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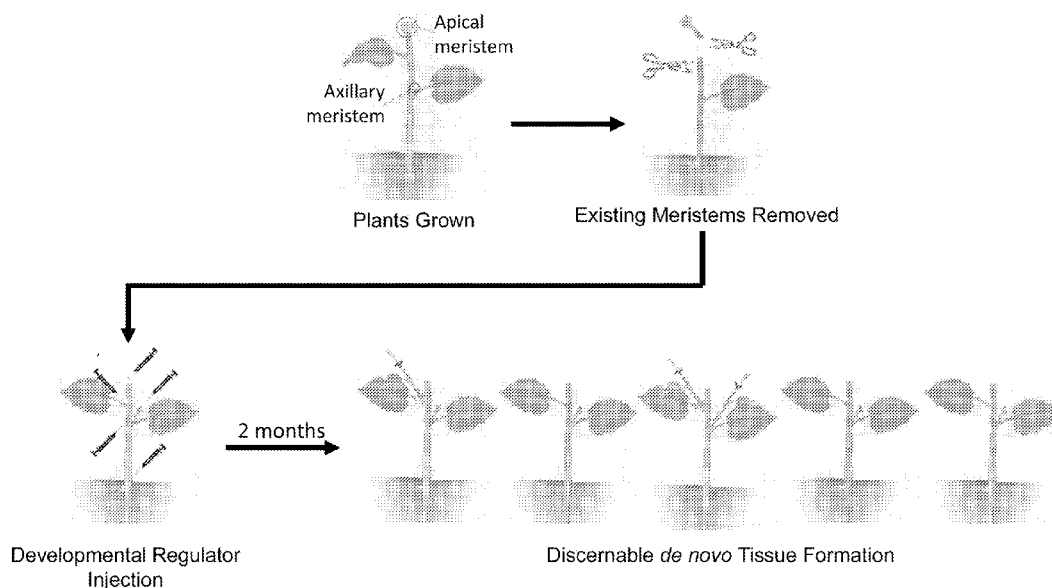
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(54) Title: DELIVERY OF DEVELOPMENTAL REGULATORS TO PLANTS FOR THE INDUCTION OF MERISTEMATIC TISSUE WITH GENETIC ALTERATIONS

FIG. 1



(57) Abstract: Materials and methods for inducing genetic alterations in meristematic plant tissue are provided herein.

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DELIVERY OF DEVELOPMENTAL REGULATORS TO PLANTS FOR THE INDUCTION OF MERISTEMATIC TISSUE WITH GENETIC ALTERATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 62/690,165, filed June 26, 2018. The disclosure of the prior application is considered part
5 of (and is incorporated by reference in) the disclosure of this application.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

This invention was made with government support under IOS-1339209 awarded
by the National Science Foundation. The government has certain rights in the invention.

TECHNICAL FIELD

This document relates to materials and methods for inducing genetic alterations in
meristematic plant tissue.

BACKGROUND

The ability to generate plants with a desired genetic makeup, whether
commercially or for basic research, is limited by two facets – the delivery of genetic
engineering (GE) reagents and the subsequent generation of edited tissues. Procedures for
delivery of GE reagents typically utilize the gene transferring bacterium *Agrobacterium*
20 *tumefaciens*, or physical means such as particle bombardment. After delivery of reagents,
many protocols require subsequent regeneration of the edited somatic tissue into whole
plants. Transformed somatic tissue often is pushed to de-differentiate (into callus) and
then re-differentiate using tissue culture media containing specific hormone ratios
required to drive shoot or root formation. Tissue culture techniques can be complicated
25 by the fact that a majority of agriculturally relevant crop lines are recalcitrant to tissue
culture regeneration. Additionally, this process can be time consuming and technically
demanding, requires sterile conditions, and may be subject to undesired genetic
alterations due to prolonged exposure to hormones. Efforts also may be confounded

because resulting tissues can present non-specific chimerism for the transgene, requiring more than one generation to fix the genetic event of interest. All of these limitations render current practices for plant genetic engineering non-ideal for large scale generation of edited plants in a high throughput manner.

5 Developmental regulatory genes have been employed to generate tissues with modifications of interest. For example, controlled expression of the maize regulatory genes WUSCHEL (WUS) and BABY BOOM (BBM) induced somatic embryogenesis from immature maize embryos, as well as other somatic tissues (Lowe et al., *Plant Cell* 28(9):1998-2015, 2016; and Mookkan et al., *Plant Cell Reports* 36(9):1477-1491, 2017).
10 Hundreds of plantlets were recovered from these somatic embryos even in lines previously recalcitrant to tissue culture, and the generated somatic embryos were capable of developing into full plants with the transgene cassette of interest. Despite this improvement to standard monocot regeneration practices, however, the generation of full plants still required tissue culture steps, sterile technique, and explant handling.
15 In addition, these techniques were only demonstrated in monocot species and have not been implemented in any dicot species.

SUMMARY

20 This document provides new techniques that avoid the constraints noted above and provide a boon for both basic research and commercial germplasm production. The combination of developmental regulators WUS and SHOOT MERISTEMLESS (STM), as well as other regulator combinations, can have an impact on patterning and formation of shoot meristems (Gallois et al., *Development* 129:3207-3217, 2002), and these developmental regulators have been ectopically expressed in *Arabidopsis thaliana* and
25 other species to create meristem-like tissue. The methods described herein are based, at least in part, on the discovery that such genes can be combined with GE reagents to promote the formation of edited meristematic tissue that can flower and produce seed. The resultant seed is derived from a single meristematic cell, and therefore represents a clonal genetic editing event that provides an abundance of edited seed after one
30 generation. The direct delivery method described herein also provides the advantage of

avoiding tissue culture, which reduces the time needed to regenerate tissues and considerably simplifies the process of generating GE events. Thus, the methods described herein can circumvent the limitations of current plant regeneration protocols, and greatly enhance the potential for development of GE plant lines for both commercial use and basic research.

Thus, this document is based, at least in part, on the development of methods for delivery of developmental regulators to whole plants to induce the transdifferentiation of somatic plant cells *in vivo* for the production of meristems. These meristems can carry transgenic insertions or genetic editing events to the next generation, creating seed with a GE event of interest in a fraction of the time needed using current standard protocols. The techniques described herein can simplify protocols for transformation, remove requirements for tissue culture, and be accessible to labs with diverse skill sets.

In a first aspect, this document features a method for generating plant tissue having one or more genetic modifications of interest. The method can include (a) introducing into plant cells (i) nucleic acid encoding one or more developmental regulators that, when expressed in the plant cells, induce meristem formation from the plant cells, and (ii) nucleic acid comprising one or more sequences that, when expressed, modify a plant cell to achieve one or more genetic modifications of interest; and (b) deriving *de novo* tissue from plant cells identified as having the one or more genetic modifications of interest. The one or more developmental regulators can include one or more of Baby Boom, Isopentenyl Transferase, Irrepressible Variants of Monopteros, Shoot Meristemless, and Wuschel. The introducing can be by Agrobacterium, and the nucleic acid encoding one or more developmental regulators and the nucleic acid comprising one or more sequences that modify a plant cell can be included on the same T-DNA or on separate T-DNAs. The method can include introducing nucleic acid encoding two or more developmental regulators into the plant cells by Agrobacterium, where the two or more developmental regulators are encoded by a single T-DNA or are encoded by separate T-DNAs. The method can include introducing nucleic acid encoding two or more developmental regulators into the plant cells by Agrobacterium, where the two or more developmental regulators are encoded by two or more strains of

Agrobacterium. The introducing can include electroporation, biolistics, particle bombardment, chemical transfection, nanoparticle delivery, or viral infection. The introducing can include transient transformation or stable transgenesis. The plant cells into which the nucleic acids are introduced can be within a differentiated tissue, within an undifferentiated tissue, within a whole plant, within a germinating seedling, or within a plant part taken from a plant. The plant cells can be cells of a monocotyledonous plant, or cells of a dicotyledonous plant. The one or more sequences that modify a plant cell can include a transgene that, when expressed in the plant cells, achieves an agriculturally relevant trait (e.g., herbicide tolerance). The one or more sequences that modify a plant cell can include a transgene that, when expressed, edits the plant DNA. For example, the one or more sequences that modify a plant cell can include a nucleotide sequence encoding a targeted endonuclease (e.g., a meganuclease, zinc finger nuclease, transcription activator-like effector nuclease, or Clustered Regularly-Interspaced Short Palindromic Repeats-associated nuclease). The one or more sequences that modify a plant cell can encode a targeted enzyme that modifies plant DNA (e.g., a cytosine deaminase or an adenosine deaminase, such as BE3 or ABE). The one or more sequences that modify a plant cell can encode a targeted endonuclease and can include a repair template to introduce one or more specific modifications into the plant genome. The *de novo* tissue can be meristematic and can be capable of deriving new tissue carrying the one or more genetic modifications of interest. The new tissue can include a branch, a flower, or a root.

In some cases, the method can include (a) using Agrobacterium, introducing into cells of a germinating seedling or a portion thereof nucleic acid encoding the one or more developmental regulators, wherein expression of the one or more developmental regulators induces meristem formation in the germinating seedling or portion thereof; (b) introducing into the cells, via the Agrobacterium, the nucleic acid comprising one or more sequences that, when expressed, modify the cells to achieve the one or more genetic modifications of interest; and (c) culturing the meristem induced by the one or more developmental regulators, to obtain modified plant tissue comprising the one or more genetic modifications of interest. The method can include introducing nucleic acid

encoding two or more developmental regulators, wherein the two or more developmental regulators are encoded by one T-DNA or by separate T-DNAs. The method can include introducing nucleic acid encoding two or more developmental regulators, where the two or more developmental regulators are encoded by separate strains of *Agrobacterium*. The germinating seedling or portion thereof can be from a monocotyledonous plant or from a dicotyledonous plant. The one or more genetic modifications can include insertion of a transgene that, when expressed, achieves an agriculturally relevant trait (e.g., herbicide tolerance). The one or more genetic modifications can include insertion of a transgene that, when expressed, edits the plant cell DNA. The nucleic acid that modifies a plant cell can encode a targeted endonuclease (e.g., a meganuclease, zinc finger nuclease, transcription activator-like effector nuclease, or Clustered Regularly-Interspaced Short Palindromic Repeats-associated nuclease). The nucleic acid that modifies a plant cell can encode a targeted enzyme that modifies plant DNA (e.g., a cytosine deaminase or an adenosine deaminase, such as BE3 or ABE). The nucleic acid that modifies a plant cell can encode a targeted endonuclease and can include a repair template to introduce a specific modification into the genetic material of the plant cell. The method can further include assaying the meristem induced by the one or more developmental regulators for the one or more genetic modifications of interest, and subsequently generating a whole plant from the meristem induced by the one or more developmental regulators. The method also can include placing the meristem induced by the one or more developmental regulators directly into culture and inducing the meristem in culture to form a plant.

In another aspect, this document features a method for generating plant tissue containing one or more genetic modifications of interest. The method can include (a) using *Agrobacterium*, introducing into cells of a germinating seedling or a portion thereof nucleic acid encoding one or more developmental regulators, wherein expression of the one or more developmental regulators induces meristem formation in the germinating seedling or portion thereof; (b) simultaneously introducing into the cells, via the *Agrobacterium*, nucleic acid that modifies genetic material within the cells to achieve one or more targeted genetic modifications of interest; and (c) culturing the meristem induced by the one or more developmental regulators, to obtain modified plant tissue containing

the one or more genetic modifications of interest. The one or more developmental regulators can include, for example, one or more of Baby Boom, Isopentenyl Transferase, Irrepressible Variants of Monopteros, Shoot Meristemless, and Wuschel. The method can include introducing two or more developmental regulators into cells of the germinating seedling or portion thereof, where the two or more developmental regulators are encoded by one T-DNA, or where the two or more developmental regulators are encoded on separate T-DNAs. The method can include introducing two or more developmental regulators into cells of the germinating seedling or portion thereof, where the two or more developmental regulators are encoded by two or more strains of Agrobacterium.

The germinating seedling or portion thereof can be from a monocotyledonous plant or from a dicotyledonous plant. The one or more targeted genetic modifications can include insertion of a transgene that, when expressed, achieves an agriculturally relevant trait (e.g., herbicide tolerance). The one or more targeted genetic modifications can include insertion of a transgene that, when expressed, edits the plant cell DNA. The nucleic acid that modifies a plant cell can encode a targeted endonuclease, such as a meganuclease, zinc finger nuclease (ZFN), transcription activator-like effector (TALE) nuclease, or clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) nuclease. The nucleic acid that modifies a plant cell can encode a targeted enzyme that modifies plant DNA. The targeted enzyme can be a cytosine deaminase or an adenosine deaminase (e.g., BE3 or ABE). The nucleic acid that modifies a plant cell can encode a targeted endonuclease and can contain a repair template to introduce a specific modification into the genetic material of the plant cell. The method can further include assaying meristem induced by the one or more developmental regulators for the one or more genetic modifications of interest, and subsequently generating a whole plant from the meristem induced by the one or more developmental regulators. The method can further include placing the meristem induced by the one or more developmental regulators directly into culture and inducing the meristem in culture to form a plant.

In another aspect, this document features a method for generating plant tissue containing one or more genetic modifications of interest. The method can include (a)

introducing into plant cells (i) nucleic acid encoding one or more developmental regulators that, when expressed in the plant cells, induce meristem formation from the plant cells, and (ii) nucleic acid comprising one or more sequences that modify a plant cell to achieve one or more genetic modifications of interest; and (b) deriving *de novo* tissue from plant cells identified as having the one or more genetic modifications of interest. The one or more developmental regulators can include one or more of Baby Boom, Isopentenyl Transferase, Irrepressible Variants of Monopteros, Shoot Meristemless, and Wuschel. The introducing can be by Agrobacterium, and the nucleic acid encoding one or more developmental regulators and the nucleic acid comprising one or more sequences that modify a plant cell can be included on the same T-DNA.

Alternatively, the nucleic acid encoding one or more developmental regulators and the nucleic acid comprising one or more sequences that modify a plant cell can be included on separate T-DNAs. The method can include introducing nucleic acid encoding two or more developmental regulators into the plant cells by Agrobacterium, where the two or more developmental regulators are encoded by a single T-DNA, or where the two or more developmental regulators are encoded by separate T-DNAs. The method can include introducing nucleic acid encoding two or more developmental regulators into the plant cells by Agrobacterium, where the two or more developmental regulators are encoded by two or more strains of Agrobacterium. The introducing can include electroporation, nanoparticle delivery, biolistics, particle bombardment, chemical transfection, or viral infection. The method can include transient delivery of the one or more developmental regulators or stable integration of genes encoding the one or more developmental regulators into the plant genome by any of the above means of delivery.

The plant cells into which the nucleic acids are introduced can be within a differentiated tissue, or within an undifferentiated tissue. The plant cells into which the nucleic acids are introduced can be within a whole plant, or within a plant part taken from a plant. The plant cells can be of a monocotyledonous plant or a dicotyledonous plant.

The one or more sequences that modify a plant cell can include a transgene that, when expressed in the plant cells, achieves an agriculturally relevant trait (e.g., herbicide tolerance). The one or more sequences that modify a plant cell can include a transgene

that, when expressed, edits the plant DNA. The one or more sequences that modify a plant cell can include a nucleotide sequence encoding a targeted endonuclease, such as a meganuclease, ZFN, TALE nuclease, or CRISPR/Cas nuclease. The one or more sequences that modify a plant cell can encode a targeted enzyme that modifies plant DNA (e.g., a cytosine deaminase or an adenosine deaminase, such as BE3 or ABE). The one or more sequences that modify a plant cell can encode a targeted endonuclease and can include a repair template to introduce one or more specific modifications into the plant genome. The *de novo* tissue can be meristematic and capable of deriving new tissue carrying the one or more genetic modifications of interest. The new tissue can include a branch, a flower, or a root.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a diagram depicting an experimental procedure for a direct injection method as provided herein. Plants can be grown to a developmental stage at which axillary shoot meristems are visualized. Removal of existing shoot meristem tissue (shoot apical meristem and axillary meristems) can be performed to increase the potential for *de novo* shoot formation. Reagents to create a genetic change and to stimulate growth of *de novo* tissue can be delivered to plant tissues by various means, including direct injection

as depicted in FIG. 1. The delivered reagents can result in induction of *de novo* shoots, as well as other somatic tissues, and the newly formed tissue(s) can contain the genetic change of interest.

FIGS. 2A-2F show the generation of *de novo* tissues from injection sites.

5 Reagents for the induction of *de novo* shoot meristems were delivered to *Nicotiana benthamiana* as outlined in FIG. 1. For this experiment, *Agrobacterium* was injected into somatic tissues. The delivered T-DNA contained a geminiviral replicon (GVR) designed to circularize and replicate within a plant cell. The replicon contained a luciferase reporter (SEQ ID NO:14) for visualization of vector presence, as well as the
10 developmental regulators WUS (SEQ ID NO:6) and STM (SEQ ID NO:7). FIGS. 2A, 2B, 2C, and 2D are representative of induced *de novo* growth observed for multiple plants. FIG. 2E and 2F are images providing visual confirmation that in some cases, newly formed tissues contained the reporter gene, as evidenced by imaging in bright field (FIG. 2E) and bioluminescence (FIG. 2F).

15 FIGS. 3A to 3G show the results of molecular analyses to assess the delivery of developmental regulators and editing of *de novo* tissue. *Agrobacterium* was used to deliver a T-DNA containing sequences encoding developmental regulators, a luciferase reporter gene, and a guide RNA (gRNA) targeting an endogenous site in the genome of *N. benthamiana* plants transgenic for 35S:Cas9. The *Agrobacterium* solution was
20 delivered to somatic tissues by injection (FIGS. 3C and 3F; injection sites are indicated by boxes). Sample tissues were harvested at distal sites of tissue generated from the injection sites (FIGS. 3C and 3F; harvest sites are indicated by circles). Tissue samples were treated with luciferin and imaged for bioluminescence (FIGS. 3A and 3D, bright field; FIGS. 3B and 3E, luminescence). Genomic DNA was isolated from the harvested
25 tissue by cetyl trimethylammonium bromide (CTAB) extraction, and the genomic region targeted by the gRNA was amplified by PCR and subjected to *NcoI* endonuclease digestion prior to electrophoresis (FIG. 3G). The Cas9 endonuclease could induce mutations to destroy the *NcoI* site at the gRNA target. Thus, the WT “un-edited” genomic sequence produced a lower molecular weight band upon *NcoI* digestion (FIG. 3G, solid
30 arrow), while undigested “edited” DNA produces a higher molecular weight band

(dashed arrow). Samples 114-2 and 155-1 showed complete cutting (lanes 3 and 5) and therefore do not have induced mutations. Samples 114-1 and 115-2 (lanes 1 and 7) demonstrated incomplete cutting by *NcoI*, denoting gRNA-induced mutations. The positive control (Pos) was obtained by leaf infiltration followed by leaf DNA isolation 7 days post infection.

FIGS. 4A to 4G show that edited shoots are discernable by phenotype due to targeted editing. Transgenic *N. benthamiana* plants expressing 35S:Cas9 were exposed to developmental regulators to induce *de novo* tissue formation. The developmental regulators were delivered either as a coinfection of *Agrobacterium* strains containing WUS or STM on separate T-DNA vectors (FIG. 4A) or as a single T-DNA vector containing WUS and IPT (FIG. 4B). All vectors included a gRNA targeting both NbPDS homologs present in the genome (SEQ ID NOS:16 and 17). A PDS knock-out phenotype was observed as white sectoring (FIG. 4A) or as completely white *de novo* meristems (FIGS. 4B and 4C) formed from the injection site. To molecularly characterize targeted mutation of both PDS homologs, genomic DNA was isolated from select tissues (white arrows), and was amplified by PCR and visualized for induced mutations at the gRNA target sites. As two homologs of the NbPDS gene exist in the *N. benthamiana* genome, primers were used that selectively amplify either homolog 1 (Niben101Scf14708g00023.1; FIG. 4D) or homolog 2 (Niben101Scf01283g02002.1; FIG. 4E). Incomplete digestion of PCR amplicons by the restriction enzyme *NcoI* denoted a mutation within the restriction recognition site. Amplification of Scf14708g00023.1 provided a band of 712 bp, while a *NcoI* digested band was visible at 648 bp. Amplification of Scf01283g02002.1 provided a band of 755 bp, and a corresponding cleaved 691 bp band after *NcoI* digest. Tissue samples assayed for the split vector system were completely modified at the Scf14708g00023.1 locus as observed by undigested restriction bands in the digest assay (FIG. 4D, lane 2), but appeared unedited (completely digested) at the Scf01283g02002.1 locus (FIG. 4E, lane 2). This pattern was reversed for the combination vector. Positive control ("Editing Cont.") was acquired by infiltrating vectors into leaf tissue and harvesting DNA 7 days post infection, and was expected to demonstrate chimerism (both cleaved and uncleaved bands; FIGS. 4D and

4E, lane 6). Negative control (“Digest Cont.”) utilized genomic DNA isolated from an uninoculated plant (FIGS. 4D and 4E, lane 8). While restriction digest assays did not show editing for some samples (FIG. 4D, lane 4; FIG. 4E, lane 2), Illumina sequencing performed on genomic amplifications of Scf14708g00023.1 validated the presence of genomic edits when WUS or STM were delivered on separate T-DNA vectors (FIG. 4F; from top to bottom, SEQ ID NOS:38, 39, 40, and 38) or when WUS and IPT were delivered via a single T-DNA vector (FIG. 4G; from top to bottom, SEQ ID NOS:38, 40, and 39). The frequency of edits inferred the tissues present were non-chimeric as compared to negative controls. In addition, edits that could not be visualized by restriction digest were confirmed as edited for both alleles of both homologs of PDS (targeted mutations involved a ‘C’ insertion that did not destroy the CCATGG *NcoI* site).

FIGS. 5A and 5B further demonstrate the ability to create targeted mutations in the *N. benthamiana* PDS2 locus (SEQ ID NO:17). Constructs introduced included a single vector containing WUS and STM (WUS/STM, SEQ ID NO:30) a single vector containing WUS and IPT (WUS/IPT; SEQ ID NO:31) a combination of co-inoculated vectors each containing a single DR (STM, IPT, WUS, MPA, and BBM; SEQ ID NOS:33, 34, 35, 36, and 37, respectively). FIG. 5A is a table providing results from these studies. Derived shoots were given individual designators to facilitate tracking of samples (FIG. 5A, column 2). Purified genomic DNA was amplified for the PDS2 locus.

Amplicons were pooled and submitted for next generation sequencing. The observed number of sequences containing the expected forward and reverse barcodes (column 3), the number of sequences observed to have non-specific DNA insertions at the sgRNA target site (column 4), the number of sequences observed to have targeted mutations at the sgRNA target site (column 5), and the total number of sequences that were observed to have targeted modifications (column 6) are provided. Mutations observed at a frequency >30% of the total are denoted in column 7. “Seed produced” (column 8) indicates whether sampled shoots were identified that produced seed. It was observed that one sample (5-14-1-08) produced seed capable of transmitting genomic modifications to the next generation. FIG. 5B shows the sequences of mutations observed by next generation sequencing for plants listed in FIG. 5A.

FIG. 6 is a graph plotting results from an injection study in *N. benthamiana*. Constructs introduced included WUS and IPT as either a single vector (WUS/IPT; SEQ ID NO:31), or a combination of co-inoculated vectors each containing a single DR (SEQ ID NOS:34 and 35), or a vector containing editing reagents but no DRs (No Dev Reg, SEQ ID NO:98). The total number of sites injected, as well as the number of shooting sites, was monitored across all plants within each group. Groups were additionally observed for the number of shoots with distorted morphology, likely induced by developmental regulators, and photobleaching due to simultaneous targeted mutations in both PDS homologs.

FIG. 7 is a representative image of an induced chimeric shoot with a WT growth pattern and sectored green and photobleached tissue, both of which produced seed (inner two circles). Below the image are the genotypes of the parental green and white tissues (outer two circles) observed by Tracking of Indels by Decomposition (TIDE) analysis of Sanger sequencing and phenotypes of the resulting progeny (*see*, also, FIGS. 8A and 10A for the resulting genotypes of individual seedlings). The -48bp mutation constituted an in-frame deletion that may have maintained a functional PDS protein, retaining the green phenotype for green sectors.

FIG. 8A is a table listing observed mutations for white tissues shown in FIG. 7 and for the resulting progeny. Genomic DNA was extracted from parental tissue and seedlings and submitted for Sanger sequencing. Sequences were assessed for mutations by TIDE sequence trace analysis. FIG. 8B is an image showing seedlings exhibiting the PDS KO phenotype.

FIG. 9 is a pair of images confirming that the plants shown FIGS. 7 and 8B were transgene-free. Genomic DNA (gDNA) was extracted from parental white (ParW) and green (ParG) tissues of a plant demonstrating targeted editing (shown in FIG. 7), as well as from ten seedlings derived from the white flower (S1-S10; FIG. 8A). Genomic DNA also was extracted from plants that did not receive the vector (Neg Ctrl1 and Neg Ctrl2), as well as from leaf tissue infiltrated with the target vector (Pos Ctrl). DNA was amplified using primers specific to the U6 promoter present on the T-DNA (expected 448 bp). Ladder = NEB 2log.

FIG. 10A is a table listing observed mutations for green tissues shown in FIG. 7 and the resulting progeny. Genomic DNA was extracted from parental tissue and seedlings and submitted for Sanger sequencing. Sequences were assessed for mutations by TIDE sequence trace analysis. FIG. 10B is an image demonstrating segregation of the PDS KO phenotype in a 3:1 ratio observed for the seedlings listed in FIG. 8A.

FIG. 11 is a table listing observed phenotypes and genotypes for PDS mutations in a T0 plant (plant designator 5-14-1-08; FIGS. 5A and 5B) induced to form meristems and next generation progeny. The parent plant was trimmed and co-inoculated with agrobacterium strains individually carrying T-DNAs harboring DRs STM (pMM131; SEQ ID NO:33), IPT (pMM134; SEQ ID NO:34), WUS (pMM135; SEQ ID NO:35), MPA (pMM136; SEQ ID NO:36), or BBM (pMM146; SEQ ID NO:37). Resulting induced *de novo* shoots were screened for PDS phenotype and genotype. Shoots were allowed to flower and set seed. Next generation progeny seed was germinated and assessed for phenotype and genotype. FIG. 11 provides information for parental shoot tissue from 5-14-1-8 and ten screened seedlings. The parent tissue was observed to harbor a -3bp mutation in PDS1 in a heterozygous state. Progeny from this event were observed to segregate this -3bp mutation.

FIGS. 12A-12I depict induced transgenic shooting for vectors containing a luciferase reporter in *Solanum tuberosum* (potato) and *Vitis vinifera* (grape). Agrobacterium strains expressing either IPT (FIGS. 12A, 12B, and 12C; SEQ ID NO:96) or co-expressing WUS and IPT (FIGS. 12D, 12E, 12F, and 12G; SEQ ID NO:94) were delivered to potato plants along with a luciferase expression cassette (SEQ ID NO:14). FIGS. 12A and 12D show abnormal shooting phenotypes induced by DRs 95 days post infection (p.i.). Transgenic shoot formation was confirmed by imaging bioluminescence of *de novo* shoots formed after injection (FIGS. 12B, 12C, 12E, 12F, and 12G) at 97 days p.i. Fully transgenic shoots were detected upon trimming away several wild-type shoots (FIGS. 12C and 12G), signifying stable T-DNA integration in those induced shoots (highlighted in FIG. 12C by white arrowheads). FIGS. 12H and 12I demonstrate that delivery of DRs to grape plants (Pixie Pinnot cultivar) induced transgenic shoot formation with normal growth phenotypes. FIG. 12H is an image showing an exemplary

grape plant 40 days after co-inoculation of agrobacterium strains individually carrying T-DNA vectors with DRs (WUS, IPT, MPΔ, STM, and BBM; SEQ ID NOs:88, 89, 90, 91, and 92, respectively). Newly formed shoots were generated at Agrobacterium inoculation sites. FIG. 12I shows that newly formed shoots removed from the grape plant of FIG.

12H panel were transgenic, as determined by a bioluminescence assay. Shoots were removed, exposed to luciferin substrate, and imaged for bioluminescence. The presence of bioluminescent-positive tissues indicated that the newly formed tissues were transgenic and expressed the luciferase reporter.

FIG. 13 is a diagram showing the steps of a Fast-TrACC delivery method as provided herein. As depicted, Fast-TrACC delivery involves three days of treatment with an *Agrobacterium tumefaciens* culture of interest, where the Agrobacteria contain one or more T-DNAs that encode one, two, or more developmental regulators, and also contain nucleic acid sequences that can result in a genetic change within a plant. The T-DNA also can include a reporter such as a luciferase gene. In the first step, Agrobacteria cultures are grown overnight to achieve confluency. Next, cultures are resuspended in AB:MES salts to increase the expression of *vir* genes, and grown overnight again. The final treatment involves combining AB:MES salts with plant growth media (1/2 MS) to promote the activity of both the bacteria and the seedlings being co-cultured. After the final treatment, the Agrobacteria are combined with seedlings roughly 2-3 days post germination. The co-culture is incubated for two days before the seedlings are washed free of the Agrobacteria. The washed seedlings are returned to liquid 1/2 MS containing the antibiotic Timentin to kill off any residual Agrobacteria. Using a reporter such as luciferase, seedlings can be analyzed for delivery of the T-DNA construct.

FIGS. 14A to 14D illustrate the generation of growths from sites of delivery for the Fast-TrACC method. T-DNAs with GVRs containing luciferase and the developmental regulators WUS and STM were incorporated into *N. benthamiana* seedlings. The sites of delivery were approximated by observing luminescence from the luciferase reporter (FIGS. 14A and 14B). *De novo* tissue growth occurred from the sites with the highest expression of luciferase (circles in FIGS. 14A and 14B). This presumably was due to high levels of developmental regulator expression coinciding with

the reporter expression. The *de novo* tissues that were generated developed into different tissue types. Many remained in an undifferentiated callus-like state (FIG. 14C, arrow), while others progressed into meristem-like tissues (FIG. 14D, circle). The meristem-like growths developed defined structures such as leaflets (FIG. 14D, arrows).

FIGS. 15A to 15E show regeneration of plantlets from developmental regulator derived shoots. Using the developmental regulator combination WUS and STM, shoot-like growths (FIGS. 15A and 15B) were formed on the cotyledons of *N. benthamiana* seedlings. These growths were transferred to auxin-rich rooting media to promote root system formation. Once full plantlets were generated, they were moved to soil (FIGS. 15C and 15D) to promote further growth. The generated plantlets exhibited a variety of whole plant phenotypes. For example, certain plantlets exhibited close to wild type appearance (FIG. 15D) while others had a far more disorganized phenotype (FIG. 15C). Three leaves from each of six generated plantlets were tested for expression of luciferase (FIG. 15E) from the original delivered T-DNA. Five of the six tested plantlets were luciferase positive in at least one leaf sample, indicating some chimeric level of transgene integration and maintenance.

FIGS. 16A to 16H show confirmation of editing occurring in generated growths. Using the Fast-TrACC method, gene editing reagents were delivered along with the developmental regulators WUS and STM to promote the formation of edited growths. Five out of twenty-four seedlings had growths (FIG. 16A) that were candidates for molecular analysis. Using primers in NbPDS (Niben101Scf14708g00023.1), the target locus was amplified and tested for edits via a RFLP assay (FIG. 16B). The growths also were tested for amplification of Rep (FIG. 16C) to indicate the presence of the T-DNA. The isolated DNA samples were submitted for next generation sequencing (NGS; FIGS. 16D to 16H) for the NbPDS locus of interest to determine the frequency and scope of edits made. A variety of different edits were detected at the target site, with a single cytosine insertion and a single base deletion being the most consistently observed edits. Sequences that likely were the product of PCR induced mutation (asterisks) also were observed; these are not likely to have been caused by the delivered editing reagents. FIG. 16D, top to bottom: SEQ ID NOS:38, 40, 41, 39, 42, 43, 44, 45, 46, 47, 48, and 49. FIG.

16E, top to bottom: SEQ ID NOS:38, 40, 39, 50, 51, N/A, 52, 45, N/A, 53, 43, 54, 41, 44, 55, 56, 57, and 58. FIG. 16F, top to bottom: SEQ ID NOS:38, 40, 44, 39, 59, N/A, 45, 49, 41, 60, 50, 48, 61, and 62. FIG. 16G, top to bottom: SEQ ID NOS:38, 40, 39, 44, 45, 42, 50, 63, 41, 64, 54, N/A, 65, and 66. FIG. 16H, top to bottom: SEQ ID NOS:38, 40, 39, 45, 67, 41, N/A, 49, 44, N/A, 50, 68, 69, 57, and 48. N/A, no sequence identifier if less than 10 nucleotides in length.

FIGS. 17A to 17I show results for mosaic plantlets derived from growths. Plantlets were regenerated from developmental regulator derived growths that received gene editing components (Cas9 and NbPDS gRNA). These plantlets exhibited a range of phenotypes, with individuals that appeared mostly wild type (FIGS. 17A and 17B) as well as individuals exhibiting abnormal leaf or branch phenotypes (FIG. 17C), or generally disorganized states. DNA isolated from leaves using CTAB was pooled and submitted for NGS sequencing at the NbPDS (Niben101Scf14708g00023.1) locus. The resulting spectrum of edited reads indicated that the derived plantlets were mosaics of different edits. The proportion of reads isolated that contained edits was fairly low (FIGS. 17D, 17E, and 17F), but clearly detectable. The most commonly induced mutations of those observed (FIGS. 17G, 17H, and 17I) were single base insertions and deletions, as expected. FIG. 17G, top to bottom: SEQ ID NOS:38, 38, 40, 39, 70, N/A, and 71. FIG. 17H, top to bottom: SEQ ID NOS:38, 38, 40, 39, 52, N/A, 47, 50, N/A, 44, 58, N/A, 72, 60, 73, 68, 45, 74, 48, and 75. FIG. 17I, top to bottom: SEQ ID NOS:38, 38, 40, 71, 39, 76, 51, N/A, 77, 45, 44, 54, 43, and 48. N/A, no sequence identifier if less than 10 nucleotides in length.

FIGS. 18A to 18D show vertical transmission of integrated T-DNA. Plants were derived after delivery of developmental regulators using the Fast-TrACC method. The original construct delivered to the progenitor plant via Agrobacterium contained 35S:Luciferase, Nos:WUS, and CmYLCV:STM on the T-DNA (pRN119, SEQ ID NO:27). Candidate plants (FIG. 18A) exhibiting high luciferase expression from leaf punches (FIG. 18B) were monitored for vertical transmission; after the plants flowered and set seed, the seeds themselves were tested for luminescence. The seedlings were found to maintain high levels of reporter expression (FIG. 18C), and luminescence was

observed in the positive seedlings at a Mendelian ratio, consistent with inheritance from a heterozygote (FIG. 18D, 119-P1-5-2).

FIG. 19A is a table listing characteristics for plants that received various combinations of DRs. To determine combinations of DRs that were most effective in creating *de novo* meristems, *A. tumefaciens* strains each carrying a single DR delivered individually or in various pools to *N. benthamiana* seedlings, which were monitored for *de novo* growth formation. Out of twelve tested options, five combinations produced *de novo* meristems and subsequent plants. The number of seedlings, growths, shoot-like growths, white shoot-like growths, and full plants for these five combinations are plotted in FIG. 19B.

FIG. 20 is an image showing a seedling that resulted from Fast-TrACC delivery of T-DNAs with developmental regulators and a gRNA that targeted the two homologs of phytoene desaturase (PDS1 and PDS2) to *N. benthamiana* seedlings constitutively expressing Cas9. When the alleles of both homologs are fully knocked-out, plants exhibited a white phenotype due to photobleaching. About 15% of the shoots were white, but could not be grown into full plants due to lack of chlorophyll.

FIG. 21A is an image showing green plants that were chimeric for edits at the PDS loci. Seedlings derived from some of these plants possessed the expected white phenotype (arrowheads). In plant 1-7, two separate flowers (designated as F4 and F6) produced white seedlings. Mutations were observed in the alleles of both PDS loci, and are listed in FIG. 21B. The sgRNA sequence is underlined, and the predicted cut site is represented as a vertical line.

FIGS. 22A-22J demonstrate results for tomato. To induce *de novo* meristems in tomato, combinations of developmental regulators that effectively induced meristems in *N. benthamiana* were tested (WUS+IPT and two combinations of WUS+STM). For both combinations of WUS and STM, no shoot-like growths were formed (FIGS. 21A and 21C), and substantial tissue necrosis was observed after delivery. In contrast, the combination of WUS and IPT promoted shoot-like growths (FIGS. 21A and 21C), which ultimately formed fully rooted plants (FIG. 21B). WUS and IPT were then delivered to tomato seedlings on either a single vector (WUS/IPT) or on separate vectors in two

different *Agrobacterium* strains (WUS&IPT). Both WUS/IPT and WUS&IPT showed an increase in the frequency of average growths per plant over the background level of growths that developed on plants that did not receive developmental regulators (FIGS. 22D and 22J). Shoot-like growths form from the WUS and IPT derived growths (FIGS. 22E and 22J), and luciferase positive, meristem-like structures were observed (FIG. 22F (boxed) and 22G, arrowhead). These structures progressed to form shoot-like growths (FIG. 22H) that were excised and assessed for luminescence (FIG. 22I). Four out of 15 shoot-like growths showed evidence of luminescence (FIGS. 22I and 22J).

DETAILED DESCRIPTION

A principle goal of GE techniques is the creation of an editing event in the germline of an organism so that the modification can be transmitted to the next generation. For plants, the germline is produced by reproductive tissues derived from the meristem, instead of from isolated gametophyte cells. Plant meristems are the developmental centers of the plant from which all ensuing plant growth is derived. If these stem cells are edited, all tissues subsequently derived from the meristem should contain the GE event(s) of interest, leading to transmission to the next generation. Direct modification of existing meristematic tissue has proven challenging, as it is a highly regulated tissue type that has historically been recalcitrant to genetic modification. This little understood barrier, among other factors, has necessitated the use of suboptimal tissue culture procedures for most agriculturally relevant crops.

The present document is based, at least in part, on the discovery that developmental regulators can be combined with GE reagents to promote the formation of edited meristematic tissue that can flower and produce seed. The methods described herein include steps for delivery of developmental regulators to whole plants to induce the transdifferentiation of somatic plant cells *in vivo*, leading to the production of meristems. These meristems can carry transgenic insertions or genetic editing events to the next generation, creating seed with a GE event of interest in a fraction of the time needed using current standard protocols. Because the seed is derived from a single meristematic cell, it represents a clonal genetic editing event that provides an abundance

of edited seed after a single generation. The direct delivery methods described herein also provide the advantage of avoiding tissue culture, which can reduce the time needed to regenerate tissues and considerably simplifies the process of generating GE events. Thus, the methods provided herein can circumvent the limitations of current plant regeneration protocols, and greatly enhance the potential for development of GE plant lines for both commercial use and basic research.

In some embodiments, this document provides methods in which plants can be grown to a desired stage in either sterile or non-sterile conditions (e.g., soil). In these methods, one or more developmental regulators can be delivered to select tissues, either by *Agrobacterium* or through ectopic means such as direct injection, electroporation, particle bombardment, biolistics, chemical transfection, viral infection, nanoparticle delivery, or any other suitable means for transient transfection or stable integration (exemplified in FIG. 1). In some cases, pre-existing meristems can be removed prior to delivery of the one or more developmental regulators. The tissues can be induced to produce meristematic tissues containing a GE event of interest. Importantly, this method can obviate the need for sterile tissue culture and advanced equipment, significantly reducing the cost and level of expertise necessary to carry out experiments.

Non-limiting examples of developmental regulators that can be used in the methods provided herein are listed in TABLE 1. As used herein, a “developmental regulator” (DR) is an agent (e.g., a transcription factor, an enzyme, or a hormone) that directs or influences a plant’s development, and may guide the differentiation of plant cells, organs, or tissues. For example, a DR can be a transcription factor (e.g., *Baby Boom*, *Irrepressible Variants of Monopteros*, *Shoot Meristemless*, or *Wuschel*) that can stimulate plant hormone biosynthesis or plant susceptibility to/sensing of cytokinins or other plant hormones that affect plant development and lead to *de novo* meristem development. In some cases, a DR can lead to increased cytokinin levels. Therefore, a DR also can be a means of increasing one or more cytokinins through ectopic application or through endogenous biogenesis, such as by increasing the expression of one or more enzymes involved in the synthesis of plant hormones. Thus, in some cases, a DR can be an enzyme involved in synthesis of plant hormones, such as *Isopentenyl Transferase*,

which is in the cytokinin biosynthesis pathway. Other examples of enzymes that can lead to increased cytokinin levels and may be useful as DRs include, without limitation, tRNA-isopentenyltransferase, cytochrome P450 monooxygenase, LONELY GUY, adenosine kinase, and adenine phosphoribosyltransferase. A nucleic acid encoding a DR also is considered to be a DR for the purposes of this document, since the nucleic acid can be delivered to plant cells (e.g., in a whole plant or plant part) in order to increase the level of the encoded DR. The DR coding sequence can be operably linked to a promoter (e.g., Nos, 35S, CmYLCV, AtUBQ10, or any other appropriate promoter) that drives expression of the DR in plant cells. Moreover, in some cases, a DR can be a means of increasing expression of genes downstream of the DRs listed in TABLE 1.

Thus, in some embodiments of the methods provided herein, one or more of the DRs listed in TABLE 1 can be delivered to a plant or a plant part.

TABLE 1
Developmental Regulators

Name	Abbreviation
Baby Boom	BBM
Isopentenyl Transferase	IPT
Irrepressible Variants of Monopteros	MPΔ
Shoot Meristemless	STM
Wuschel	WUS
Leafy Cotyledon 1	LEC1
Wound Induced Dedifferentiation 1	WIND1

Exemplary sequences for at least some of the above-referenced DRs and promoters are provided in the attached sequence listing. It is to be noted, however, that homologs of these DRs exist in numerous plant species, and the methods provided herein are not limited to use of the listed DRs or to DRs having 100% identity to the provided sequences. In some cases, for example, a DR coding sequence can have at least 80% (e.g., at least 85%, at least 90%, or at least 95%) identity to the WUS sequence set forth in SEQ ID NO:6, the STM sequence set forth in SEQ ID NO:7, the MPΔ sequence set forth in SEQ ID NO:8, the BBM sequence set forth in SEQ ID NO:9 or SEQ ID NO:10, or the IPT sequence set forth in SEQ ID NO:11. Further, in some cases, a DR can have an

amino acid sequence that is at least 80% (e.g., at least 85%, at least 90%, or at least 95%) identical to the WUS sequence set forth in SEQ ID NO:118, the STM sequence set forth in SEQ ID NO:119, the MPΔ sequence set forth in SEQ ID NO:120, the BBM sequence set forth in SEQ ID NO:121, or the IPT sequence set forth in SEQ ID NO:122.

5 The terms “percent identity” or “identity” in the context of two or more nucleic acids or polypeptides refer to two or more sequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same. The percent identity can be measured using sequence comparison software or algorithms or by visual inspection.

10 In general, percent sequence identity is calculated by determining the number of matched positions in aligned nucleic acid or polypeptide sequences, dividing the number of matched positions by the total number of aligned nucleotides or amino acids, respectively, and multiplying by 100. A matched position refers to a position in which identical nucleotides or amino acids occur at the same position in aligned sequences.

15 With regard to DR sequences, the total number of aligned nucleotides or amino acids refers to the minimum number of DR nucleotides or amino acids that are necessary to align the second sequence, and does not include alignment (e.g., forced alignment) with non-DR sequences. The total number of aligned nucleotides or amino acids may correspond to the entire DR sequence or may correspond to fragments of a full-length DR
20 sequence.

 Sequences can be aligned using the algorithm described by Altschul et al. (*Nucleic Acids Res*, 25:3389-3402, 1997) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches or alignments can be performed to determine percent sequence identity
25 between a DR nucleic acid or amino acid sequence and any other sequence or portion thereof using the Altschul et al. algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a NOTCH sequence and another
30 sequence, the default parameters of the respective programs are used.

This document also provides methods that are referred to herein as “Fast-TrACC” (Treated Agrobacterium Co-Culture) methods (exemplified in FIG. 13). Fast-TrACC methods involve the co-culture of germinating seedlings with treated Agrobacterium to deliver gene cassettes encoding developmental regulators. The developmental regulators can be broadly delivered to somatic cells in a variety of tissue types within the germinating seedlings. Cells expressing the developmental regulators, and their surrounding neighbors, can then be induced into a meristematic growth pattern that subsequently derives plant tissues of interest (e.g., shoots). In addition to the cassettes encoding the developmental regulators that promote shoot formation, one or more transgenes can be co-delivered. In some cases, the one or more transgenes themselves can create a GE event of interest. Alternatively, the transgene(s) can encode one or more gene editing reagents that can make precise alterations to the developing meristem, or to somatic cells prior to transdifferentiation, to create the desired GE event.

Both types of methods described herein can offer new and broadly applicable approaches to solve current bottlenecks in delivery of GE reagents, as well as the regeneration of tissues carrying GE events of interest. Inherently, *in vivo* delivery of developmental regulators provides a means to easily deliver reagents to somatic tissues, and also provides positive selection for cells receiving GE reagents. By inducing transdifferentiation of somatic cells and subsequent growth on existing tissues, the significant periods of time that otherwise would be necessary for regeneration and development of whole plant tissues can be avoided. Additionally, as these developmental regulators are evolutionarily conserved, these methods are amenable to use across a variety of species. Finally, the methods may avoid regulatory hurdles in the development of agricultural crops, as there is potential for transient delivery of reagents and subsequent recovery of non-transgenic progeny carrying a GE event of interest.

Thus, in some embodiments, this document provides methods for generating plant cells, plant parts, plant tissues, or plants that contain one or more genetic modifications of interest, where the methods can include removing existing meristems from a plant, and then introducing nucleic acids into cells of the remaining plant. The introduced nucleic acid sequences can (1) encode one or more (e.g., two, three, four, or more) developmental

regulators such as WUS, BBM, IPT, MPA, and/or STM to induce meristem formation, and (2) edit endogenous sequences within the plant cells, or encode polypeptides that act to edit endogenous sequences within the plant cells, to result in a genetic modification of interest. As a result of introducing these nucleic acid sequences, *de novo* tissue
5 subsequently derived from the plant can carry the genetic modification of interest. In some cases, the *de novo* tissue can be meristematic, and capable of deriving new tissue (e.g., branch, flower, or root tissue) carrying the genetic modification(s) of interest.

The nucleic acids provided to the plant (e.g., after removal of existing meristems) can be delivered by any suitable method, including by *Agrobacterium* – in which case the
10 developmental regulator(s) and the editing sequence(s) can be delivered on the same T-DNA or on separate T-DNAs. In some cases, the nucleic acids can be delivered by direct injection, electroporation, biolistics, nanoparticle delivery, particle bombardment, chemical transfection, viral infection, or any other useful method that can result in transient expression or stable integration of the delivered nucleic acid sequences. When
15 two or more developmental regulators are delivered by *Agrobacterium*, they can be present on the same T-DNA or on separate T-DNAs. In some cases, different strains of *Agrobacterium* can be used to deliver the developmental regulator(s) and the gene editing component(s). In addition, it is to be noted that the T-DNA(s) used in the methods provided herein can include any suitable replicon. In some cases, for example, a T-DNA
20 can include a viral replicon (e.g., a geminivirus replicon), which can include any appropriate virus component (e.g., RepA) to enable the generation of meristems.

In some embodiments, this document also provides methods for generating plant cells, plant parts, plant tissues, or plants that contain a genetic modifications of interest, where the methods include using *Agrobacterium* to introduce nucleic acids into
25 germinating seedlings. The nucleic acids can (1) encode one or more (e.g., two, three, four, or more) developmental regulators such as WUS, BBM, IPT, MPA, and/or STM to induce meristem formation in the germinating seedling, and (2) edit endogenous sequences within cells of the seedlings, or encode polypeptides that act to edit endogenous sequences within cells of the seedlings, to result in a genetic modification of
30 interest. The methods can further include culturing meristem generated as a result of

expression of the developmental regulator(s), to yield modified plant cells, plant tissue, plant parts, and/or plants that contain the genetic modification of interest.

The developmental regulator(s) and the editing nucleic acid(s) can be introduced into the seedling via the same T-DNA or via separate T-DNAs, or even via different strains of *Agrobacterium*. Similarly, when two or more developmental regulators are used, they can be introduced into the seedling via the same T-DNA, or via separate T-DNAs or different strains of *Agrobacterium*.

The methods provided herein can be used with monocotyledonous plants, plant cells, plant tissues, and plant parts (e.g., banana, grasses such as *Brachypodium distachyon*), wheat, oats, barley, maize, *Haynaldia villosa*, millet, palms, orchids, onions, pineapple, rice, rye, sorghum, and sugarcane) and dicotyledonous plants, plant cells, plant tissues, and plant parts (e.g., alfalfa, amaranth, *Arabidopsis*, beans, Brassica, carnations, chrysanthemums, citrus plants, coffee, cotton, eucalyptus, grape, impatiens, melons, peanuts, peas, peppers, Petunia, poplars, potatoes, rapeseed, roses, safflower, soybeans, squash, strawberry, sugar beets, sunflower, tobacco, tomatoes, and woody tree species).

In some cases, the methods provided herein can be used to obtain plants, plant tissues, plant parts, and plant cells having a desired trait, such as an agriculturally relevant trait. Agriculturally relevant traits can include, without limitation, herbicide tolerance, resistance to diseases and pests, growth rate, size, shape, color, and flavor of harvested products. For example, the methods provided herein can be used to insert a transgene into the genomic sequence of a plant cell, where expression of the transgene yields the desired trait. In some cases, expression of an inserted transgene can produce a polypeptide that edits the plant DNA. Examples of such polypeptides include targeted rare-cutting endonucleases (e.g., meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector (TALE) endonucleases, and RNA-guided endonucleases such as clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) endonucleases), as well as targeted cytosine or adenosine deaminases (e.g., apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-CRISPR/Cas fusions such as BE3, and ABE). Methods for making and using such targeted DNA modifying enzymes are described elsewhere. *See,*

e.g., Sander et al., *Nature Methods*, 8:67-69, 2011; Jacoby et al., *Nucl. Acids Res.*, 10.1093/nar/gkr1303, 2012); Christian et al., *Genetics*, 186:757-761, 2010; U.S. Publication No. 2011/0145940; Cong et al., *Science* 339:819-823, 2013; and Mali et al., *Science* 339:823-826, 2013. For example, CRISPR/Cas systems use RNA base pairing to
5 direct DNA or RNA cleavage by a Cas endonuclease. CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) sequences direct the Cas enzyme to a specific target DNA sequence (Makarova et al., *Nat Rev Microbiol*, 9(6):467-477, 2011). The modification of a single targeting RNA can be sufficient to alter the nucleotide target of a Cas protein. In some cases, crRNA and tracrRNA can be engineered as a single
10 cr/tracrRNA hybrid to direct Cas9 cleavage activity (Jinek et al., *Science*, 337(6096):816-821, 2012).

In some embodiments, a repair template also can be delivered to plant cells along with a targeted endonuclease. When the endonuclease cleaves the plant cell DNA, the repair template can become integrated into the plant cell's genomic DNA, thus
15 introducing a specific modification into the plant genome.

The methods provided herein also can include culturing the meristem induced by the developmental regulator(s) to give rise to a plant. In some cases, before a plant is generated from the new meristem, *de novo* derived tissue resulting from expression of the developmental regulator(s) can be assessed to determine whether it includes the genetic
20 modification of interest. For example, DNA from newly derived tissue can be isolated and assessed by restriction digest, hybridization methods (e.g., Southern blotting), or sequencing to determine whether a genetic modification has occurred at the target site. In some embodiments, the expression of a reporter delivered with the developmental regulator(s) and the editing sequence(s) can first be detected, to identify tissues that are
25 likely to carry the genetic modification.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Vectors for Expressing Developmental Regulators

To facilitate the expression of developmental regulators in plants, T-DNA vectors encoding different arrangements of developmental regulators were generated (SEQ ID NOS:6-11. These T-DNA vectors were designed to have two developmental regulators combined on one T-DNA (SEQ ID NOS:26-32) or to contain a single developmental
5 regulator (SEQ ID NOS:33-37). Construct descriptions are provided in TABLE 2. Developmental regulators were expressed using the 35S (SEQ ID NO:1), CmYLCV (SEQ ID NO:3), AtUBQ10 (SEQ ID NO:2), or Nos (SEQ ID NO:5) promoter. For Fast-TrACC experiments, these vectors often coexpressed the RNA guided endonuclease Cas9 (SEQ ID NO:24) driven by the 35S promoter. For many of the experiments, a gRNA was
10 expressed under the control of an AtU6 promoter (SEQ ID NO:4) targeting both of the duplicated PDS1 homologs (Niben101Scf14708g00023.1, SEQ ID NO:16; and Niben101Scf01283g02002.1, SEQ ID NO:17) in the *N. benthamiana* genome. A luciferase reporter gene (SEQ ID NO:14) driven by either the 35S or the CmYLCV promoter was used as a visual confirmation of construct delivery. These constructs were
15 cloned into the T-DNA backbone of pTRANS_201 (SEQ ID NO:18) or pTRANS_221 (SEQ ID NO:19) as described elsewhere (Cermak et al., *Plant Cell* 29(6):1196-1217, 2017). This *Agrobacterium* vector was designed to deliver a T-DNA containing a modified bean yellow dwarf virus (BeYDV) capable of circularization and replication upon delivery and expression of the encoded replication protein (Rep; SEQ ID NO:25) *in*
20 *vivo*. Replication of the replicon can enable an increased copy number of the vector, and consequently high levels of gene expression. Additionally, this vector has the potential to replicate regardless of whether it integrates into the genome, enabling transient high copy delivery of vector constructs.

TABLE 2
Construct descriptions

Construct	Promoter: Reporter	Promoter:gRNA	Promoter: 1st DR	Promoter: 2nd DR	Base Vector	SEQ ID:
pRN114	35S:Luc+	---	Nos:WUS	CmCLYV:STM	T-DNA w/ BeYDV Replicon, Kan Resistance	26
pRN119	CmYLCV:Luc+	---	Nos:WUS	35S:STM	T-DNA w/ BeYDV Replicon, Kan Resistance	27
pRN120	CmYLCV:Luc+	---	Nos:WUS	AtUBQ10:STM	T-DNA w/ BeYDV Replicon, Kan Resistance	28
pRN227	35S:Cas9	AtU6: NbPDS gRNA1	Nos:WUS	CmCLYV:STM	T-DNA w/ BeYDV Replicon, Kan Resistance	29
pMM113	35S:Luc+	AtU6: NbPDS gRNA1	Nos:WUS	CmCLYV:STM	T-DNA w/ BeYDV Replicon, Kan Resistance	30
pMM114	CmYLCV:Luc+	AtU6: NbPDS gRNA1	Nos:WUS	35S:IPT	T-DNA w/ BeYDV Replicon, Kan Resistance	31
pMM115	CmYLCV:Luc+	AtU6: NbPDS gRNA1	Nos:WUS	35S:MPA	T-DNA w/ BeYDV Replicon, Kan Resistance	32
pMM131	35S:Luc+	AtU6: NbPDS gRNA1	---	CmCLYV:STM	T-DNA w/ BeYDV Replicon	33
pMM134	CmYLCV:Luc+	AtU6: NbPDS gRNA1	35S:IPT	---	T-DNA w/ BeYDV Replicon	34
pMM135	CmYLCV:Luc+	AtU6: NbPDS gRNA1	Nos:WUS	---	T-DNA w/ BeYDV Replicon	35
pMM136	CmYLCV:Luc+	AtU6: NbPDS gRNA1	---	35S:MPA	T-DNA w/ BeYDV Replicon	36
pMM146	CmYLCV:Luc+	AtU6: NbPDS gRNA1	---	Ubi1:BBM	T-DNA w/ BeYDV Replicon	37
pMM230	35S:AtCas9	AtU6:gRNA (VvMLO)	Nos:ZmWUS2	AtUbi10:LUC	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	88
pMM231	35S:AtCas9	AtU6:gRNA (VvMLO)	35S:IPT	AtUbi10:LUC	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	89

pMM232	35S:AtCas9	AtU6:gRNA (V _v MLO)	AtUbi10:LUC	35S:MPA	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	90
pMM233	35S:AtCas9	AtU6:gRNA (V _v MLO)	AtUbi10:LUC	35S:STM	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	91
pMM234	35S:AtCas9	AtU6:gRNA (V _v MLO)	AtUbi10:LUC	AtUbi10:BBM	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	92
pMM235	35S:AtCas9	AtU6:gRNA (V _v MLO)	AtUbi10:LUC	---	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	93
pMVK057	---	AtUbi10:Luc	Nos:ZmWUS2	35S:IPT	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	94
pMVK058	---	AtUbi10:Luc	Nos:ZmWUS2	---	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	95
pMVK059	---	AtUbi10:Luc	---	35S:IPT	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	96
pMVK060	---	AtUbi10:Luc	---	---	pCambia T-DNA w/ BeYDV Replicon, Kan Resistance	97

Example 2 – Generation of Transgenic Branches from Direct Injection

Plant cells are inherently totipotent and can be transdifferentiated into other cell types. Thus, studies were conducted to determine whether *de novo* meristems could be induced *in vivo* by ectopically expressing DRs in plant somatic cells, and by co-delivering gene editing reagents with the DRs, whether it might be possible to create edited meristems that ultimately produce seed and transmit induced genetic changes to the next generation.

Young (10-12 week-old) transgenic *N. benthamiana* plants that constitutively expressed Cas9 were pruned to remove all visibly discernible shoot meristems (FIG. 1). Agrobacterium strains with vectors encoding various DR combinations and a luciferase reporter were grown overnight and resuspended in infiltration buffer (150 μ M acetosyringone, 10 mM MgCl₂, pH 5.6) to an OD₆₀₀ of 0.2. Selected meristematic tissues (shoot apical and axillary) were removed from plants using a razor blade. Agrobacterium cultures were injected into wound sites created by the removal of meristematic tissue, and also into other tissues such as nodes and internodes. Plants were monitored for 2 weeks to remove residual unerupted premature axillary meristems, as well as those that spontaneously developed from the stem base. After 3-5 weeks, various tissue types developed. Some shoots appeared phenotypically normal (FIGS. 2A and 3F), whereas others displayed abnormal and/or adverse phenotypes, such as an abundance of leaves or other developmental abnormalities, likely due to persistent expression of the DRs (FIGS. 2B-2D and 3C).

To demonstrate that transgenic tissues were generated *de novo* from injected tissues, samples were visualized for luciferase activity. Tissues were isolated from newly formed shoots arising from Agrobacterium injection sites. To visualize luciferase expression, tissues were immersed in water containing 5 mM luciferin for 5 minutes prior to bright field imaging or imaging in the dark using a CCD camera. Some tissues demonstrated luciferase expression, indicating that the newly formed tissues were derived from cells that had received the T-DNA from Agrobacterium (FIGS. 2E, and 2F, 3A, 3B, 3D, and 3E). Thus, direct injection of Agrobacterium strains delivering DRs was able to induce transgenic shoots.

In subsequent studies, transgenic shoots are allowed to develop and produce flowers. At some frequency, transgenes are transmitted to progeny, thereby creating stably transgenic plants.

Example 3 – Generation of Tissues with Gene Edits from Direct Injection

The T-DNA delivered to the transgenic Cas9 plants also expressed a gRNA targeting *N. benthamiana* phytoene desaturase (PDS) genes. There are two PDS homologs in *N. benthamiana* (NbPDS1; Niben101Scf14708g00023.1 and NbPDS2; Niben101Scf01283g02002.1). The gRNA was selected to target conserved sequences in both genes. In the absence of PDS, photobleaching occurs due to lack of photoprotective carotenoids, giving rise to a readily discernible phenotype (Qin et al., *Cell Res* 17:471-482, 2007). A subset of the shoots that emerged in the experiments described in Example 2 were white, suggesting biallelic inactivation of the two PDS homologs (FIGS. 4A-4C). To assess gene editing, genomic DNA was isolated from white tissue samples, amplified by PCR, and analyzed for NHEJ induced mutations at the gRNA target sites. Primers were used to selectively amplify either gene: TGGGAACTGAAAGTCAAGATGGTC (oCS1200; SEQ ID NO:20) and ACAATAAATGGGATGGGCCTGG (oCS1202; SEQ ID NO:21) for Niben101Scf14708g00023.1, and TGGGAACTGAAAGTCAAGATGTTT (oMM299; SEQ ID NO:22) and CAAAAGCTAGCTTATGAGGTGAAGC (oMM300; SEQ ID NO:23) for Niben101Scf01283g02002.1). Incomplete digestion of PCR amplicons by the restriction enzyme *NcoI* indicated that mutations had occurred within the restriction recognition site as a result of gRNA-directed cutting and error prone NHEJ mediated repair (FIGS. 3G, 4D, and 4E). These results indicated that *de novo* derived tissues received the expression cassettes containing the developmental regulators and gene editing reagents.

To confirm the creation of non-chimeric, genetically modified *de novo* tissue, genomic DNA was isolated from tissues exhibiting the PDS phenotype. NGS primers specific to the Scf14708g00023.1 homolog (NbPDS1, SEQ ID NOS:78-87) were used to amplify the locus in the genomic DNA sample, and the resulting amplicon was submitted for Illumina sequencing. The results demonstrated a mutation profile consistent with a

single editing event at the target locus as compared to negative controls (FIGS. 4F and 4G). Thus, the activity of developmental regulators strongly increased the potential for deriving editing events in *de novo* meristems.

Example 4 – Vertical Transmission of GE from *de novo* Derived Tissues

It was desired to determine whether GE in induced shoots could transmit the edits to the next generation. However, none of the shoots with developmental abnormalities or the PDS phenotype set seed. Because all shoots were molecularly surveyed for mutations at the PDS targets, however, one green shoot that produced viable seed and had a 3 bp deletion in one PDS allele was identified (FIG. 5A, shaded row; FIG. 5B). To determine if additional gene edited shoots could be obtained, a second experiment was performed in which WUS and IPT were delivered on the same T-DNA or on separate T-DNAs (again, a mixed infection with separate strains). Rather than monitoring the total number of shoots produced, the number of shoots that emerged from each injection site was monitored. Previous experiments had suggested that initial shoots were often not transgenic and, as such, shoots appearing in the first 20 days were removed and discarded. Abundant shoots emerged regardless of whether the developmental regulators were on the same T-DNA or on T-DNAs in different strains (FIG 6). When on the same T-DNA, for example, 46 shoots were recovered from 76 injection sites. Of these, 16 shoots had a distorted phenotype and four were white or had white sectors, indicative of transgene overexpression and PDS targeting, respectively. In contrast, the negative control produced no white shoots, although some shoots were initially distorted due to trimming but then progressed with a WT growth pattern.

One shoot emerged that was chimeric for white and green tissue, but otherwise was phenotypically normal and non-bioluminescent (FIG. 7). From the white tissue, a flower was produced that set seed, which produced completely white seedlings when germinated (FIGS. 8A and 8B). The seedlings had biallelic mutations in both PDS homologs, and the frameshift mutations transmitted to the progeny were present in the parental white tissue. Neither the parental tissues nor the seedlings were transgenic, as indicated by the lack of luciferase expression and the inability to detect the transgene

cassette by PCR (FIG. 9). Seed and tissue also were harvested from the associated green chimeric sector. Germinated seed segregated in an approximately 3:1 ratio for the PDS phenotype (FIGS. 10A and 10B). The mutations in the seedlings were the same as those observed in the parental green tissue, but they were distinct from those observed in white sectors. The green shoot that was produced in the initial experiment also was shown to transmit mutations to progeny (FIG. 11). Thus, in three independent studies, non-transgenic tissues were produced in *N. benthamiana* with multiple targeted mutations and within a single generation, without the use of plant selection. Importantly, gene edited plants that lacked a transgene were recovered, obviating the need to segregate away the transgene in subsequent generations.

Example 5 – Generation of Transgenic Tissues in Grape and Potato after Direct Injection of Developmental Regulators

To determine if *de novo* meristems could be induced on agronomically important species, additional experiments were performed in *Vitis vinifera* (grape) and *Solanum tuberosum* (potato). Cuttings from asexually propagated potato (FIGS. 12A and 12D) and grape (FIG. 12H) were injected in sterile culture jars with *Agrobacterium* strains delivering individual or a combination of DRs. Vectors in both experiments contained a luciferase reporter. For both grape and potato, a subset of plants produced bioluminescent shoots (FIGS. 12B, 12C, 12E, 12F, 12G, and 12I). In grape, the shoots were produced after combined delivery of vectors expressing WUS, IPT, STM, MPA, and BBM (pMM230 through 234), as well as after individual delivery of *Agrobacterium* expressing IPT (pMM231). In potato, shoots were induced after delivery of WUS and IPT (pMKV057) or IPT alone (pMVK059). These results demonstrated that DRs can induce transgenic shoots on diverse dicot species.

In further studies, transgenic shoots are created in potato, grape, or other species that express Cas9 and a gRNA targeting an endogenous gene. Cas9 and the gRNA create mutations in somatic cells, which are induced by the DRs to form meristems and shoots. Some shoots have edited genes that produce flowers and transmit gene edits to the next

generation. Other shoots have edited genes but lack the transgene and produce edited, transgene-free progeny (as described in Example 4 for *N. benthamiana*, for example).

Example 6 – Generation of Growths from Sites of Delivery after Fast-TrACC

Examples 1-5 describe studies using methods to generate *de novo* meristems in whole plants that either carry transgenes or have GE events. This Example and the following Examples describe studies showing that *de novo* meristem-like tissue can be generated out of somatic tissue via Fast-TrACC delivery of developmental regulators (FIG. 13).

Fast-TrACC involves treating *A. tumefaciens* cultures (GV3101 was used in the studies described herein, but any other suitable strain can be used) for three days prior to a two day co-culture with newly germinated seedlings. The first step is to grow the cultures overnight (8-12 hours) at 28°C. Next, cells are harvested by centrifugation and re-suspended to an OD₆₀₀ of 0.3 in AB:MES salts (17.2 mM K₂HPO₄, 8.3 mM NaH₂PO₄, 18.7 mM NH₄Cl, 2 mM KCl, 1.25 mM MgSO₄, 100 µM CaCl₂, 10 µM FeSO₄, 50 mM MES, 2% glucose (w/v), 200 µM acetosyringone, pH 5.5) (Wu et al., *Plant Methods* 10:19, 2014), with the intent to increase the expression of *vir* genes, and then grown overnight. The culture is again centrifuged and resuspended to OD₆₀₀ within the range of 0.10 to 0.18 in a 50:50 (v/v) mix of AB:MES salts and ½ MS liquid plant growth medium (½ MS salt supplemented with 0.5% sucrose (w/v), pH 5.5). The *A. tumefaciens* culture is now ready for incubating with seedlings.

Seeds are sterilized using 70% ethanol for 1 minute and 50% bleach (v/v) for 5 minutes. The seeds are then rinsed 5 times with sterile water, and transferred to 6-well plates (~5 seeds per well in 2 mL ½ MS), where they are subsequently germinated and maintained in growth chambers for 2-3 days at 24°C under a 16 hour / 8 hour light/dark cycle. *A. tumefaciens* is added and the co-culture is incubated for two days before the seedlings are washed free of *A. tumefaciens* using sterile water. The washed seedlings are returned to liquid ½ MS containing 100 µM of antibiotic timentin to effectively counter-select against residual *A. tumefaciens*.

The Fast-TrACC method was used to deliver *Agrobacterium tumefaciens* T-DNA constructs (SEQ ID NOS:26 and 27) encoding developmental regulators into the cotyledons of *N. benthamiana* seedlings. These constructs contained a luciferase reporter (CmYLCV:Luc, SEQ ID NO:3:SEQ ID NO:14) and the developmental regulators WUS (Nos:WUS, SEQ ID NO:5:SEQ ID NO:6) and STM (SEQ ID NO:7). STM was expressed using three different promoters (35S, SEQ ID NO:1; AtUBQ10, SEQ ID NO:2; and CmYLCV, SEQ ID NO:3) to determine if one had optimal meristem patterning potential. All three promoters formed growths but at slightly different frequencies.

Using the luciferase reporter expression as a proxy for T-DNA delivery, sectors of the plant that received the developmental regulators were identified. From regions exhibiting high levels of localized luciferase expression (FIGS. 14A and 14B, circled) developmental regulator-derived growths were formed. The derived growths appeared to be of two types: one subset included globular callus-like growths (FIG. 14C, arrow), while others formed into meristem-like structures (FIG. 14D, circled). The globular growths continued as undifferentiated tissue, but the meristem-like growths formed structures such as leaflets (FIG. 14D, arrows) that indicated differentiation of the tissue. Observing *de novo* formation of meristem-like tissues suggested that developmental regulators can be implemented to generate new plant tissues out of entirely separate tissue types, which allows for the potential to be used for the generation of whole plants.

Example 7 – Generation of Transgenic Plants from Fast-TrACC Treatment

To determine the ability to produce plants from *de novo* growths, *N. benthamiana* seedlings with meristem-like growths were selected as candidates for the generation of transgenic plants. The meristem-like growths were derived using the developmental regulator combination WUS and STM. Efforts were made to ensure that the newly formed meristem-like tissue could be propagated. The meristem-like tissues were grown for about 3 weeks, until they started to form secondary leaves (FIGS. 15A and 15B). The shoot-like growths were then transferred to auxin-rich media to establish a root network. Once roots formed (about 1.5 weeks later), the plantlets were transferred to soil (FIGS. 15C and 15D). Among the derived plants, some looked essentially wild type (FIG. 15D),

some exhibited excessive shooting and slight leaf abnormalities (FIG. 15C), and others were completely disfigured (not shown). To assess transgenesis, leaf punches were taken from each recovered plant and tested for luciferase expression (FIG. 15E). Varying levels of luciferase expression were observed, ranging from completely luciferase positive to specific sectoring of expression, to overall absence of luciferase.

One purpose of generating transgenic plants, whether of mosaic or uniform genetic constitution, was to produce flowers. These flowers can be derived from the same tissues that lead to the formation of luciferase positive leaves, implying that at some frequency, the flowers have the T-DNA and therefore will be transgenic themselves. The transgenic flowers will produce seed, some of which will be transgenic. Seeds from transgene-positive plants can be collected and tested for luciferase expression (i.e., presence of the transgene) and for the presence of GE events. Demonstration of heritable transmission of transgenes is described below in Example 10; demonstration of heritable transmission of GE events is described below in Example 12.

Example 8 – Confirmation of Gene Editing in Developmental Regulator-Derived Growths

To demonstrate that GE events can occur in *de novo* growths, gene editing reagents were combined with developmental regulators and delivered to seedlings. The construct that was used (SEQ ID NO:29) contained 35S:Cas9 (SEQ ID NO:1:SEQ ID NO:24), AtU6:gRNA (SEQ ID NO:4:SEQ ID NO:12), Nos:WUS (SEQ ID NO:5:SEQ ID NO:6), and CmYLCV:STM (SEQ ID NO:3:SEQ ID NO:7). Limits on construct size prevented the inclusion of luciferase as a delivery reporter. Seedlings were screened for production of growths. Out of twenty-four seedlings, five seedlings exhibited growth formations (FIG. 16A) suitable to test for edits.

The growths were excised and DNA was isolated from each. From the isolated DNA, the target region in NbPDS1 was PCR amplified. The gRNA used in these studies targeted a locus with a *NcoI* restriction site that, when edited, will not allow for digestion in a RFLP assay. Four of the five tested samples contained the “protected” band indicative of editing (FIG. 16B). These same samples contained the T-DNA, as indicated

by the presence of Rep (FIG. 16C). The samples were then submitted for NGS. The resulting sequences (FIGS. 16D-16H) indicated that the tissues were considerably edited, with as high as 42% of reads from a given growth being edited. This observation indicated that the delivery of gene editing reagents with developmental regulators
5 allowed for editing within the tissues generated by developmental regulators.

Example 9 – Generation of Edited Plants After Fast-TrACC Treatment

To illustrate the capability to regenerate edited plants in a fashion similar to the generation of transgenic plants, whole plants were derived from edited growths. The
10 same construct (SEQ ID NO:29) used to generate edits within undifferentiated growths was used to promote the formation of plantlets with edits. A new set of growths were developed on *N. benthamiana* cotyledons that then formed meristem-like growths. Once the growths established a shoot-like structure, they were transferred to rooting medium to initiate a root network. Full plants were considered formed once the root system was
15 established (FIGS. 17A-17C), and the plants were subsequently moved to soil.

Leaf tissue samples were taken from the generated plants and submitted for NGS. The sequencing results from individual tissue samples resulted in a small proportion of edited reads (FIGS. 17D-17F), revealing that the generated plantlets were chimeric but did contain edits derived from the gene editing reagents. Since the plants were chimeric, a
20 variety of different mutations were isolated from the NGS reads (FIGS. 17G-17I). The most common mutations were single base insertions or deletions. Reads that were likely due to aberrant mutations from PCR amplification (denoted with asterisks) were also isolated, indicating that the overall editing frequencies highlighted (FIGS. 17D-17F) were over estimates. The amount of mosaicism differed between plants, with the presence of
25 the most common two edits ranging from 9% (FIG. 17H) to as low as 2% (FIG. 17G).

Ideally, these chimeric plants will contain mutations within a floral meristem, which would cause the flower to produce edited seeds at some frequency. As described below in Example 12, plants are grown and seeds are collected and tested for gene editing. The isolation of edited plants derived from these seeds demonstrates that edited

plants can be obtained in a single generation through the creation of developmental regulator-derived plants.

Example 10 – Vertical Transmission of Transgenes from Fast-TrACC Derived Plants

5 The ultimate goal for DR-based plant generation is to create genetic changes that can be transmitted to subsequent generations. Several of the DR-derived *N. benthamiana* plants grew seed-bearing flowers. These plants were created using the constructs pRN114, pRN119 and pRN120 (SEQ ID NOS:26-28) and exhibited different levels of luciferase expression (FIG. 15E). Seeds from the most luciferase-positive plant, RN119-
10 P1-5-2 (FIG. 18A), were collected and tested for luminescence (FIG. 18B). A large proportion of the seeds from this plant were positive for luciferase (FIG. 18C). The inheritance pattern of luciferase (~75% of seedlings) was in line with the expected Mendelian inheritance pattern of a hemizygous transgene insertion (FIG. 18D). Not all plants tested had a luciferase-positive signal in their derived seeds (FIG. 18D, RN119-4-
15 5), which could be due to mosaicism in the parent, segregation of the transgene, or silencing of the T-DNA. It also is possible that transient delivery of the developmental regulators primed a micro-environment that promoted meristem formation without transgene integration. Regardless, the observation that transgenes could be transmitted to the next generation demonstrated that a heritable transgenic event was created through *de*
20 *novo* induction of a meristem.

Example 11 – Optimizing Combinations of Developmental Regulators for Meristem

Induction and the Recovery of Plants

Fast-TrACC was used to test different combinations of developmental regulators
25 in order to identify those that could best induce growths that give rise to full plants. Separate *A. tumefaciens* strains, each carrying expression cassettes for a unique DR, were pooled for seedling co-culture. Twelve combinations of DRs were tested, and five of those combinations resulted in growths from which plants could be derived (FIGS. 19A and 19B). Two combinations, WUS & STM and WUS & IPT, produced up to five times
30 as many shoot-like growths and roughly four times more full plants than the other

treatments. Thus, Fast-TrACC can be used to determine the best combination of developmental regulators for meristem induction in a given plant species.

Example 12 – Vertical Transmission of GE Events from Fast-TrACC Derived Plants

5 Studies were conducted to determine if Fast-TrACC could be used to generate meristems with gene edits and subsequently plants that transmit mutations to progeny. In the experiment described in Example 11, transgenic *N. benthamiana* seedlings constitutively expressing Cas9 were treated with Fast-TrACC. In addition to a DR, the T-DNAs carried a cassette that expressed a gRNA targeting NbPDS1 and NbPDS2.

10 Biallelic knockouts of both PDS homologs are expected to result in a white phenotype due to chlorophyll photobleaching (Qin et al., *supra*). About 15% of the generated shoots showed evidence of photobleaching, but these shoots did not form full plants; they were likely compromised by lack of chlorophyll (FIG. 20). Nonetheless, white shoots were evaluated molecularly and found to have biallelic mutations in both PDS homologs. Thus
15 Fast-TrACC can generate meristems with gene edits.

 Of 27 total plants recovered in the experiment described in Example 11, five phenotypically normal green plants were found to show considerable amounts of editing in somatic cells (FIG. 19A). For one of these plants, seed collected from two flowers (F4 and F6) produced green and white seedlings (FIG. 21A). gRNA target sites for both PDS
20 homologs were assessed molecularly for two white seedlings derived from each flower, and mutations were observed in both alleles of each PDS gene (FIG. 21B). Based on this data, it was concluded that co-delivery of DRs and gene editing reagents can produce shoots with mutations, and these shoots can transmit mutations to the next generation.

Example 13 – Generation of Transgenic Tomato Shoots Using Fast-TrACC

25 As DRs are evolutionarily conserved, studies were conducted to determine whether the approach for seedling transformation would be applicable to other plant species. Combinations of DRs that generated *de novo* meristems on *N. benthamiana* seedlings were therefore tested to determine whether they could induce shoots on other
30 dicots, such as tomato. In particular, Fast-TrACC was used to deliver three combinations

of developmental regulators (Nos:WUS&35S:STM, Nos:WUS&CmYLCV:STM, and Nos:WUS&35S:IPT) to tomato seedlings. For both combinations of WUS & STM, no shoot-like growths formed (FIGS. 22A and 22C). In contrast, WUS & IPT promoted shoot-like growths (FIG. 22A and 22C), which ultimately formed fully rooted plants (FIG. 22B).

Next, WUS and IPT were delivered to tomato seedlings on either a single vector (WUS/IPT) or on separate vectors in two different *Agrobacterium* strains (WUS&IPT). Both WUS/IPT and WUS&IPT showed an increase in the frequency of average growths per plant over the background level of callus-like growths that developed on plants that did not receive developmental regulators (FIG. 22D). From the WUS and IPT derived growths, shoot-like growths formed that were luciferase positive (FIGS. 22E, 22F, and 22G). These structures progressed to form shoots (FIG. 22H), which were excised and assessed for luminescence (FIG. 22I). Four out of 15 shoots showed evidence of luminescence (FIG. 22J). Thus FAST-TrACC can be used to determine the optimal combination of developmental regulators for meristem induction in other plant species, which indicates that Fast-TrACC has utility outside of the *N. benthamiana* model to induce transgenic shoots.

In subsequent experiments, transgenic shoots are placed on root-inducing medium to promote root formation. Resulting plantlets are transferred to soil where they continue to grow, flower, and produce fruit and seed. Progeny are assessed to for transmission of the transgene, as demonstrated in Example 10. Gene edited tomato plants are generated and assessed through an approach similar to that described in detail in Example 12 for *N. benthamiana*.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for generating plant tissue comprising one or more genetic modifications of interest, the method comprising:
 - (a) introducing into plant cells (i) nucleic acid encoding one or more developmental regulators that, when expressed in the plant cells, induce meristem formation from the plant cells, and (ii) nucleic acid comprising one or more sequences that, when expressed, modify a plant cell to achieve one or more genetic modifications of interest; and
 - (b) deriving *de novo* tissue from plant cells identified as having the one or more genetic modifications of interest.
2. The method of claim 1, wherein the one or more developmental regulators comprise one or more of Baby Boom, Isopentenyl Transferase, Irrepressible Variants of Monopteros, Shoot Meristemless, and Wuschel.
3. The method of claim 1 or claim 2, wherein the introducing is by *Agrobacterium*, and wherein the nucleic acid encoding one or more developmental regulators and the nucleic acid comprising one or more sequences that modify a plant cell are included on the same T-DNA.
4. The method of claim 1 or claim 2, wherein the introducing is by *Agrobacterium*, and wherein the nucleic acid encoding one or more developmental regulators and the nucleic acid comprising one or more sequences that modify a plant cell are included on separate T-DNAs.
5. The method of any one of claims 1 to 4, comprising introducing nucleic acid encoding two or more developmental regulators into the plant cells by *Agrobacterium*, wherein the two or more developmental regulators are encoded by a single T-DNA.

6. The method of claim 1, 2, or 4, comprising introducing nucleic acid encoding two or more developmental regulators into the plant cells by *Agrobacterium*, wherein the two or more developmental regulators are encoded by separate T-DNAs.
7. The method of claim 1, 2, or 4, comprising introducing nucleic acid encoding two or more developmental regulators into the plant cells by *Agrobacterium*, wherein the two or more developmental regulators are encoded by two or more strains of *Agrobacterium*.
8. The method of claim 1 or claim 2, wherein the introducing comprises electroporation, biolistics, particle bombardment, chemical transfection, nanoparticle delivery, or viral infection.
9. The method of any one of claims 1 to 8, wherein the introducing comprises transient transformation.
10. The method of any one of claims 1 to 8, wherein the introducing comprises stable transgenesis.
11. The method of any one of claims 1 to 10, wherein the plant cells into which the nucleic acids are introduced are within a differentiated tissue.
12. The method of any one of claims 1 to 10, wherein the plant cells into which the nucleic acids are introduced are within an undifferentiated tissue.
13. The method of any one of claims 1 to 10, wherein the plant cells into which the nucleic acids are introduced are within a whole plant.
14. The method of any one of claims 1 to 10, wherein the plant cells into which the nucleic acids are introduced are within a germinating seedling.
15. The method of any one of claims 1 to 10, wherein the plant cells into which the nucleic acids are introduced are within a plant part taken from a plant.

16. The method of any one of claims 1 to 15, wherein the plant cells are of a monocotyledonous plant.
17. The method of any one of claims 1 to 15, wherein the plant cells are of a dicotyledonous plant.
18. The method of any one of claims 1 to 17, wherein the one or more sequences that modify a plant cell comprise a transgene that, when expressed in the plant cells, achieves an agriculturally relevant trait.
19. The method of claim 18, wherein the agriculturally relevant trait is herbicide tolerance.
20. The method of any one of claims 1 to 17, wherein the one or more sequences that modify a plant cell comprise a transgene that, when expressed, edits the plant DNA.
21. The method of claim 20, wherein the one or more sequences that modify a plant cell comprise a nucleotide sequence encoding a targeted endonuclease.
22. The method of claim 21, wherein the targeted endonuclease comprises a meganuclease, zinc finger nuclease, transcription activator-like effector nuclease, or Clustered Regularly-Interspaced Short Palindromic Repeats-associated nuclease.
23. The method of any one of claims 1 to 17, wherein the one or more sequences that modify a plant cell encode a targeted enzyme that modifies plant DNA.
24. The method of claim 23, wherein the targeted enzyme is a cytosine deaminase or an adenosine deaminase.
25. The method of claim 24, wherein the cytosine deaminase or adenosine deaminase is BE3 or ABE.

26. The method of any one of claims 1 to 17, wherein the one or more sequences that modify a plant cell encode a targeted endonuclease and comprise a repair template to introduce one or more specific modifications into the plant genome.
27. The method of any one of claims 1 to 26, wherein the *de novo* tissue is meristematic and is capable of deriving new tissue carrying the one or more genetic modifications of interest.
28. The method of claim 27, wherein the new tissue comprises a branch, a flower, or a root.
29. The method of claim 1 or claim 2, comprising:
- (a) using *Agrobacterium*, introducing into cells of a germinating seedling or a portion thereof nucleic acid encoding the one or more developmental regulators, wherein expression of the one or more developmental regulators induces meristem formation in the germinating seedling or portion thereof;
 - (b) introducing into the cells, via the *Agrobacterium*, the nucleic acid comprising one or more sequences that, when expressed, modify the cells to achieve the one or more genetic modifications of interest; and
 - (c) culturing the meristem induced by the one or more developmental regulators, to obtain modified plant tissue comprising the one or more genetic modifications of interest.
30. The method of claim 29, comprising introducing nucleic acid encoding two or more developmental regulators, wherein the two or more developmental regulators are encoded by one T-DNA.
31. The method of claim 29, comprising introducing nucleic acid encoding two or more developmental regulators, wherein the two or more developmental regulators are encoded on separate T-DNAs.

32. The method of claim 29, comprising introducing nucleic acid encoding two or more developmental regulators, wherein the two or more developmental regulators are encoded by separate strains of *Agrobacterium*.
33. The method of any one of claims 29 to 32, wherein the germinating seedling or portion thereof is from a monocotyledonous plant.
34. The method of any one of claims 29 to 32, wherein the germinating seedling or portion thereof is from a dicotyledonous plant.
35. The method of any one of claims 29 to 34, wherein the one or more genetic modifications comprise insertion of a transgene that, when expressed, achieves an agriculturally relevant trait.
36. The method of claim 35, wherein the agriculturally relevant trait is herbicide tolerance.
37. The method of any one of claims 29 to 34, wherein the one or more genetic modifications comprise insertion of a transgene that, when expressed, edits the plant cell DNA.
38. The method of claim 37, wherein the nucleic acid that modifies a plant cell encodes a targeted endonuclease.
39. The method of claim 38, wherein the targeted endonuclease comprises a meganuclease, zinc finger nuclease, transcription activator-like effector nuclease, or Clustered Regularly-Interspaced Short Palindromic Repeats-associated nuclease.
40. The method of any one of claims 29 to 34, wherein the nucleic acid that modifies a plant cell encodes a targeted enzyme that modifies plant DNA.
41. The method of claim 40, wherein the targeted enzyme is a cytosine deaminase or an adenosine deaminase.

42. The method of claim 41, wherein the cytosine deaminase or adenosine deaminase is BE3 or ABE.

43. The method of any one of claims 29 to 34, wherein the nucleic acid that modifies a plant cell encodes a targeted endonuclease and comprises a repair template to introduce a specific modification into the genetic material of the plant cell.

44. The method of any one of claims 29 to 43, further comprising assaying the meristem induced by the one or more developmental regulators for the one or more genetic modifications of interest, and subsequently generating a whole plant from the meristem induced by the one or more developmental regulators.

45. The method of any one of claims 29 to 43, comprising placing the meristem induced by the one or more developmental regulators directly into culture and inducing the meristem in culture to form a plant.

FIG. 1

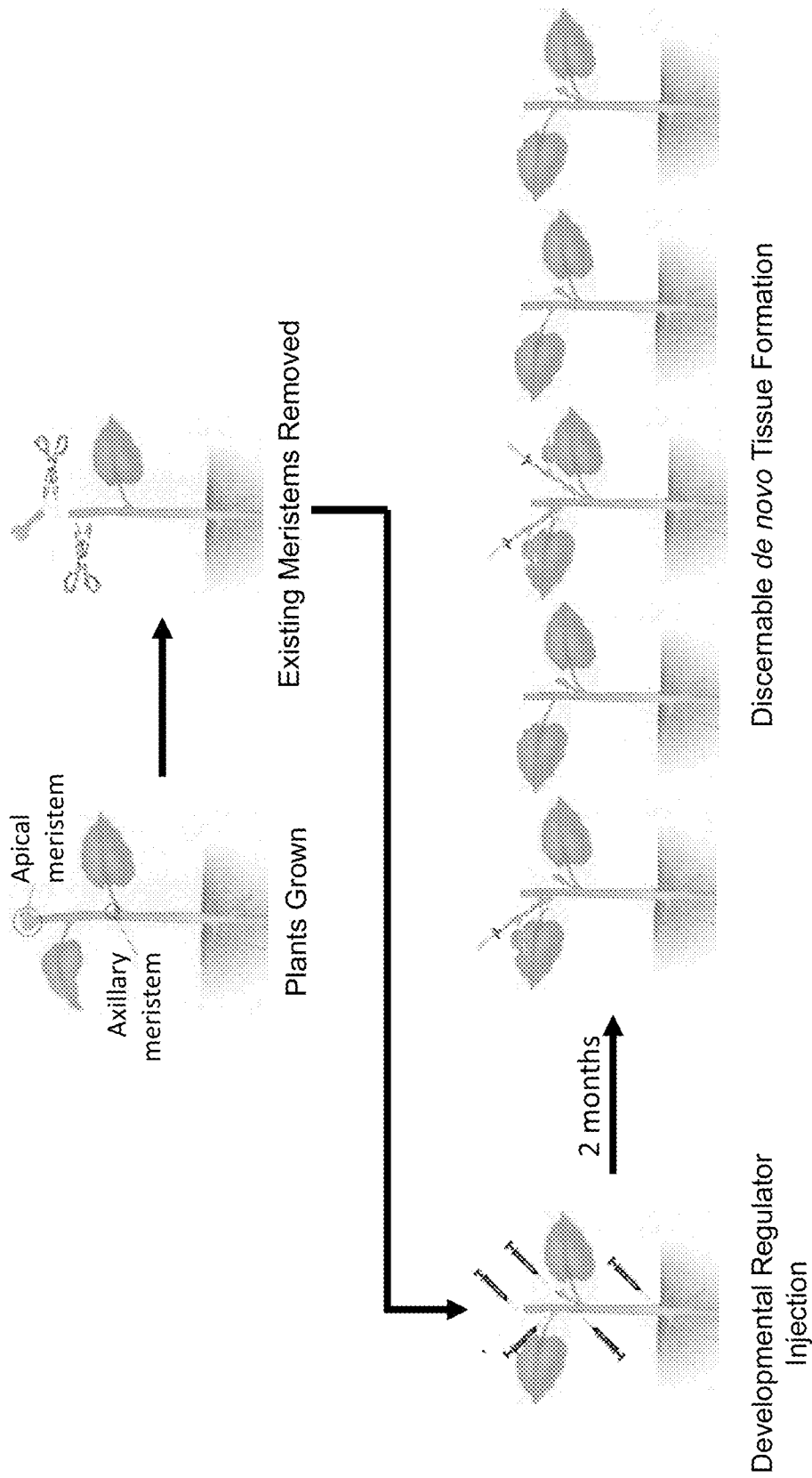


FIG. 2E

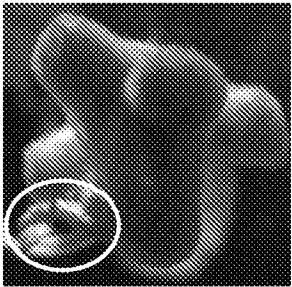


FIG. 2D

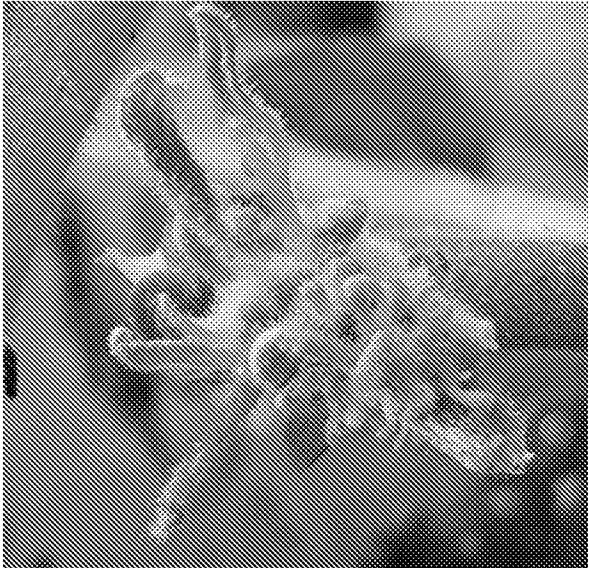


FIG. 2B

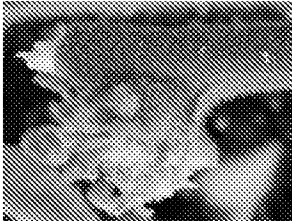


FIG. 2A

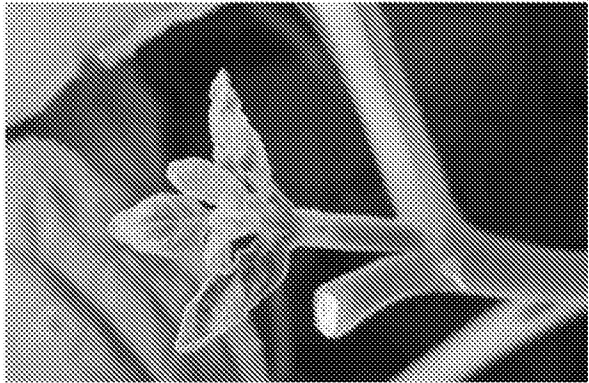


FIG. 2F

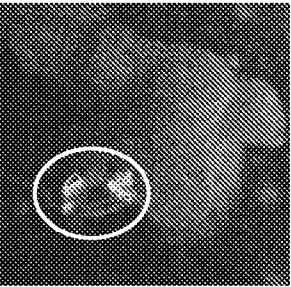


FIG. 2C



FIG. 3A

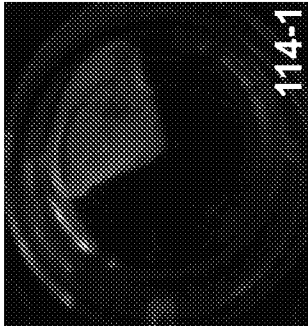


FIG. 3B

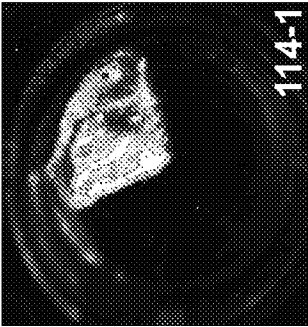


FIG. 3D



FIG. 3E

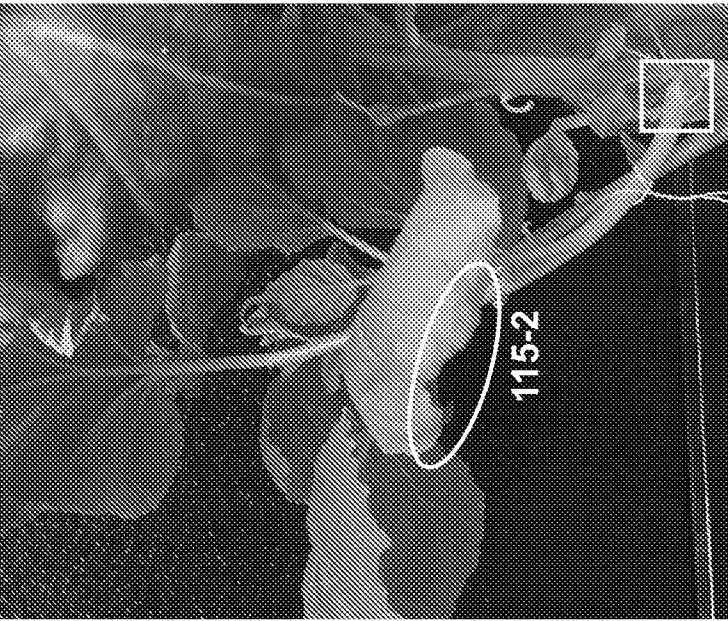
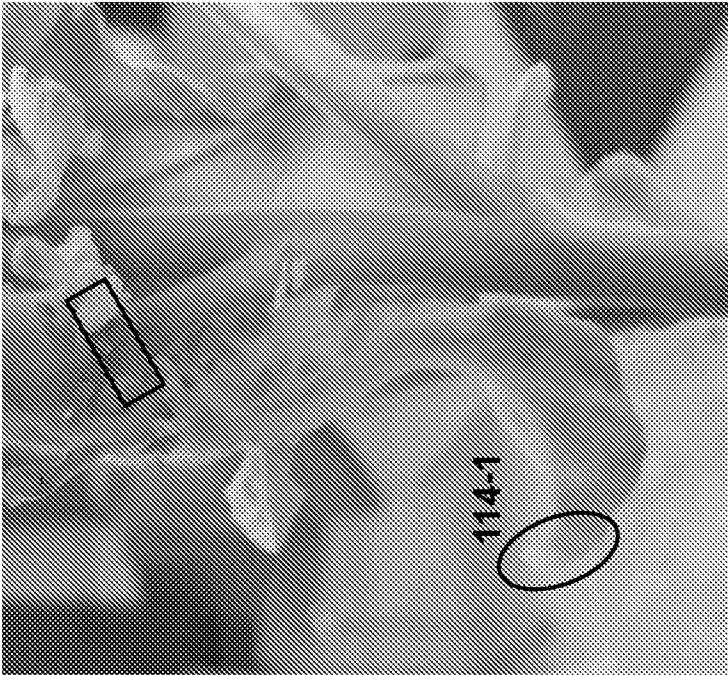
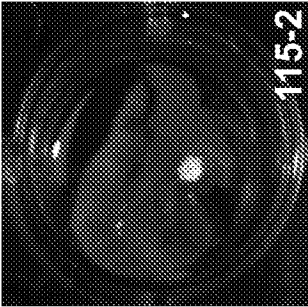


FIG. 3C

FIG. 3F

FIG. 3G

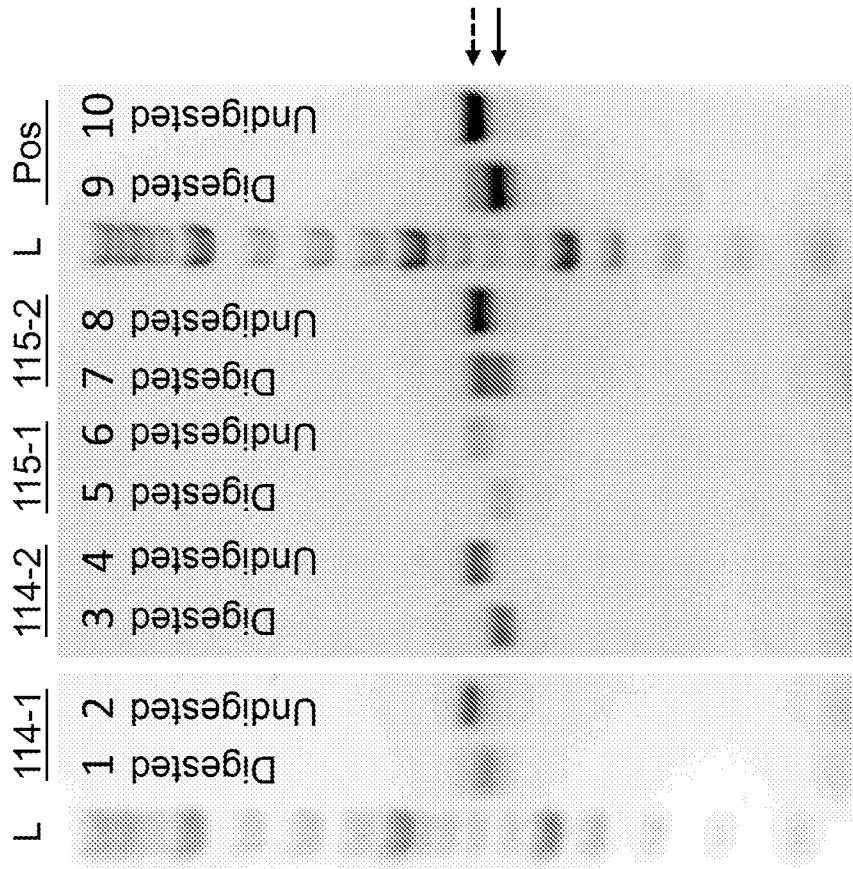


FIG. 4C

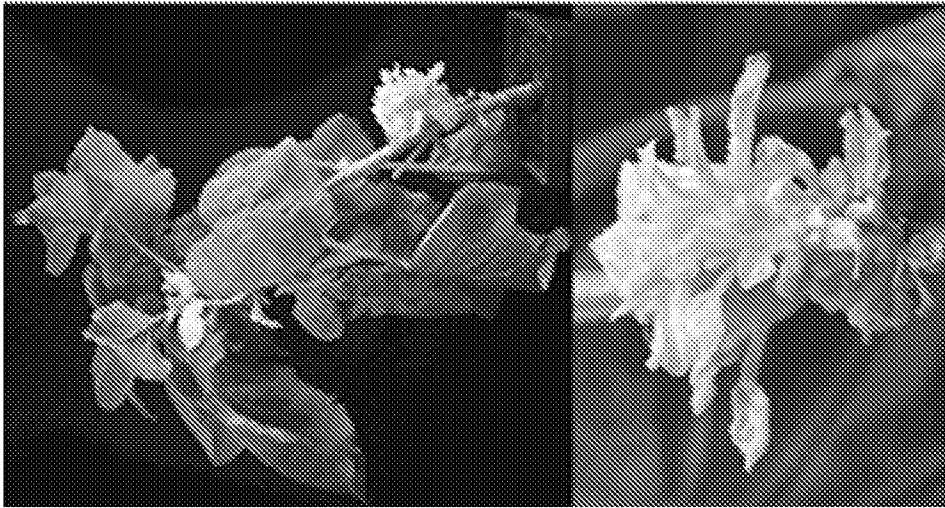


FIG. 4B

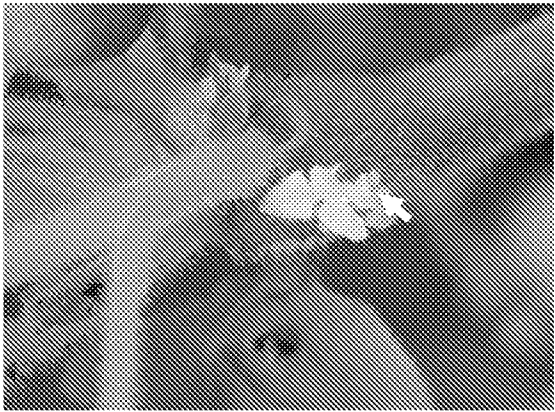


FIG. 4A

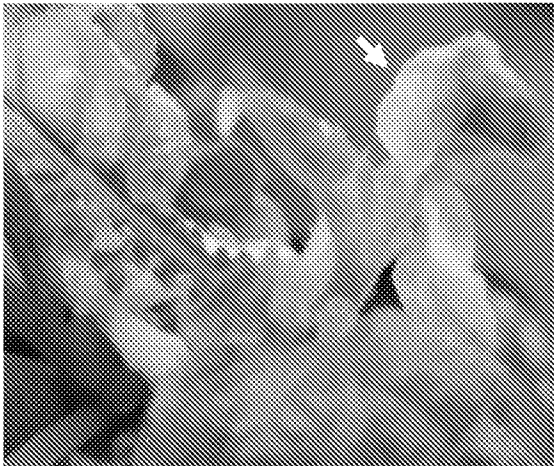


FIG. 4D

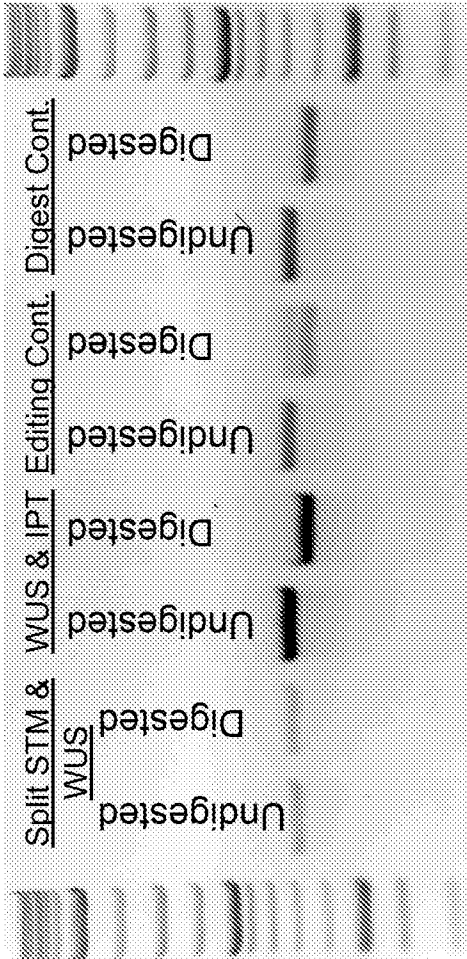


FIG. 4E

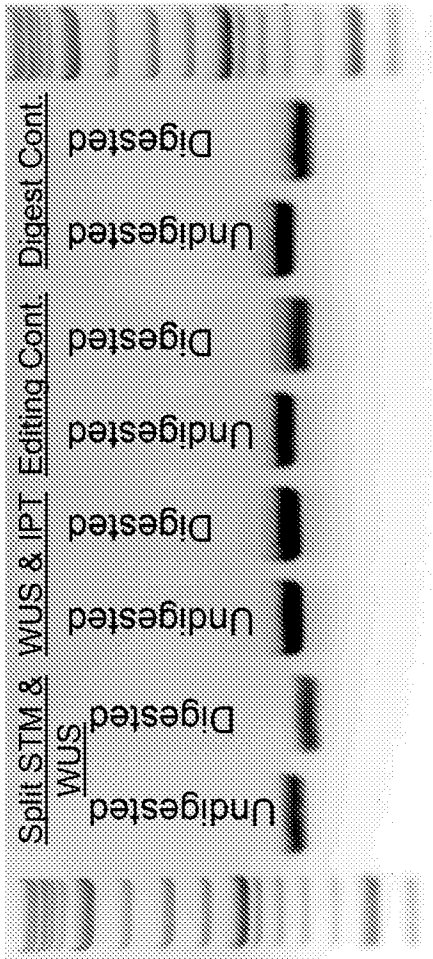


FIG. 4F

Split STM & WUS

G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	Reference
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	93.43% (14997 reads)
T	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	4.60% (739 reads)
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.21% (34 reads)

FIG. 4G

WUS & IPT

G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	Reference
T	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	96.92% (2488 reads)
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	1.52% (39 reads)

FIG. 5A

Construct	Plant designator	Sequences analyzed	Insertions	Deletions	Indel frequency	Observed mutations	Seed produced?
WUS/STM	5-13-3-12	1628	0	821	821 (50.4%)	-1bp & WT	No
WUS/IPT	7-19-2-11**	1089	0	1072	1072 (98.4%)	-10bp & -29bp	No
WUS/IPT	7-19-2-12**	2431	0	2399	2399 (98.7%)	-10bp & -29bp	No
WUS/IPT	7-19-2-13**	528	0	493	493 (93.4%)	-10bp & -29bp	No
All Combo	5-14-1-08	258	0	104	104 (40.3%)	-3bp & WT	Yes
All Combo	1-1-5	1057	111	892	1003 (94.9%)	Chimeric: spectrum of +1bp, -3bp, -1bp or larger	No

** Shoots derived from the same injection site had the same mutation profile, suggesting they were derived from common, edited progenitor cells.

FIG. 5B

WT:	(SEQ ID NO:)
GGAATTTGTTATGTTTGGTAGCGACTC-CATGGGGCATAAGTTTAGAATTCGTACTCCC	(99)
5-13-3-12: GGAATTTGTTATGTTTGGTAGCGACT--CATGGGGCATAAGTTTAGAATTCGTACTCCC	(100)
GGAATTTGTTATGTTTGGTAGCGACTC-CATGGGGCATAAGTTTAGAATTCGTACTCCC	(99)
7-19-2-11: GGAATTTGTTATGTTTGGTAG--ATGGGGCATAAGTTTAGAATTCGTACTCCC	(101)
GGAATTTGTTATGTTTGGTAGCG--ACTCCC	(102)
7-19-2-12: GGAATTTGTTATGTTTGGTAG--ATGGGGCATAAGTTTAGAATTCGTACTCCC	(101)
GGAATTTGTTATGTTTGGTAGCG--ACTCCC	(102)
7-19-2-13: GGAATTTGTTATGTTTGGTAG--ATGGGGCATAAGTTTAGAATTCGTACTCCC	(101)
GGAATTTGTTATGTTTGGTAGCG--ACTCCC	(102)
5-14-1-08: GGAATTTGTTATGTTTGGTAGCGA--CATGGGGCATAAGTTTAGAATTCGTACTCCC	(103)
GGAATTTGTTATGTTTGGTAGCGACTC-CATGGGGCATAAGTTTAGAATTCGTACTCCC	(99)
1-1-5 #1: GGAATTTGTTATGTTTGGTAGCGA---CATGGGGCATAAGTTTAGAATTCGTACTCCC	(104)
1-1-5 #2: GGAATTTGTTATGTTTGGTAGCGACT--CATGGGGCATAAGTTTAGAATTCGTACTCCC	(100)
1-1-5 #3: GGAATTTGTTATGTTTGGTAGCGACTCCCATGGGGCATAAGTTTAGAATTCGTACTCCC	(105)
1-1-5 #4: GGAATTTGTTATGTTTGGTAGCGACTC-CATGGGGCATAAGTTTAGAATTCGTACTCCC	(99)
1-1-5 #5: GGAATTTGTTATGTTTGGTAGCGA---ATGGGGCATAAGTTTAGAATTCGTACTCCC	(106)
1-1-5 #6: GGAATTTGTTATGTTTGGTAG--CATGGGGCATAAGTTTAGAATTCGTACTCCC	(107)
1-1-5 #7: GGAATTTGTTATGTTTGGTAGCG--ACTCCC	(108)
1-1-5 #8: GGAATTTGTTATGTTTGGT---ATGGGGCATAAGTTTAGAATTCGTACTCCC	(109)
1-1-5 #9: GGAATTTGTTATGTTTGGTAGT---ATGGGGCATAAGTTTAGAATTCGTACTCCC	(110)
1-1-5 #10: GGAATTTGTTATGTTTGGTAG--ATGGGGCATAAGTTTAGAATTCGTACTCCC	(111)
1-1-5 #11: GGAATTTGTTATGTTTGGTAGTA---GGGCATAAGTTTAGAATTCGTACTCCC	(112)
1-1-5 #12: ---ATGGGGCATAAGTTTAGAATTCGTACTCCC	(113)
1-1-5 #13: ---	---

FIG. 6

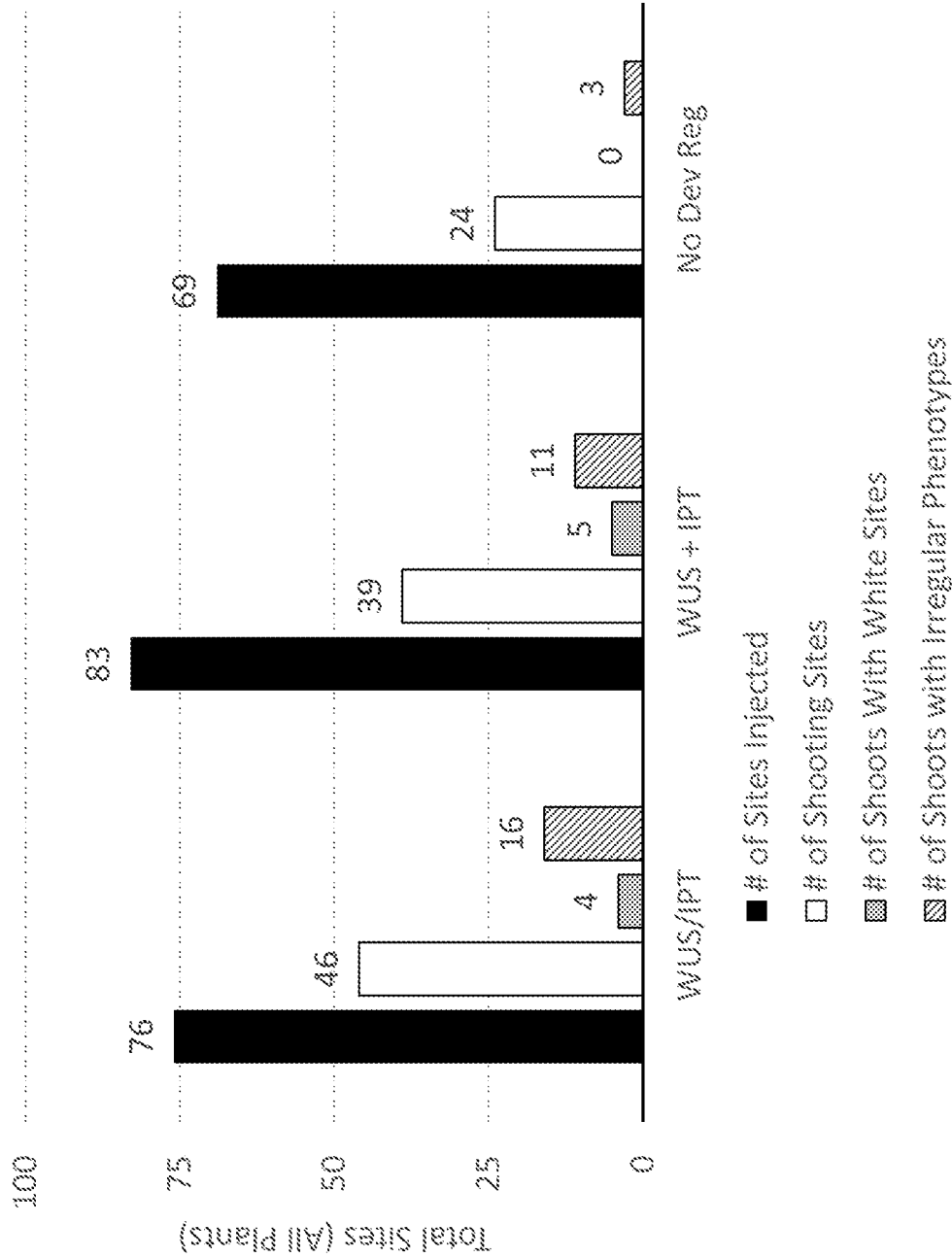


FIG. 7



Parent Tissue: White

<u>PDS1</u>
+1 / -1 / -2 / -3
<u>PDS2</u>
-48 / +1 / -1

Phenotype:

100% (10/10)
White Seedlings

3:1 (27:9)
Green:White Seedlings

Parent Tissue: Green

<u>PDS1</u>
-1
<u>PDS2</u>
-48 / -1

FIG. 8A

Sample	Phenotype	PDS1*	PDS2*
Parental white tissue	White	+1 (18%) / -1 (34%) -2 (21%) / -3 (16%)	-48 / +1 / -1
Seedling 1	White	+1 (42%) / -2 (49%)	-1 (98%)
Seedling 2	White	+1 (43%) / -2 (48%)	-1 (96%)
Seedling 3	White	+1 (39%) / -2 (51%)	+1 (44%) / -1 (46%)
Seedling 4	White	-2 (95%)	+1 (49%) / -1 (37%)
Seedling 5	White	+1 (46%) / -2 (46%)	+1 (99%)
Seedling 6	White	+1 (51%) / -1 (41%)	+1 (42%) / -1 (45%)
Seedling 7	White	No data	+1 (58%) / -1 (35%)
Seedling 8	White	+1 (55%) / -2 (35%)	+1 (36%) / -1 (35%)
Seedling 9	White	+1 (100%)	+1 (49%) / -1 (42%)
Seedling 10	White	+1 (41%) / -2 (48%)	+1 (98%)

FIG. 8B



FIG. 9

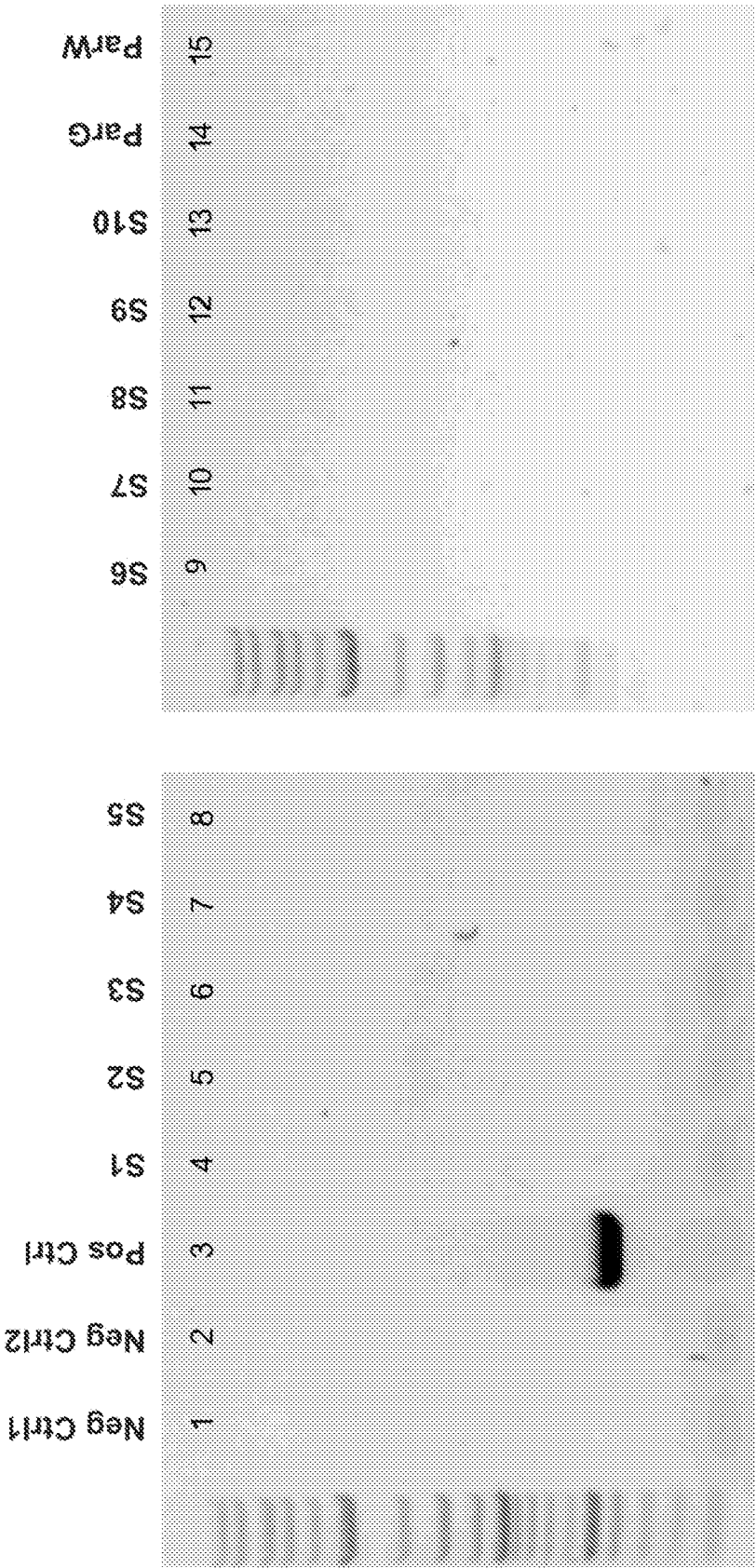


FIG. 10A

Sample	Phenotype	PDS1*	PDS2*
Parental green tissue	Green	-1bp (100%)	Heterozygous -48/-1
Seedling 1	Green	-1 (99%)	Heterozygous -48/-1
Seedling 2	Green	-1 (99%)	Homozygous -48
Seedling 3	Green	-1 (100%)	Heterozygous -48/-1
Seedling 4	Green	No data	No data
Seedling 5	Green	-1 (100%)	Heterozygous -48/-1
Seedling 6	Green	-1 (100%)	Heterozygous -48/-1
Seedling 7	Green	-1 (100%)	Heterozygous -48
Seedling 8	Green	-1 (98%)	Heterozygous -1/-48
Seedling 9	Green	-1 (100%)	Homozygous -48
Seedling 10	Green	-1 (100%)	Heterozygous -48/-1
Seedling 28	White	-1 (100%)	Homozygous -1
Seedling 29	White	-1 (100%)	Homozygous -1
Seedling 30	White	-1 (100%)	Homozygous -1
Seedling 31	White	-1 (100%)	Homozygous -1
Seedling 32	White	-1 (100%)	Homozygous -1

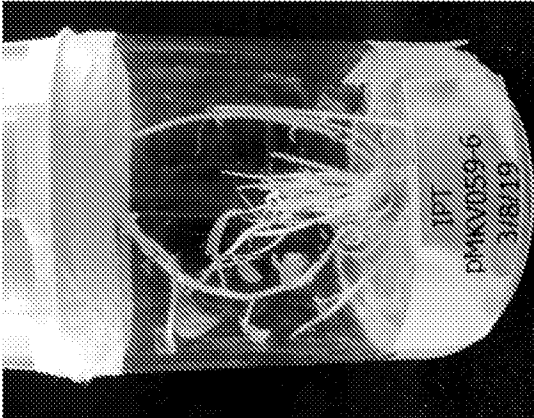
FIG. 10B



FIG. 11

Seedling Number	Phenotype	PDS1*	PDS2*
Parental tissue	WT	-3bp, WT	WT (100%)
S1	WT	WT (100%)	WT (100%)
S3	WT	WT (100%)	WT (100%)
S5	WT	WT (48%), -3bp (46%)	WT (98%)
S7	WT	WT (48%), -3bp (45%)	WT (99%)
S9	WT	WT (100%)	WT (96%)
S11	WT	WT (46%), -3bp (48%)	WT (99%)
S13	WT	-3bp (99.6%)	WT (99%)
S15	WT	WT (43%), -3bp (55%)	WT (97%)
S17	WT	-3bp (99.4%)	WT (100%)
S19	WT	-3bp (99%)	WT (98%)

FIG. 12A



IPT

FIG. 12B

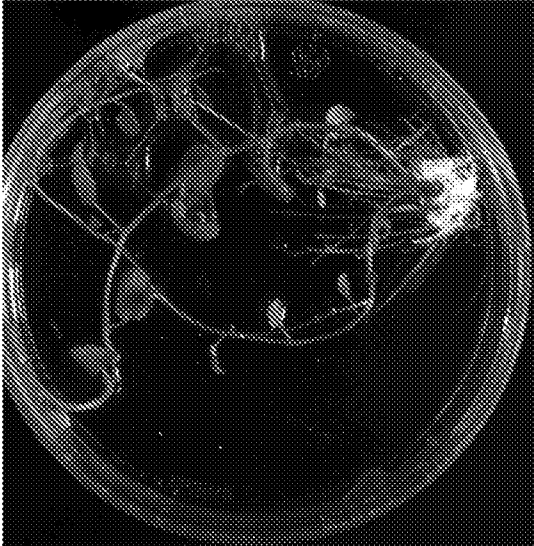
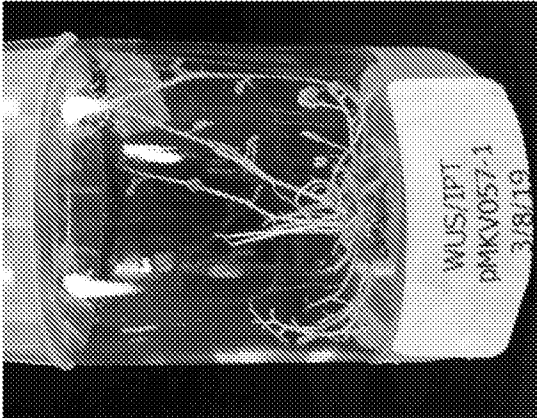
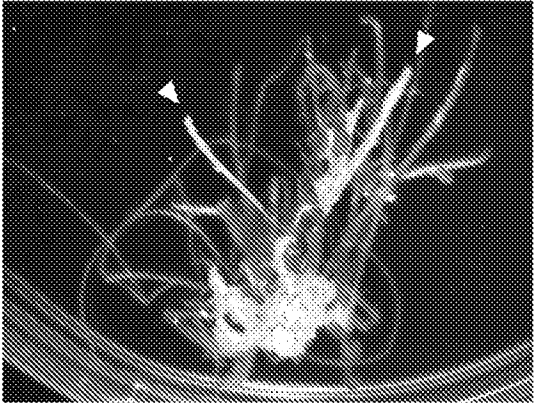


FIG. 12C



WUS/IPT

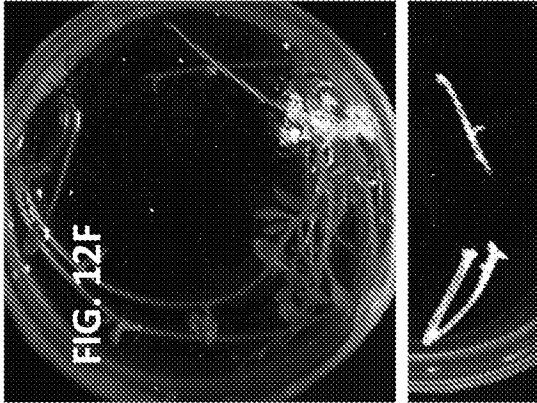
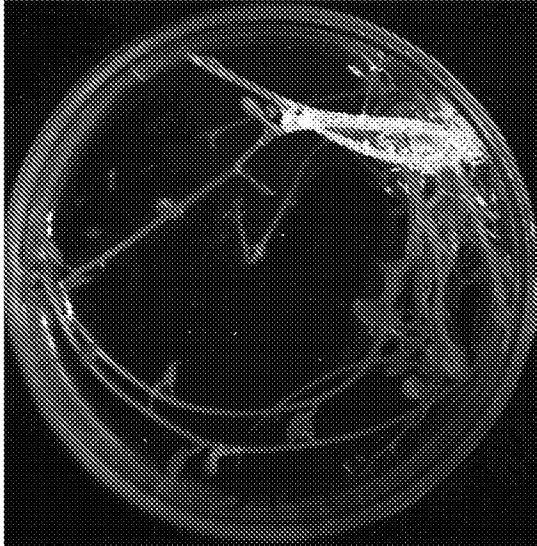


FIG. 12D

FIG. 12E

FIG. 12G

FIG. 12I



FIG. 12H

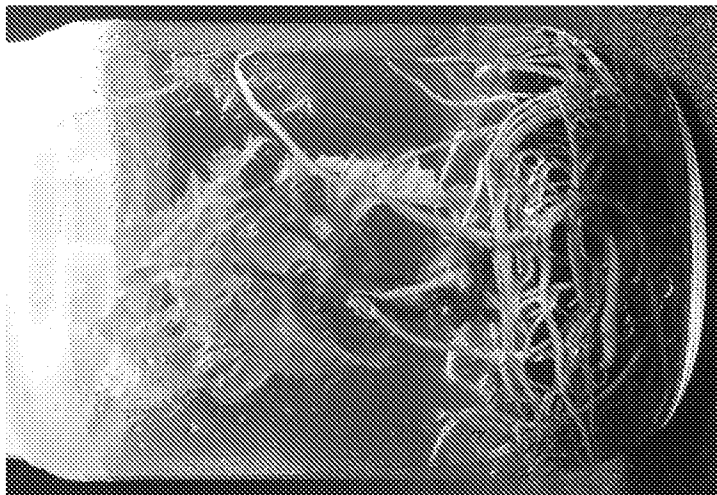


FIG. 13

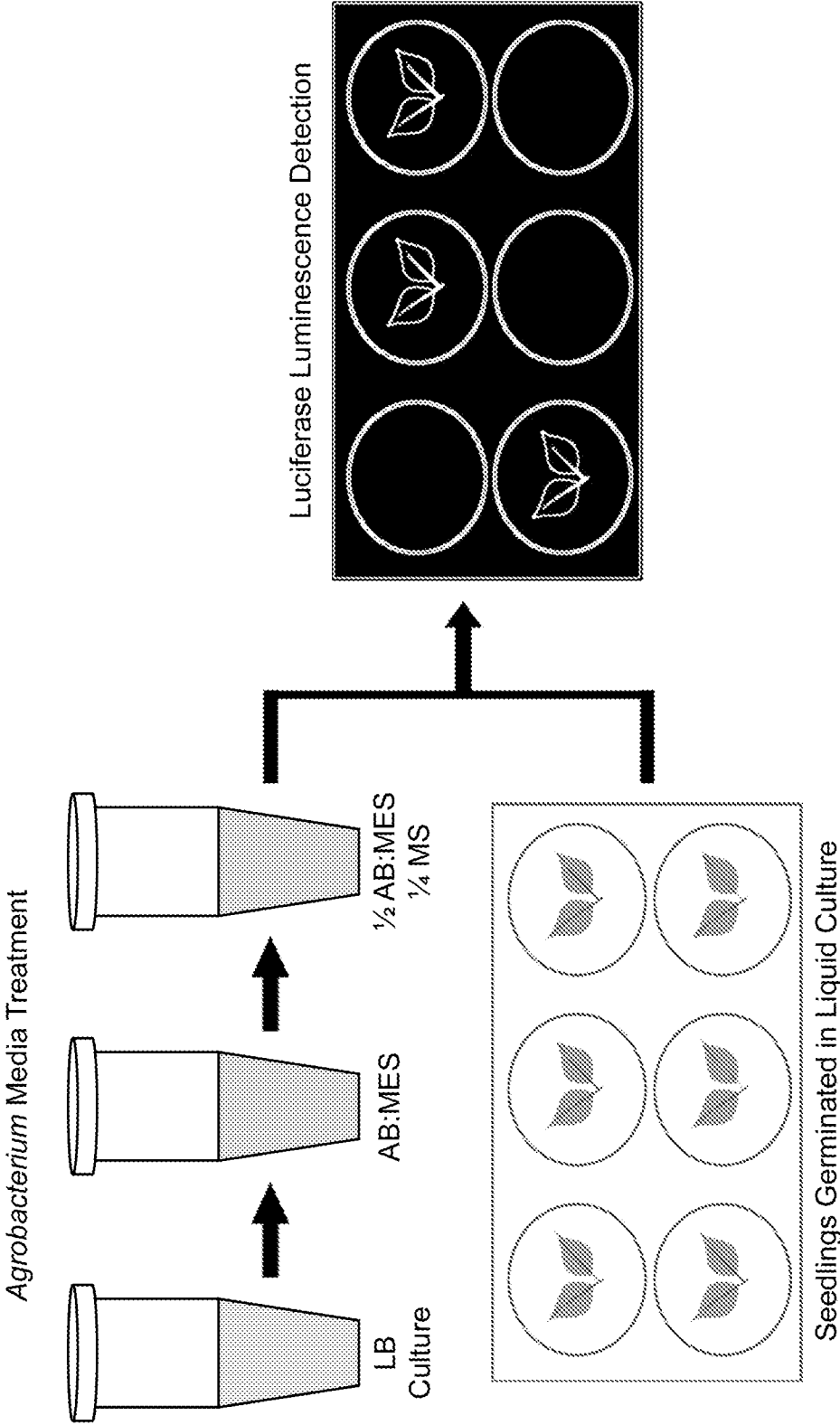
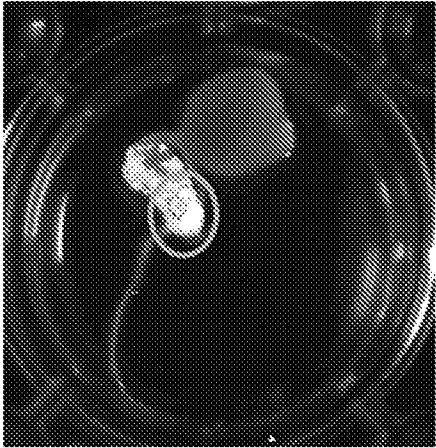


FIG. 14A

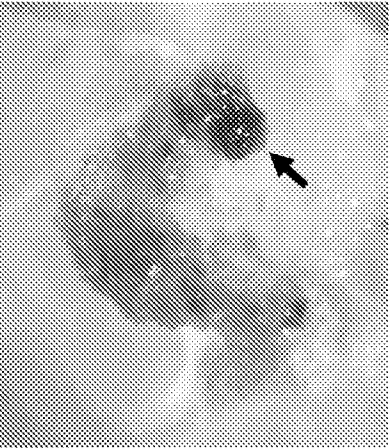
pRN119 (Nos-WUS & 35S-STM,
BeYDV Replicon), P1 #7



Initial Luciferase
Fluorescence

FIG. 14C

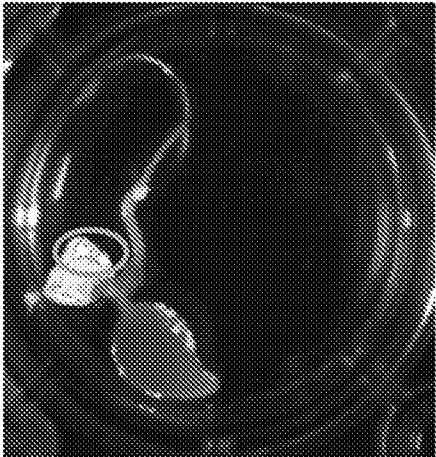
pRN119 (Nos-WUS & 35S-STM,
BeYDV Replicon), P1 #7



24 Days Post
Imaging

FIG. 14B

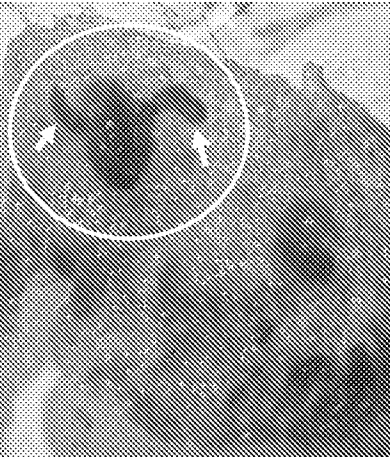
pRN114 (Nos-WUS & CmYLCV-STM,
BeYDV Replicon), P1 #2



Initial Luciferase
Fluorescence

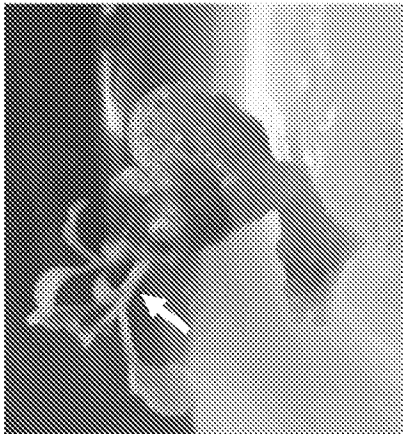
FIG. 14D

pRN114 (Nos-WUS & CmYLCV-STM,
BeYDV Replicon), P1 #2



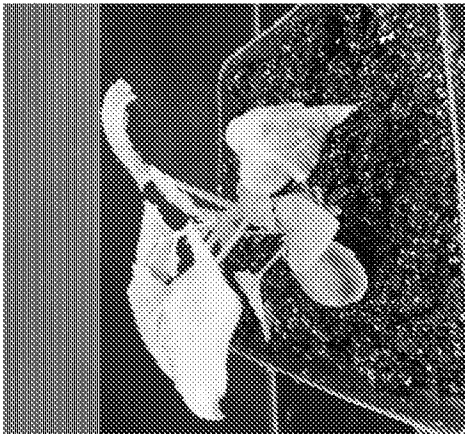
24 Days Post
Imaging

FIG. 15B
pRN119-4-5



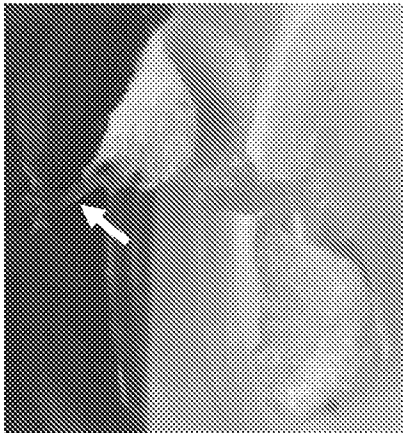
Original Growth

FIG. 15D
pRN119-4-5



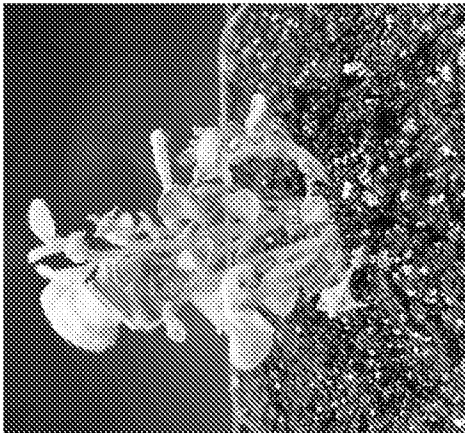
Regenerated
Plantlets

FIG. 15A
pRN120-1-3



Original Growth

FIG. 15C
pRN120-1-3



Regenerated
Plantlets

FIG. 15E

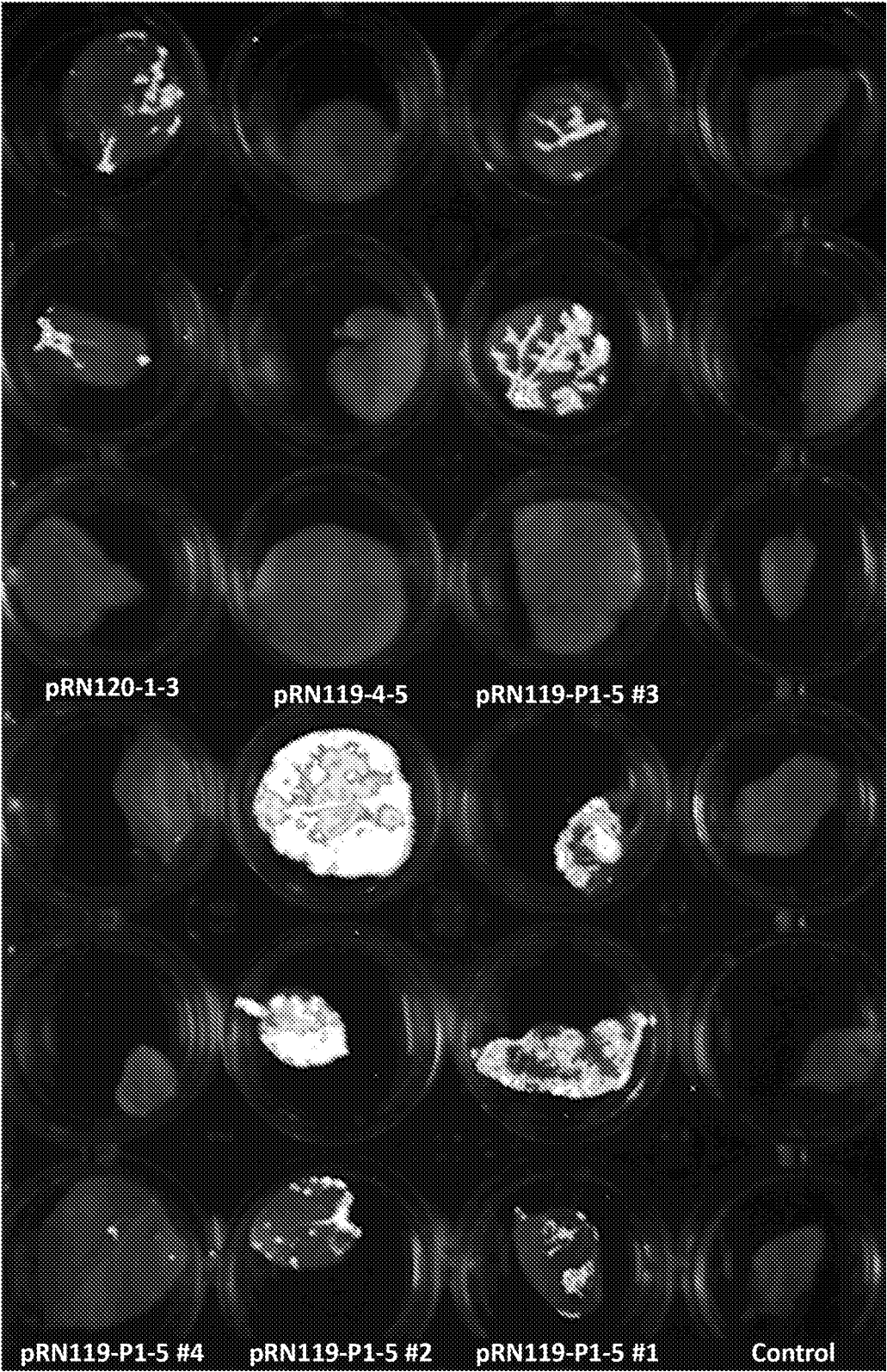


FIG. 16B

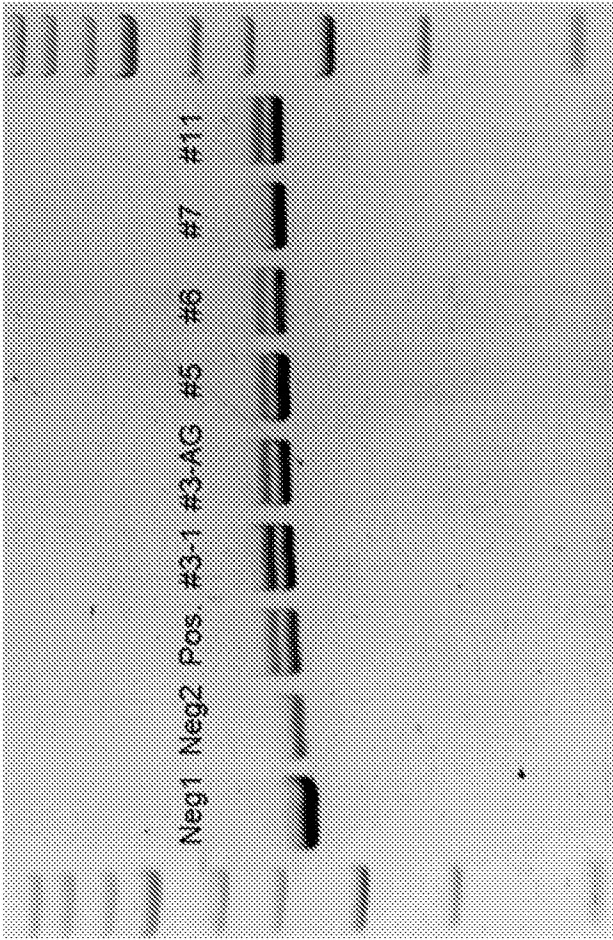


FIG. 16C

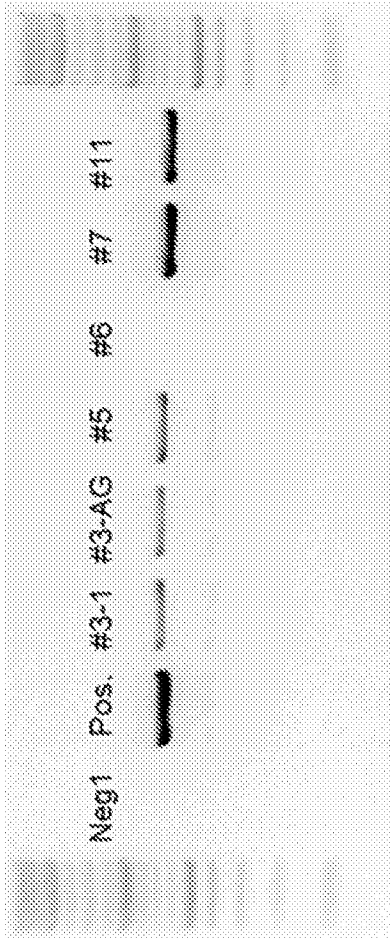


FIG. 16A

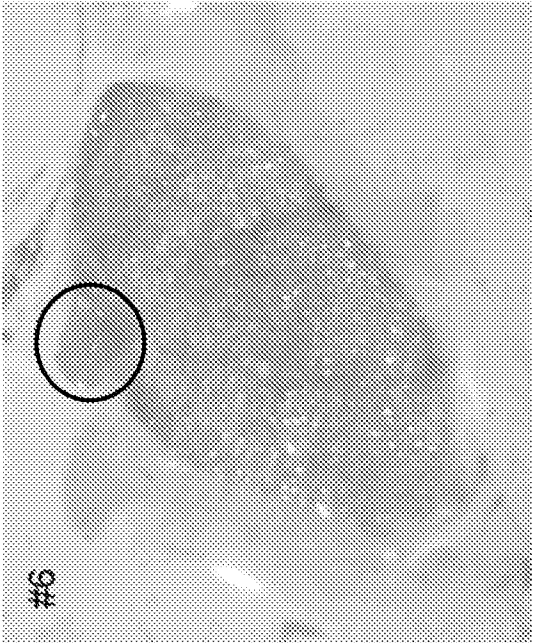


FIG. 16E

#3-AG 32% Editing

G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	C	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	67.76% (4005 reads)		
T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	C	C	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	13.21% (781 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	-	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	7.22% (427 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	-	-	-	-	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.91% (54 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.83% (49 reads)			
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.68% (40 reads)				
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.63% (37 reads)			
G	T	T	T	T	G	G	T	A	G	T	A	G	C	G	A	-	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.61% (36 reads)		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.52% (31 reads)				
G	T	T	T	T	G	G	T	-	-	-	-	-	-	-	-	-	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.52% (31 reads)			
G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	-	-	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.51% (30 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	T	C	C	T	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	0.49% (29 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	-	-	-	-	-	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.46% (27 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	C	C	C	A	-	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.27% (16 reads)		
C	T	T	C	T	G	G	T	C	A	T	G	G	-	-	-	-	C	A	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.25% (15 reads)		
G	T	T	T	T	G	G	T	A	G	T	A	G	C	-	-	-	-	-	-	-	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.25% (15 reads)	
G	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.24% (14 reads)		
T	C	A	A	T	C	A	T	A	G	T	-	G	C	G	A	G	A	C	A	T	C	G	G	T	G	A	G	A	T	T	G	T	C	A	T	G	T	0.20% (12 reads)

FIG. 16G

#6 27.3% Editing

G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	72.40% (6366 reads)		
T	T	T	T	G	G	T	A	G	C	G	A	C	T	C	C	C	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	12.51% (1100 reads)	
G	T	T	T	G	G	T	A	G	C	G	A	C	T	-	C	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	4.75% (418 reads)		
G	T	T	T	G	G	T	A	G	C	G	A	C	T	C	C	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	1.74% (153 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	-	C	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	1.01% (89 reads)	
G	T	T	T	G	G	T	A	G	T	-	-	-	-	-	-	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	0.63% (55 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	-	-	-	-	C	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	0.41% (36 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	-	-	-	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	0.36% (32 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	-	-	-	-	-	A	T	A	A	G	T	T	T	A	G	A	T	A	A	G	T	T	A	G	A	T	0.30% (26 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	-	-	G	C	A	T	A	A	G	T	T	A	G	A	T	T	A	G	A	T	0.28% (25 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	T	A	T	G	G	G	C	A	T	A	G	T	T	A	G	T	T	A	G	A	0.25% (22 reads)
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.25% (22 reads)			
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	-	T	G	G	G	C	A	T	A	A	G	T	T	T	A	G	A	T	0.22% (19 reads)		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	A	T	A	A	G	T	T	A	G	A	T	0.22% (19 reads)		

FIG. 16H

#11	28.0% Editing																				
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	71.71% (8333 reads)
	T	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	11.40% (1325 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	5.06% (588 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	1.73% (201 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.95% (110 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.74% (86 reads)
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.73% (85 reads)	
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.66% (77 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.49% (57 reads)
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.46% (54 reads)	
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.45% (52 reads)
	T	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.40% (46 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.36% (42 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.32% (37 reads)
	G	T	T	T	G	G	T	A	G	T	A	G	T	A	G	T	A	G	T	A	0.22% (26 reads)

FIG. 17C



FIG. 17B



FIG. 17A

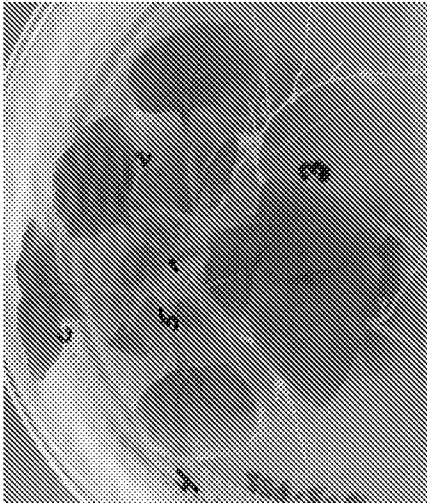


FIG. 17D

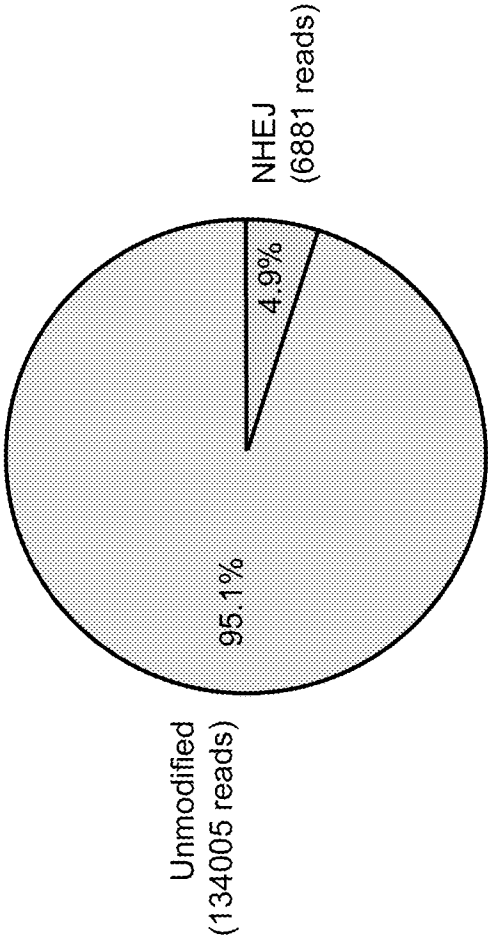


FIG. 17E

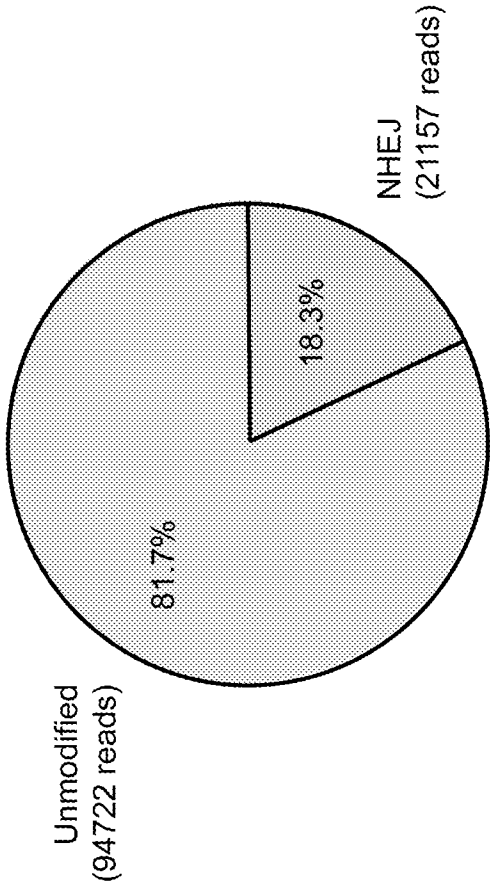


FIG. 17F

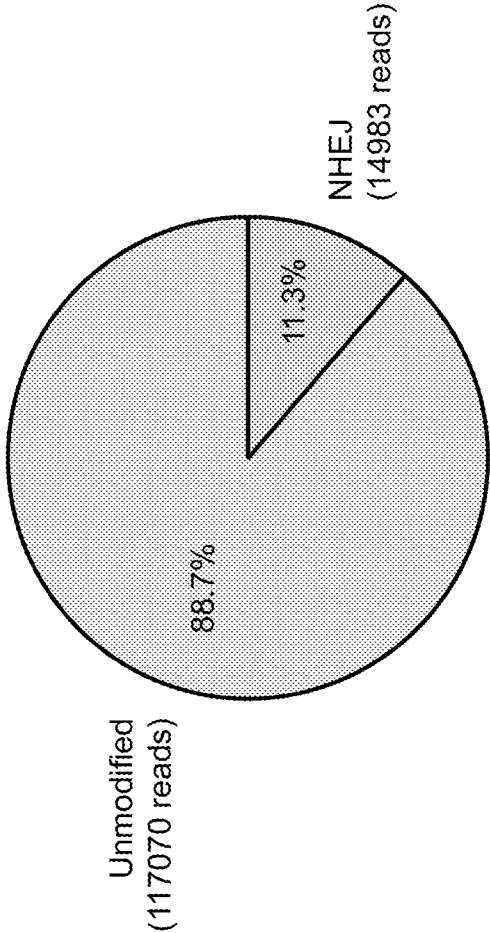


FIG. 17G

G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	A	G	T	T	A	G	A	T	Reference				
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	A	G	T	T	A	G	A	T	92.83% (130789 reads)				
T	T	T	G	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	C	A	T	A	G	T	T	A	G	A	T	1.45% (2036 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	C	A	T	G	G	G	C	A	T	A	G	A	T	0.68% (955 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	A	G	T	G	G	C	A	T	A	G	A	T	0.65% (912 reads)*
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.31% (431 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	A	G	T	T	A	G	A	A	G	0.29% (402 reads)*			

FIG. 17I

G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	G	T	T	A	G	A	T	Reference		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	G	T	T	A	G	A	T	85.53% (112946 reads)		
T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	3.27% (4321 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	G	T	A	G	A	T	2.40% (3170 reads) *
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	2.06% (2718 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	G	T	T	A	G	A	T	0.92% (1210 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.81% (1068 reads)		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	T	T	A	G	A	T	0.58% (766 reads)		
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.42% (559 reads) *	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	-	-	-	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.36% (481 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.35% (465 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	C	C	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.35% (460 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	C	T	-	-	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.32% (426 reads)	
G	T	T	T	G	G	T	A	G	T	A	G	C	G	A	-	-	-	-	A	T	G	G	G	G	C	A	T	A	A	G	T	T	A	G	A	T	0.25% (327 reads)	

FIG. 18A



FIG. 18B

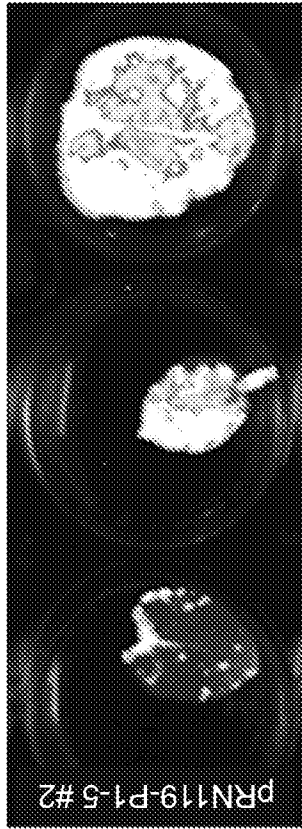


FIG. 18C

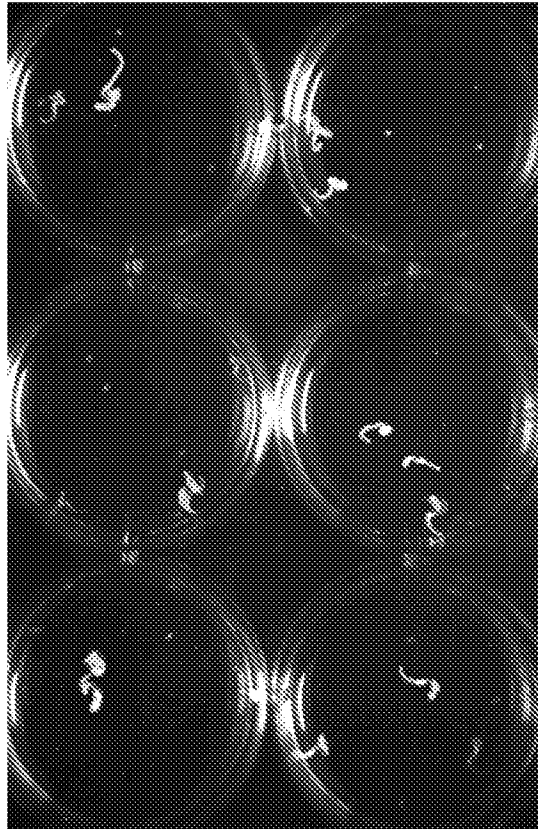


FIG. 18D

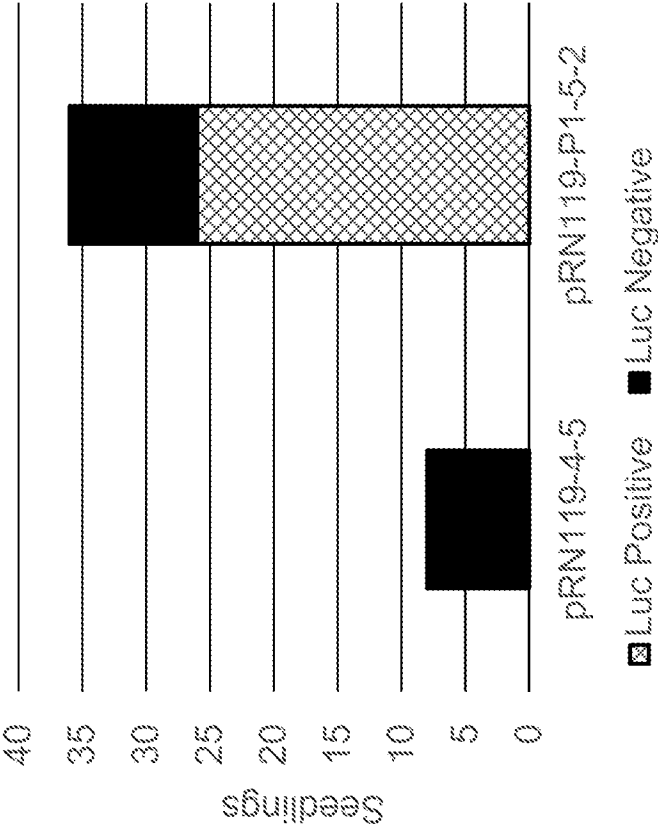


FIG. 19A

Developmental regulator combinations	Constructs co-cultured	Starting # seedlings	Total # growths	# shoot-like growths	# white shoot-like growths	Full plants formed	# edited plants	# plants with developmental abnormalities
BBM	pMM146	24	0	0	0	0	0	0
IPT	pMM134	24	0	0	0	0	0	0
MPΔ	pMM136	24	0	0	0	0	0	0
STM	pMM131	24	0	0	0	0	0	0
WUS	pMM135	24	20	4	0	1	1	1
All	pMM131, 134, 135, 136, 146	30	17	4	0	1	0	1
BBM & IPT	pMM134, 146	36	0	0	0	0	0	0
BBM & WUS	pMM135, 146	31	12	4	0	3	0	0
IPT & MPΔ	pMM134, 136	34	0	0	0	0	0	0
STM & MPΔ	pMM131, 136	29	0	0	0	0	0	0
WUS & IPT	pMM134, 135	27	46	23	3.5	11	2	0
WUS & STM	pMM131, 135	36	29	17	3	11	2	7

FIG. 19B

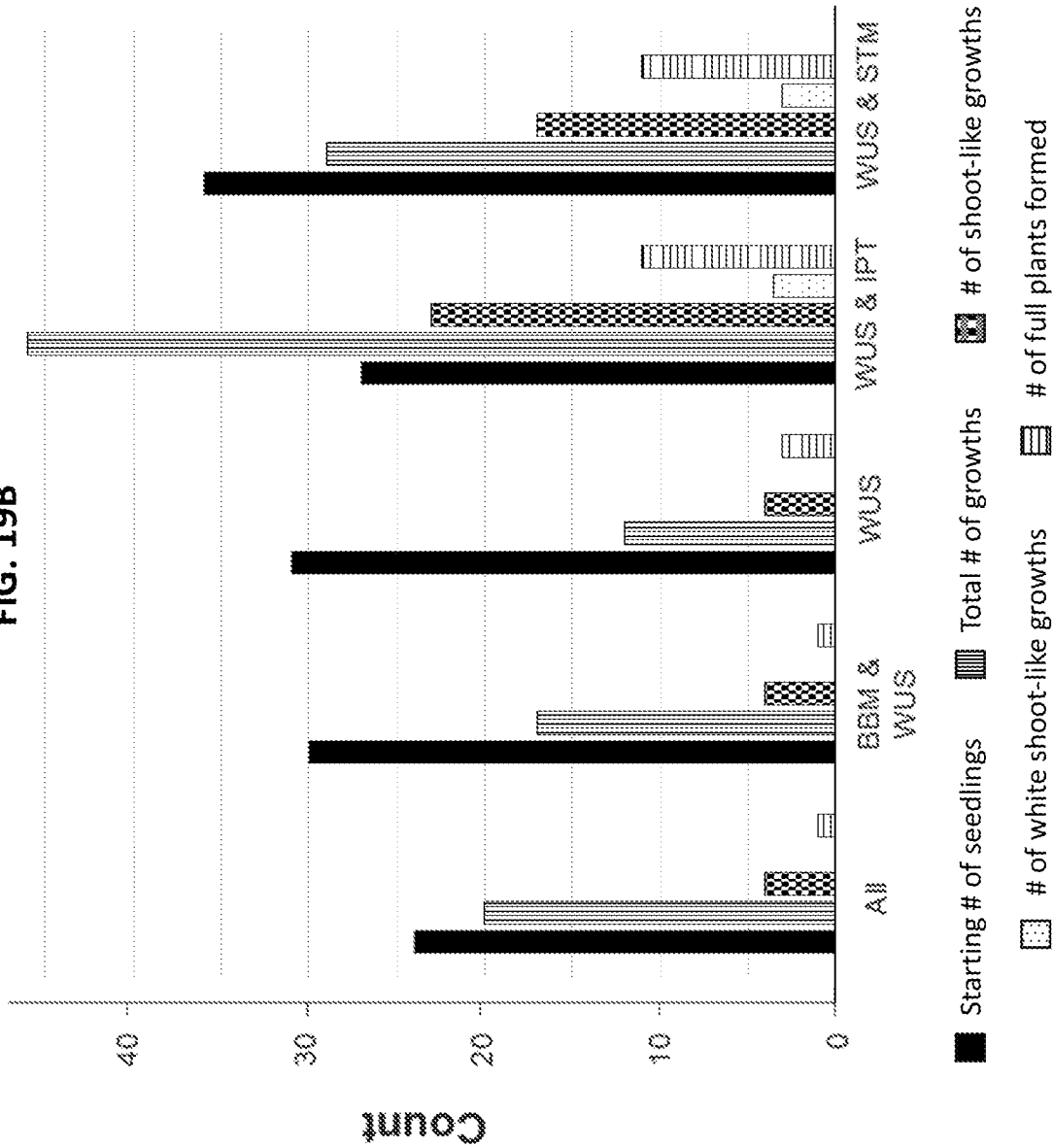


FIG. 20

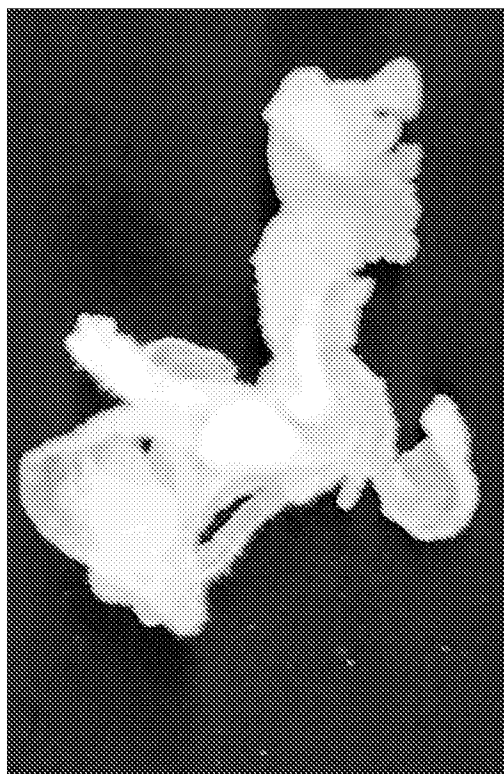


FIG. 21A



FIG. 21B

		(SEQ ID NO:)
<u>PDS1</u>		
WT:	GGAATTTGTTATGTTTGGTAGTAGCGACTC CATGGGGCA	(114)
F4:	#5-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGAC CATGGGGCA	(116)
F4:	#6-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGAC CATGGGGCA	(116)
F6:	#9-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
F6:	#10-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGAC CATGGGGCA	(116)
<u>PDS2</u>		
WT:	GGAATTTGTTATGTTTGGTAGTAGCGACTC CATGGGGCA	(114)
F4:	#5-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)
F4:	#6-GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)
	GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)
F6:	#9-GGAATTTGTTATGTTTGGTAGTAGCGACTCC CATGGGGCA	(115)
	GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)
F6:	#10-GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)
	GGAATTTGTTATGTTTGGTAGTAGCGACT CATGGGGCA	(117)

FIG. 22A

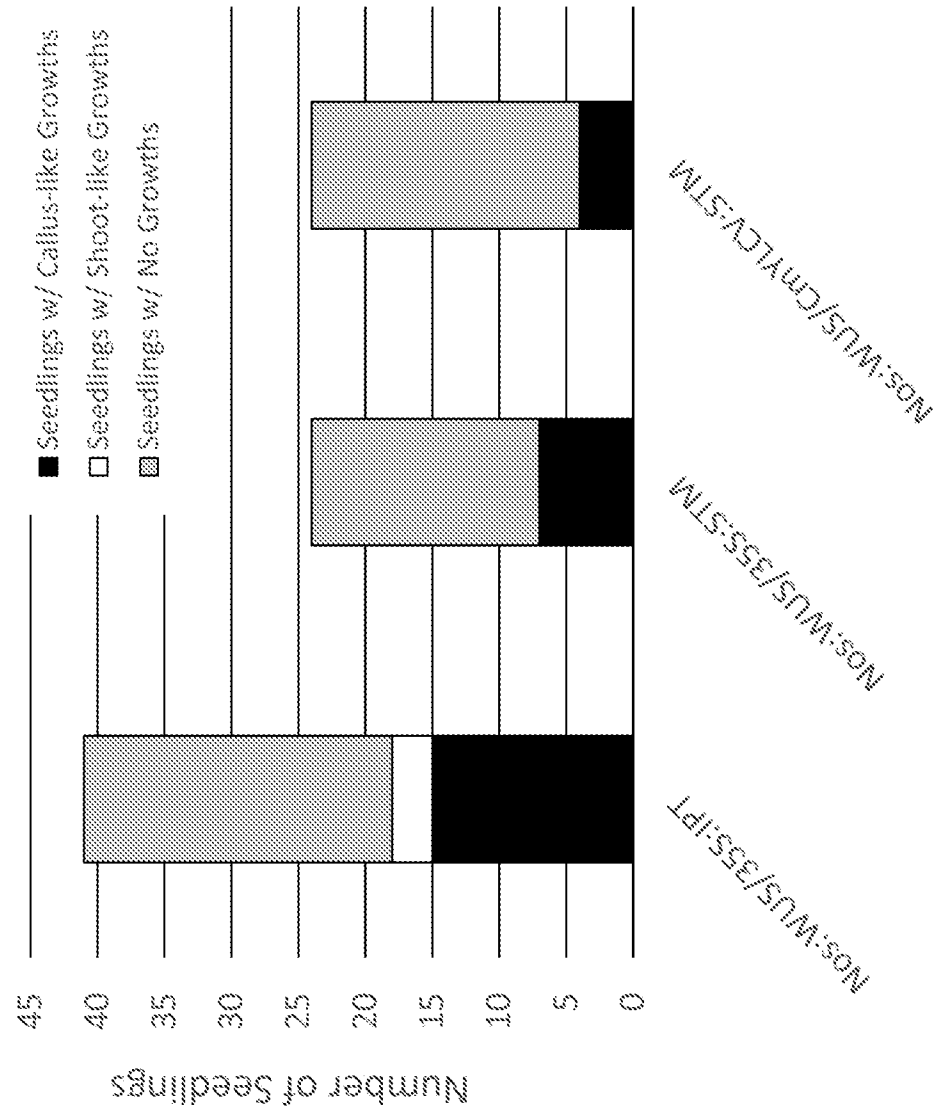


FIG. 22B



FIG. 22C

Developmental Regulators	Total Seedlings	Number w/ Callus-like Growths	Number w/ Shoot-like Growths
Nos:WUS/CmYLCV:STM	24	4	0
Nos:WUS/35S:IPT	41	15	3
Nos:WUS/35S:STM	24	7	0

FIG. 22D

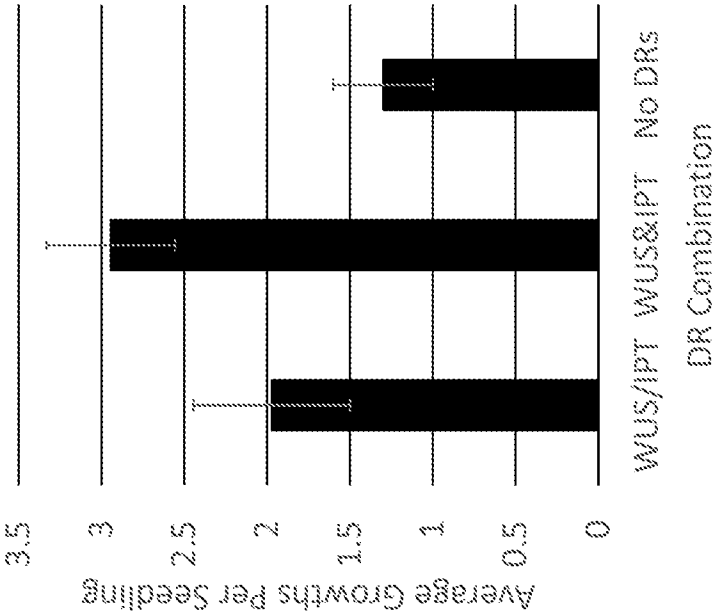


FIG. 22E

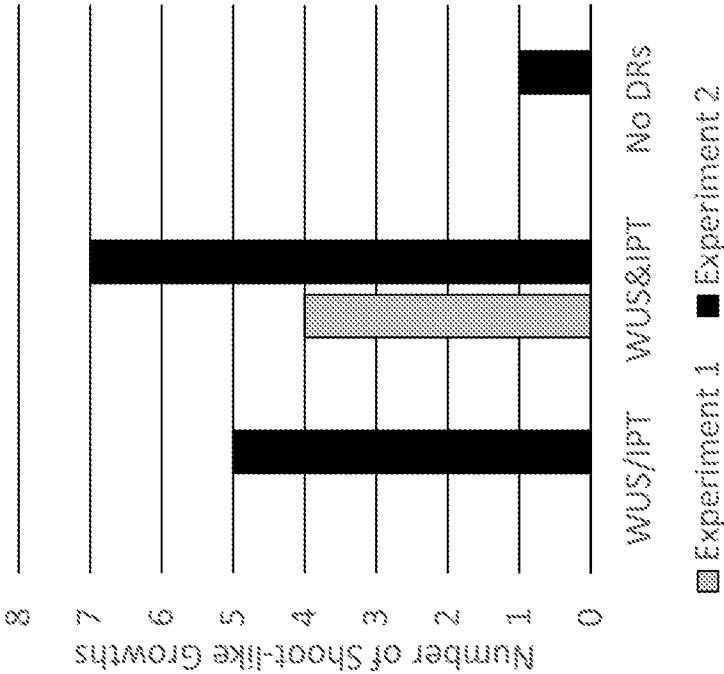


FIG. 22G

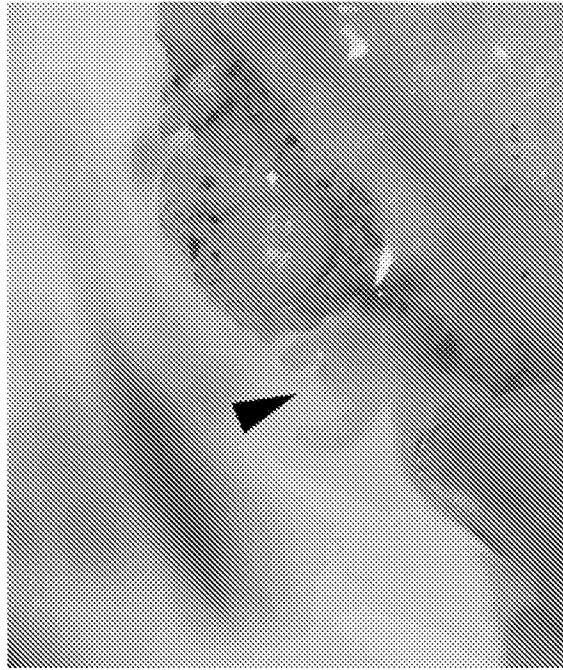


FIG. 22F

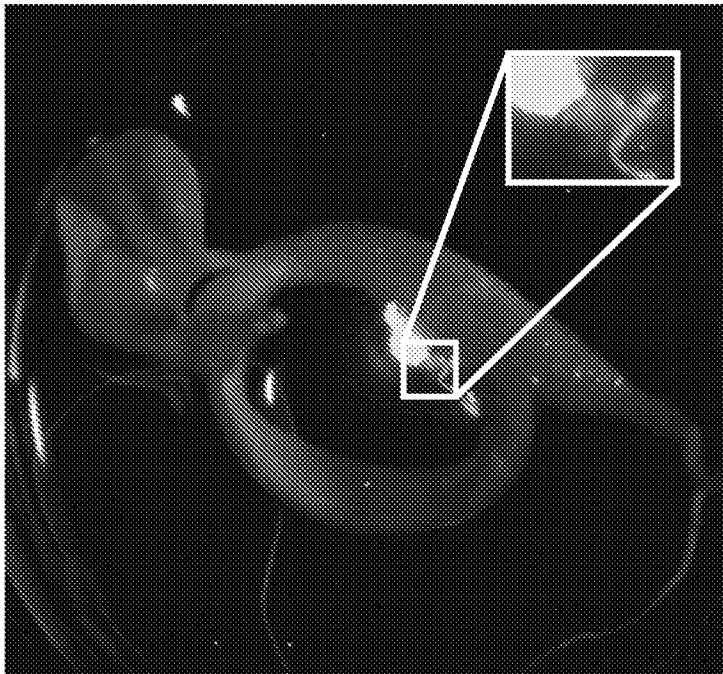


FIG. 22I

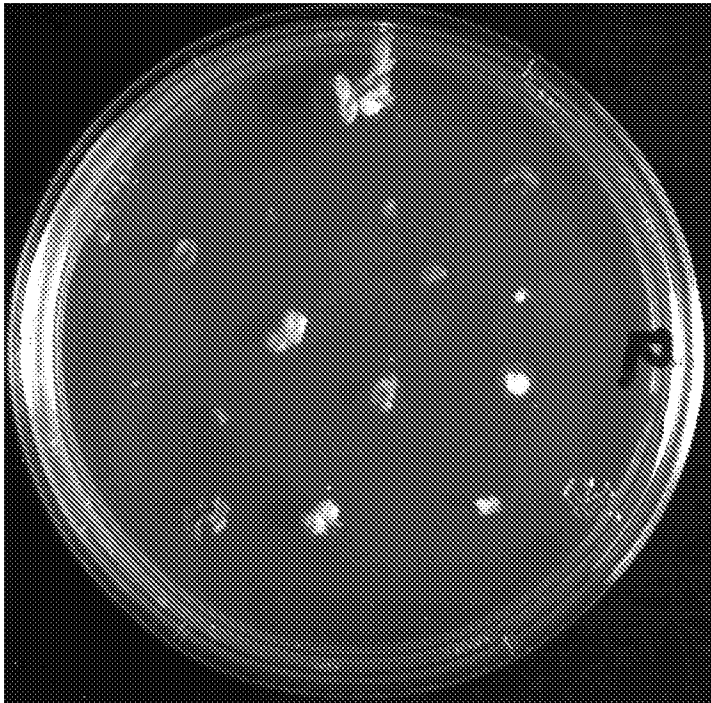


FIG. 22H

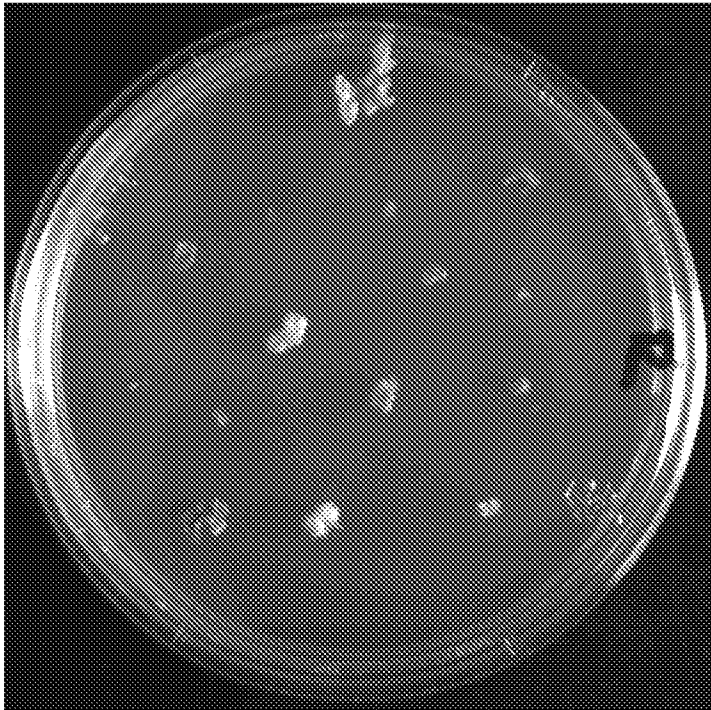


FIG. 22J

Constructs and DR combinations	Expt.	Starting # of seedlings	# of growths	Avg. growths per plant	# Shoot-like growths	Luc + shoot- like growths
WUS/IPT (MKV57)	1	6	9	1.5	0	0
	2	18	44	2.444444	3	3
WUS & IPT (MKV58 & MKV59)	1	6	20	3.333333	4	1
	2	18	46	2.555556	7	3
No DRs (MKV60)	1	5	8	1.6	0	0
	2	18	18	1	1	0

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/39297

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A01H 1/04, 4/00; C12N 15/11, 15/82 (2019.01)

CPC - A01H 1/04, 4/00; C12N 15/11, 15/82, 15/8205, 15/8229, 15/8261

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2018/080389 A1 (SWETREE TECHNOLOGIES AB) 3 May 2018; page 8, lines 18-27; page 35, lines 10-12	1-2, 3/1-2, 4/1-2, 8/1-2, 29/1-2, 30-32/29/1-2
A	WO 2017/123772 A1 (REGENTS OF THE UNIVERSITY OF MINNESOTA, et al.) 20 July 2017; page 3, lines 1-5; page 12, lines 13-23; page 22, lines 1-4; page 26, lines 12-16	1-2, 3/1-2, 4/1-2, 8/1-2, 29/1-2, 30-32/29/1-2
A	US 2018/0051267 A1 (REGENTS OF THE UNIVERSITY OF MINNESOTA, et al.) 22 February 2018; paragraphs [0017], [0079], [0115], [0117]	1-2, 3/1-2, 4/1-2, 8/1-2, 29/1-2, 30-32/29/1-2
A	(MOOKKAN, M et al.) Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators BABY BOOM and WUSCHEL2. Plant Cell Reports. September 2017, Epub 5 July 2017, Vol. 36, No. 9; pages 1477-1491; page 1478, 2nd column, 3rd paragraph; page 1484, 1st column, 1st paragraph; DOI: 10.1007/s00299-017-2169-1	1-2, 3/1-2, 4/1-2, 8/1-2, 29/1-2, 30-32/29/1-2

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

17 September 2019 (17.09.2019)

Date of mailing of the international search report

09 OCT 2019

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/39297

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-7, 9-28 and 33-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.