassa*), sugar beet (*Beta vulgaris*), sugarcane (*Saccharum* spp.), sunflower (*Helianthus annuus*), sweet potato (*Ipomoea batatas*), tangerine (*Citrus tangerina*), tea (*Camellia sinensis*), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum*), tulips (*Tulipa* spp.), turnip (*Brassica rapa rapa*), walnuts (*Juglans* spp. L.), watermelon (*Citrullus lanatus*), wheat (*Triticum aestivum*), and yams (*Discorea* spp.)). Non-limiting embodiments include microparticle or

nanoparticle bombardment delivery of DNA or RNP editing reagents to embryos, seeds, seedlings, meristematic tissue, or callus of maize, rice, wheat, barley, rye, millet, sorghum, soybean, cotton, brassicas (including oilseed brassicas and sugar beet), solanaceous plants (including tomato, pepper, potato, and eggplant), strawberry, banana, plantain, citrus fruits, coffee, cacao, and sugarcane.

Example 8

[00134] This example illustrates a method of delivering at least one effector molecule to a plant cell wherein the plant cell is located in a plant or plant part. This example illustrates a method of delivering a polynucleotide composition including at least one crRNA or gRNA or sgRNA to a non-epidermal plant cell in a seed of a plant, resulting in editing of at least one endogenous plant gene: in this case, wheat phytoene desaturase (PDS) genes in germline cells of wheat (*Triticum aestivum*) seeds. More specifically, this example illustrates a method of effecting a genetic alteration in the genome of a whole seed or part of a seed, comprising imbibition of the whole seed or part of a seed in an aqueous solution that comprises: (a) an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease, and (b) at least one guide RNA or polynucleotide encoding a guide RNA; wherein the at least one guide RNA is capable of directing the RNA-guided nuclease to a defined location in the genome, thereby effecting a genetic alteration at the defined location in the genome; and wherein the genetic alteration is at least one alteration selected from the group consisting of insertion of at least one nucleotide, deletion of at least one nucleotide, or replacement of at least one nucleotide at the defined location in the genome. This non-limiting example demonstrates direct delivery by imbibition of effector molecules including polynucleotides encoding single-guide RNAs (sgRNAs) and a polynucleotide that encodes an RNA-guided nuclease, through multiple barriers (*e. g.*, multiple cell layers, seed coat, cell walls, plasma membrane) directly into germline cells of *Triticum aestivum* seeds, resulting in an alteration of the target nucleotide sequence, PDS. The methods described do not employ the common techniques of bacterially mediated transformation (*e. g.*, by *Agrobacterium* sp.) or biolistics.

[00135] Common or bread wheat, *Triticum aestivum*, is an allohexaploid. The three wheat genomes, *i. e.*, the wheat 4A, 4B, and 4D genomes each contain a phytoene desaturase (PDS) gene, respectively: TaPDS-4A (**SEQ ID NO:45**), TaPDS-4B (**SEQ ID NO:46**), and TaPDS-4D (**SEQ ID NO:47**). Three vectors were designed for editing the endogenous wheat PDS genes. One vector (**SEQ ID NO:48**, **Table 6**) was designed for expression of Cas9 nuclease. Two vectors (**SEQ ID NO:49**, **Table 7** and **SEQ ID NO:50**, **Table 8**) were designed for expression of sgRNAs; one of skill would understand that other sgRNA sequences for alternative target genes could be substituted in the plasmid.

Table 6: pVL40.52 Cas9 vector (SEQ ID NO:48), 9453 base pairs DNA

Nucleotide position in SEQ ID NO:	Description	Comment
1-9453	Intact plasmid	SEQ ID NO:48
378-395	M13 forward primer for sequencing	
439-1557	<i>Oryza sativa</i> Actin 1 promoter	

1558-1620	<i>Oryza sativa</i> Actin 1, 5' - untranslated leader sequence	Also includes exon 1
1621-1703	<i>Oryza sativa</i> Actin 1 intron 1	
1719-6161	Monocot codon optimized Cas9 with an intron	
3258-3446	Potato IV2 intron	Disrupts the Cas9 coding sequence
6168-6414	<i>Oryza sativa</i> Actin 1, 3' - untranslated sequence	
6415-7168	<i>Oryza sativa</i> Actin 1 terminator	
7228-7248	M13 reverse primer for sequencing	Complement
7280-7310	Lac promoter for the <i>E. Coli</i> lac operon	
7616-8298	High copy number ColE1/pMB1/pBR322/pUC origin of replication	Complement
8396-9055	CDS for bla, beta-lactamase, AmpR	Complement, ampicillin selection

Table 7: pVL40.30 “PDS guide 2” sgRNA vector (SEQ ID NO:49), 3493 base pairs DNA

Nucleotide position in SEQ ID NO:	Description	Comment
1-3493	Intact plasmid	SEQ ID NO:49
272-1462	Tet	Tetracycline resistance marker
1637-1655	T7 promoter for sequencing	
1765-2063	<i>Oryza sativa</i> U6 promoter	
2064-2083	<i>Oryza sativa</i> phytoene desaturase targeting sequence (gRNA, not showing the PAM sequence)	either “PDS guide 2” for TaPDS-4A, SEQ ID NO:52 or “PDS guide 2” for TaPDS-4B or TaPDS-4D, SEQ ID NO:53)
2084-2159	Guide RNA scaffold sequence for <i>S. pyogenes</i> CRISPR/Cas9 system	SEQ ID NO:3
2326-2945	pBB322 origin of replication	

Table 8: pVL40.23 “PDS guide 1” sgRNA vector (SEQ ID NO:50), 3493 base pairs DNA

Nucleotide position in SEQ ID NO:	Description	Comment
1-3493	Intact plasmid	SEQ ID NO:50
272-1462	Tet	Tetracycline resistance marker
1637-1655	T7 promoter for sequencing	
1765-2063	<i>Oryza sativa</i> U6 promoter	
2064-2083	<i>Oryza sativa</i> phytoene desaturase targeting sequence (gRNA, not showing the PAM sequence)	“PDS guide 1”, SEQ ID NO:51
2084-2159	Guide RNA scaffold sequence for <i>S. pyogenes</i> CRISPR/Cas9 system	SEQ ID NO:3
2326-2945	pBB322 origin of replication	

[00136] Glenn hard red spring wheat seed (product ID 292G, Johnny's Selected Seed, Fairfield, ME) were surface sterilized by wetting in 95% ethanol for 1 minute at room temperature followed by 20 minutes in sterilization solution (20% bleach, 0.1% Tween-20) on a rocker at room temperature. Seed were rinsed 5 times with sterile water and air dried in a sterile laminar flow hood, then stored in the dark in a low humidity environment until use.

[00137] Twenty seed were rehydrated in 2 milliliters of sterile imbibition solution (15 millimolar sodium chloride, 1.5 millimolar sodium citrate, 20% dimethylsulfoxide) containing 100 micrograms each of: (1) pVL40.52 Cas9 vector (**SEQ ID NO:48**) (**Table 6**), (2) pVL40.23 "PDS guide 1" sgRNA vector (**SEQ ID NO:50**) (**Table 8**) including the TaPDS "PDS guide 1" sgRNA sequence (as the DNA equivalent, not showing the PAM sequence) TTTGCCATGCCAAACAAACC ("PDS guide 1" common to TaPDS-4A, TaPDS-4B, and TaPDS-4D, **SEQ ID NO:51**), and (3) pVL40.30 "PDS guide 2" sgRNA vector (**SEQ ID NO:49**) (**Table 7**) including one of the TaPDS "PDS guide 2" sgRNA sequences (as the DNA equivalent, not showing the PAM sequence), *i. e.*, TCCTGATCGGGTCAACGACG ("PDS guide 2" for TaPDS-4A, **SEQ ID NO:52**) or TCCTGATCGAGTCAACGACG ("PDS guide 2" for TaPDS-4B or TaPDS-4D, **SEQ ID NO:53**), for 42-58 hours in darkness at room temperature (22 degrees Celsius). A control treatment was treated similarly with an imbibition solution lacking any plasmid DNA. After imbibition, seeds were washed with sterile water then placed in sterile petri dishes in a growth cabinet (16/8 hour light/dark cycle; 24/20 degrees Celsius) to germinate for approximately 7 days.

[00138] The first two leaves from each germinated seedling were excised and pooled appropriately into treated or control groups. Genomic DNA (gDNA) was extracted using the CTAB procedure (Doyle, J. J. and J. L. Doyle (1987) *Phytochem. Bull.*, 19:11 – 15). The gDNA template was subjected to PCR amplification using primers O-696 (CTTTTCAGTTGGAGCTTATCCCA, **SEQ ID NO:54**) and O-697 (CCTGCTGAAAAGAAGGTGGTCATAC, **SEQ ID NO:55**) at 0.5 micromolar, with 100 nanograms gDNA template, 25 microliters of Phusion 2X Master Mix (New England Biolabs, Ipswich, MA) in a 50 microliter reaction mix. The thermocycling program consisted of 98 degrees Celsius for 1 minute followed by 30 cycles of 98 degrees Celsius for 10 seconds, 55 degrees Celsius for 10 seconds and 72 degrees Celsius for 105 seconds. The final extension was 72 degrees Celsius for 10 minutes. Products were resolved by loading 20 microliters of each reaction on a 1% E-gel (Invitrogen, Carlsbad, CA). The wild-type or non-edited (1778 base pair) band was excised and the DNA purified using the Monarch gel extraction kit (New England Biolabs). The DNA was subjected to Sanger sequence analysis using the same primers used for the PCR amplification (**SEQ ID NO:54** and **SEQ ID NO:55**).

[00139] The sequencing results showed evidence of Cas9 activity at the TaPDS "PDS guide 1" sgRNA cut site. To further refine results, PDS primers (**Table 9**) specific to the wheat 4A, 4B and 4D genome were used to amplify each copy in isolation for sequence analysis. The gDNA from 15 plants was prepared using the CTAB method. PCR conditions were identical to those used in the previous amplification. Products were resolved by loading 20 microliters of each reaction on a 1% E-gel (Invitrogen).

Table 9: Genome-specific primers for wheat phytoene desaturase

Genome	Primer 1	SEQ ID NO:	Primer 2	SEQ ID NO:
4A	MZ139_TaPDS_4A-F	SEQ ID NO:56	MZ142_TaPDS-4a,4b,4d-R	SEQ ID NO:57
4B	MZ155-TaPDS-4B-F	SEQ ID NO:58	MZ142_TaPDS-4a,4b,4d-R	SEQ ID NO:57
4D	MZ148_TaPDS_4D-F	SEQ ID NO:59	MZ142_TaPDS-4a,4b,4d-R	SEQ ID NO:57

[00140] The ~1.8 kb DNA bands were excised and purified using the Monarch gel extraction kit (New England Biolabs) and the DNA was subjected to Sanger sequence analysis. The PDS 4A band was sequenced using primer MZ139_TaPDS_4A-F (**SEQ ID NO:56**), the PDS 4B band was sequenced using primer MZ164 (NNNNNNCAGTTGGAGCTTATCCCAATGTAC, **SEQ ID NO:60**) and the PDS 4D band was sequenced using MZ148_TaPDS_4D-F (**SEQ ID NO:59**). The results are shown in **Table 10**. Under these experimental conditions, the majority of Cas9 editing activity (genomic alterations) was detected at the 4B-PDS gene; no Cas9 editing activity (genomic alterations) was detected at the 4A-PDS gene and in one line (#9) at the 4D-PDS gene.

Table 10

Plant	4A-PDS	4B-PDS	4D-PDS
Control	wt	wt	wt
1	wt	Edited at guide#1	wt
2	wt	Edited at guide#1	wt
3	wt	wt	wt
4	wt	wt	wt
5	wt	wt	wt
6	wt	wt	wt
7	wt	wt	wt
8	wt	wt	wt
9	wt	Edited at guide#1	Edited at guide#1
10	wt	Edited at guide#1	wt
11	wt	PCR failed	wt
12	wt	Edited at guide#1	wt
13	wt	Not analyzed*	wt
14	wt	Not analyzed*	wt
15	wt	Not analyzed*	wt

wt = wild-type sequence (unedited)

*not enough gDNA left for analysis

[00141] Wheat seeds that have been subjected to this imbibition/editing treatment can also be grown out for observation of a visible bleached phenotype due to modification of the endogenous PDS gene(s) results in a plant having an observable bleached phenotype. Plants that survive to reproductive maturity are allowed to set seed, and progeny seed are subjected to molecular analysis for the presence of heritable alterations to one or more of the endogenous PDS genes(s).

[00142] One of skill in the art would recognize that there are alternative reagents and compositions (*e. g.*, DNA encoding a nuclease or RNPs including a nuclease) including such reagents that are useful for introducing alterations or edits into the genome (*e. g.*, use of CRISPR nucleases other than Cas9, such as

CasX, CasY, and Cpf1, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALE effectors or TALENs), Argonaute proteins, or a meganuclease or engineered meganuclease) and thus similar embodiments of the imbibition technique described herein include use of any of these reagents or compositions. Similarly, the imbibition technique described herein is generally applicable to any plant part, plant tissue, or whole plant, seed, seedling, or embryo (*e. g.*, whole seed or part of a seed, excised embryos, callus, leaf or other plant part, meristematic tissue), and of any plant species (*e. g.*, alfalfa (*Medicago sativa*), almonds (*Prunus dulcis*), apples (*Malus x domestica*), apricots (*Prunus armeniaca*, *P. brigantiae*, *P. mandshurica*, *P. mume*, *P. sibirica*), asparagus (*Asparagus officinalis*), bananas (*Musa* spp.), barley (*Hordeum vulgare*), beans (*Phaseolus* spp.), blueberries and cranberries (*Vaccinium* spp.), cacao (*Theobroma cacao*), canola and rapeseed or oilseed rape, (*Brassica napus*), carnation (*Dianthus caryophyllus*), carrots (*Daucus carota sativus*), cassava (*Manihot esculentum*), cherry (*Prunus avium*), chickpea (*Cicer arietinum*), chicory (*Cichorium intybus*), chili peppers and other capsicum peppers (*Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, *C. baccatum*), chrysanthemums (*Chrysanthemum* spp.), coconut (*Cocos nucifera*), coffee (*Coffea* spp. including *Coffea arabica* and *Coffea canephora*), cotton (*Gossypium hirsutum* L.), cowpea (*Vigna unguiculata*), cucumber (*Cucumis sativus*), currants and gooseberries (*Ribes* spp.), eggplant or aubergine (*Solanum melongena*), eucalyptus (*Eucalyptus* spp.), flax (*Linum usitatissimum* L.), geraniums (*Pelargonium* spp.), grapefruit (*Citrus x paradisi*), grapes (*Vitis* spp.) including wine grapes (*Vitis vinifera*), guava (*Psidium guajava*), irises (*Iris* spp.), lemon (*Citrus limon*), lettuce (*Lactuca sativa*), limes (*Citrus* spp.), maize (*Zea mays* L.), mango (*Mangifera indica*), mangosteen (*Garcinia mangostana*), melon (*Cucumis melo*), millets (*Setaria* spp, *Echinochloa* spp, *Eleusine* spp, *Panicum* spp., *Pennisetum* spp.), oats (*Avena sativa*), oil palm (*Ellis quineensis*), olive (*Olea europaea*), onion (*Allium cepa*), orange (*Citrus sinensis*), papaya (*Carica papaya*), peaches and nectarines (*Prunus persica*), pear (*Pyrus* spp.), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), peonies (*Paeonia* spp.), petunias (*Petunia* spp.), pineapple (*Ananas comosus*), plantains (*Musa* spp.), plum (*Prunus domestica*), poinsettia (*Euphorbia pulcherrima*), Polish canola (*Brassica rapa*), poplar (*Populus* spp.), potato (*Solanum tuberosum*), pumpkin (*Cucurbita pepo*), rice (*Oryza sativa* L.), roses (*Rosa* spp.), rubber (*Hevea brasiliensis*), rye (*Secale cereale*), safflower (*Carthamus tinctorius* L), sesame seed (*Sesame indicum*), sorghum (*Sorghum bicolor*), soybean (*Glycine max* L.), squash (*Cucurbita pepo*), strawberries (*Fragaria* spp., *Fragaria x ananassa*), sugar beet (*Beta vulgaris*), sugarcane (*Saccharum* spp.), sunflower (*Helianthus annuus*), sweet potato (*Ipomoea batatas*), tangerine (*Citrus tangerina*), tea (*Camellia sinensis*), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum*), tulips (*Tulipa* spp.), turnip (*Brassica rapa rapa*), walnuts (*Juglans* spp. L.), watermelon (*Citrullus lanatus*), wheat (*Triticum aestivum*), and yams (*Discorea* spp.)). Non-limiting embodiments include imbibition delivery of DNA or RNP editing reagents to whole seed or part of a seed, embryos, callus, pollen, anthers, stamens, leaf or other plant part, and meristematic tissue of maize, rice, wheat, barley, rye, millet, sorghum, soybean, cotton, brassicas (including oilseed brassicas and sugar beet), solanaceous plants (including tomato, pepper, potato, and eggplant), strawberry, banana, plantain, citrus fruits, coffee, cacao, and sugarcane.

[00143] All cited patents and patent publications referred to in this application are incorporated herein by reference in their entirety. All of the materials and methods disclosed and claimed herein can be made and used without undue experimentation as instructed by the above disclosure and illustrated by the examples. Although the materials and methods of this invention have been described in terms of embodiments and illustrative examples, it will be apparent to those of skill in the art that substitutions and variations can be applied to the materials and methods described herein without departing from the concept, spirit, and scope of the invention. For instance, while the particular examples provided illustrate the methods and embodiments described herein using a specific plant, the principles in these examples are applicable to any plant of interest; similarly, while the particular examples provided illustrate the methods and embodiments described herein using a particular sequence-specific nuclease such as Cas9, one of skill in the art would recognize that alternative sequence-specific nucleases (*e. g.*, CRISPR nucleases other than Cas9, such as CasX, CasY, and Cpf1, zinc-finger nucleases, transcription activator-like effector nucleases, Argonaute proteins, and meganucleases) are useful in various embodiments. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the invention as encompassed by the embodiments of the inventions recited herein and the specification and appended claims.