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] Disclosed is 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 embodiments 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] Disclosed is 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 embodiments 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] Disclosed is 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.

[0009a] In one aspect, there is provided 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 a polynucleotide composition 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;

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.

[0009b] In another aspect, there is provided a whole seed or part of a seed comprising a genetic alteration in its genome, provided by the method as described herein, or a plant grown or regenerated from the whole seed or part of a seed, wherein the plant comprises cells comprising the genetic alteration in

their genome and optionally is grown directly from the whole seed or part of a seed without a callus stage.

[0009c] In another aspect, there is provided a method of effecting a genetic alteration in the genome of embryonic plant tissue, comprising bombardment of the embryonic plant tissue with a polynucleotide composition comprising a particle complexed with a ribonucleoprotein (RNP) comprising an RNA-guided nuclease and at least one guide RNA (gRNA) having a nucleotide sequence designed to alter a target nucleotide sequence in the genome, thereby effecting the genetic alteration.

[0009d] In another aspect, there is provided 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 comprising:

(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 comprises at least one treatment selected from the group consisting of: microinjection and mechanical cell wall breakage; and wherein the non-epidermal plant cell, or the plant or plant part in which the non-epidermal plant cell is located, is subjected to heating or heat stress;

whereby the gRNA is delivered to the non-epidermal plant cell and the target nucleotide sequence in the non-epidermal plant cell is altered.

[0009e] In another aspect, there is provided a non-epidermal plant cell comprising an altered target nucleotide sequence, provided by the method as described herein, or a plant grown or regenerated from the non-epidermal plant cell comprising an altered target nucleotide sequence, wherein the plant comprises cells having the altered target nucleotide sequence, or seed or plant parts of the plant.

[0009f] In another aspect, there is provided the doubled haploid cell that is homozygous for the altered target nucleotide sequence, provided by the method as described herein.

[0009g] In another aspect, there is provided the regenerated doubled haploid plant provided by the method as described herein, or seed or plant parts of the regenerated doubled haploid plant, or a hybrid plant having at least one parent plant that is the regenerated doubled haploid plant.

[0009h] In another aspect, there is provided a plant grown or regenerated from the embryonic plant tissue having the genetic alteration in its genome provided by the method as described herein, or a seed of the plant, wherein the plant or seed comprises cells comprising the genetic alteration in their genome and wherein the plant optionally is grown directly from the whole seed or part of a seed without a callus stage.

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-

What is claimed:

1. 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 a polynucleotide composition 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;

   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.

2. A whole seed or part of a seed comprising a genetic alteration in its genome, provided by the method of claim 1, or a plant grown or regenerated from the whole seed or part of a seed, wherein the plant comprises cells comprising the genetic alteration in their genome and optionally is grown directly from the whole seed or part of a seed without a callus stage.

3. A method of effecting a genetic alteration in the genome of embryonic plant tissue, comprising bombardment of the embryonic plant tissue with a polynucleotide composition comprising a particle complexed with a ribonucleoprotein (RNP) comprising an RNA-guided nuclease and at least one guide RNA (gRNA) having a nucleotide sequence designed to alter a target nucleotide sequence in the genome, thereby effecting the genetic alteration.

4. 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 comprising:

      (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 comprises at least one treatment selected from the group consisting of: microinjection and mechanical cell wall breakage; and wherein the non-epidermal plant cell, or the plant or plant part in which the non-epidermal plant cell is located, is subjected to heating or heat stress;

   whereby the gRNA is delivered to the non-epidermal plant cell and the target nucleotide sequence in the non-epidermal plant cell is altered.

5. The method of claim 4, 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.

6. The method of claim 4, wherein:
   (a) the polynucleotide composition optionally comprises 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 comprises an RNA-guided nuclease or a polynucleotide that encodes the RNA-guided nuclease.
7. The method of claims 1, 3, or 4, wherein
   (a) the polynucleotide composition further comprises a chemical agent or a physical agent or a combination of both chemical and physical agents, or
   (b) the method further comprises 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.
8. The method of claim 4, 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 comprises 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.
9. The method of claim 4, 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 comprises: (a) double-stranded RNA, (b) single-stranded RNA; (c) chemically modified RNA; (d) a combination of (a) – (c).
10. The method of claim 6, wherein the RNA-guided nuclease or polynucleotide that encodes the RNA-guided nuclease is provided:
    (a) as a ribonucleoprotein complex comprising the crRNA and the RNA-guided nuclease;

(b) as a complex comprising 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 comprising 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;

(f) using a combination of any of (a) – (e).

11. The method of claim 6, 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.

12. The method of claim 4, wherein the polynucleotide composition comprises 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.

13. A non-epidermal plant cell comprising an altered target nucleotide sequence, provided by the method of claim 4, or a plant grown or regenerated from the non-epidermal plant cell comprising an altered target nucleotide sequence, wherein the plant comprises cells having the altered target nucleotide sequence, or seed or plant parts of the plant.

14. The method of claim 4, wherein the non-epidermal plant cell is haploid, wherein the method further comprises the step of chromosome doubling in the non-epidermal plant cell comprising the altered target nucleotide sequence to produce a doubled haploid cell that is homozygous for the altered target nucleotide sequence.

15. The doubled haploid cell that is homozygous for the altered target nucleotide sequence, provided by the method of 14.

16. The method of claim 14, further comprising regeneration of a doubled haploid plant from the doubled haploid cell, wherein the regenerated doubled haploid plant comprises cells that are homozygous for the altered target nucleotide sequence.

17. The regenerated doubled haploid plant provided by the method of claim 16, or seed or plant parts of the regenerated doubled haploid plant, or a hybrid plant having at least one parent plant that is the regenerated doubled haploid plant.

18. The method of any of claims 1, 3, or 6, 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.
19. The method of claims 1, 3, or 4, wherein the genetic alteration is heritable to succeeding generations.
20. A plant grown or regenerated from the embryonic plant tissue having the genetic alteration in its genome provided by the method of claim 3, or a seed of the plant, wherein the plant or seed comprises cells comprising the genetic alteration in their genome and wherein the plant optionally is grown directly from the whole seed or part of a seed without a callus stage.