Plant gene editing through *de novo* induction of meristems

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Abstract

Plant gene editing is usually carried out by delivering reagents such as Cas9 and sgRNAs to explants in culture. Edited cells are then induced to differentiate into whole plants by exposure to various hormones. Creating edited plants through tissue culture is often inefficient, requires considerable time, only works with limited species and genotypes and causes unintended changes to the genome and epigenome. We report methods to generate gene edited dicotyledonous plants through *de novo* meristem induction. Developmental regulators and gene editing reagents are delivered to somatic cells on whole plants. Meristems are induced that produce shoots with targeted DNA modifications, and gene edits are transmitted to the next generation. The *de novo* induction of gene edited meristems sidesteps the need for tissue culture, promising to overcome a bottleneck in plant gene-editing.

Editors summary

Methods to induce edited somatic plant cells to form meristems circumvent tissue culture and enable genome editing of a wider set of plant species.

Plant growth is perpetuated by a stem cell niche located in growing apices, termed meristems. The shoot apical meristem is the progenitor to all above-ground organs such as leaves and flowers. Meristem identity is dictated, in part, by developmental regulators (DRs) – transcription factors, which in *Arabidopsis thaliana* include *WUSCHEL* (*WUS*), *SHOOT*

MERISTEMLESS (*STM*) and *MONOPTEROS* (*MP*)[1]. Because plant cells are totipotent and can be trans-differentiated into other cell types, ectopic expression of specific combinations of DRs in somatic cells has the potential to induce meristems. In *A. thaliana*, for example, meristem-like structures are generated when *WUS* and *STM* or the irrepressible variant of *MP* (ΔMP) are expressed in leaf cells[2,3]. DRs work in conjunction with plant growth regulators – particularly the hormones cytokinin and auxin – to establish and maintain meristem identity[1]. In some dicots, ectopic expression of the cytokinin biosynthesis gene, isopentenyl transferase (*ipt*), is sufficient to induce shoot organogenesis[4,5].

Expression of specific DRs in plant somatic cells can induce other developmental programs. In monocots, such as maize and sorghum, expression of maize *Wuschel2* (*Wus2*) and *Baby Boom* (*Bbm*) promotes somatic cells to form embryos, which develop into whole plants[6-8]. Co-delivering transgenes with *Wus2* and *Bbm* expedites the production of transgenic plants – an approach that avoids the use of traditional tissue culture, wherein DNA is delivered to cells in culture, and plants are regenerated by exposing cells to various hormones. Tissue culture is one of the biggest bottlenecks in creating transgenic and gene edited plants, because it can only be performed with a handful of species, takes from a few to several months, and often causes undesired and unpredictable changes to genomes[9]. The use of molecular reagents such as DRs, which induce specific developmental programs, is a compelling avenue to circumvent traditional tissue culture methods.

Here, we report that concomitant expression of DRs and gene editing reagents creates transgenic and gene edited shoots through *de novo* meristem induction. Further, these shoots produce flowers and seeds and ultimately transmit transgenes and gene edits to the next generation.

Results

Induction of genetically modified meristems on seedlings.

For any given dicot plant species, we reasoned that meristems would be optimally induced by different combinations of DRs. To determine these combinations, we developed a high throughput platform in which constructs expressing different DRs under various promoters are delivered to young seedlings by *A. tumefaciens*. We chose *Nicotiana benthamiana* as a model plant because it is easy to grow, has a short lifespan (~3 months), and DNA delivery methods are well established[10]. To infect seedlings, we modified a protocol (AGROBEST) that was developed for transient transformation of *A. thaliana* seedlings by *A. tumefaciens*[11]. Our protocol, called Fast-TrACC (Fast-Treated Agrobacterium Co-Culture), involves treating *A. tumefaciens* cultures for two days with two types of media prior to culturing seedlings with *A. tumefaciens* for an additional two days (Fig. 1a). Fast-TrACC effectively delivered transgenes to seedlings, as evidenced by expression of a luciferase reporter, particularly within cells of the cotyledons (Fig. 1b).

Fast-TrACC was used to deliver maize *Wus2* and *A. thaliana STM* to *N. benthamiana* seedlings along with a luciferase reporter (Supplementary Table 1; Supplementary Fig. 1a). *Wus2* and *STM* were chosen because their respective roles in meristem cell division and

patterning have been established[12], and ectopic expression of these DRs in *A. thaliana* promotes *de novo* growth formation[2]. *Wus2* was expressed from the weak *nos* promoter and *STM* from one of three strong promoters (*35S*, *CmYLCV*, At*Ubi10*).

From regions exhibiting high levels of localized luciferase expression, callus-like growths formed, presumably due to expression of the DRs (Fig. 1c, Supplementary Fig. 1b-d). Many growths remained in an undifferentiated callus state; however, a subset progressed to form meristem-like structures, as indicated by the production of leaflets (Fig. 1d) and ultimately stems with leaflets (Fig. 1e, Supplementary Fig. 1e-h). These shoot-like growths were transferred to rooting medium, and within approximately two weeks, roots formed, enabling the plants to be transferred to soil.

Having demonstrated that Fast-TrACC can be used to induce meristems, we next tested different combinations of DRs expressed from promoters of varying strength to determine the best combination for producing full plants. Separate *A. tumefaciens* strains, each carrying expression cassettes for a unique DR, were pooled for seedling co-culture. Of twelve tested combinations, only five generated growths from which plants could be derived (Fig. 1f, Supplementary Table 2). Two combinations, *Wus2* and *STM* and *Wus2* and *ipt*, produced up to five times as many shoot-like growths and roughly four times more full plants when compared to other treatments.

We sought to introduce genetic changes in meristems that would then produce flowers and transmit the genetic changes to seeds. Plants generated from *de novo* growths induced by *Wus2* and *STM* (Fig. 2a) were tested for luciferase expression in leaf punches, and luciferase expression was observed in some plants (Fig. 2b). A few transgenic plants showed developmental abnormalities, such as curled leaves, likely due to persistent expression of the DRs (Supplementary Fig. 2, Supplementary Table 2). This was particularly true for plants overexpressing *Wus2* and *STM*. The majority of plants, regardless of their transgene status, produced seed-bearing flowers. Seeds from transgene positive plants were collected, and luciferase expression was observed in the seedlings (Fig. 2c-d). This demonstrates that a heritable transgenic event can be created through *de novo* induction of a meristem.

Fast-TrACC, as a delivery method, provides the opportunity to optimize combinations of DRs for meristem induction in other dicot plants. For example, we tested combinations of *Wus2*, *ipt* and *STM* on tomato seedlings for their ability to induce meristems (Supplementary Fig. 3a). Shoot like growths were induced by *Wus2* and *ipt*, and whole tomato plants could be recovered (Supplementary Fig. 3b). Additionally, shoot-like growths were created that maintained luciferase expression (Supplementary Fig. 3c-d). We expect Fast-TrACC could be used for other species to define the DRs needed for meristem induction and formation of genetically modified shoots.

In addition to creating transgenic plants, we wanted to determine if Fast-TrACC could be used to generate meristems with gene edits and plants that transmit targeted mutations to progeny. In the experiment used to optimize DRs for shoot induction (Fig. 1f), the treated *N. benthamiana* seedlings were transgenic and constitutively express Cas9[13]. In addition to a DR, T-DNAs carried a cassette that expresses a sgRNA targeting a gene involved in

carotenoid biosynthesis, phytoene desaturase (*PDS*), which has two homologs in *N. benthamiana* (Niben101Scf14708g00023.1 and Niben101Scf01283g02002.1, hereafter referred to as *PDS1* and *PDS2*, respectively)[14]. Biallelic knockouts of both *PDS* homologs are expected to result in a white phenotype due to chlorophyll photobleaching[15]. Approximately 15% of the generated shoots showed evidence of photobleaching, but these shoots did not form full plants; their vitality was likely compromised by lack of chlorophyll (Fig. 3a, Supplementary Table 2). Nonetheless, white shoots were evaluated molecularly and found to have biallelic mutations in both *PDS* homologs (Fig. 3c). Thus, Fast-TrACC can generate meristems with gene edits.

Twenty-seven plants were recovered after treatment with various DR combinations (Fig. 1f); five phenotypically normal green plants showed considerable amounts of editing in somatic cells (Supplementary Fig. 4). This frequency of gene editing (i.e. ~18% of plants) is comparable to that attained in *N. benthamiana* in transgenic plants that express Cas9 and sgRNAs[16]; however, our frequency is likely an underestimate, as 15% of the original shoots had lethal biallelic mutations in both *PDS* homologs. For one of the green plants (1–7), seed collected from two flowers (F4 and F6) produced green, white and phenotypically chimeric seedlings (Fig. 3b and 3d, Supplementary Fig. 5a). Target sites for both *PDS* homologs were assessed molecularly for two white seedlings from each flower, and mutations were observed in both alleles of each gene (Fig. 3c). The green/white chimeric seedlings contained the transgene (Supplementary Fig. 5b), suggesting that chimerism is due to ongoing mutagenesis at *PDS*; this is consistent with DNA sequencing data showing new mutations emerging in the chimeric plants (Fig. 3e). Based on this collective data, we conclude that co-delivery of DRs and gene editing reagents can produce shoots with mutations, and these mutations can be transmitted to the next generation.

Induction of genetically modified meristems on soil-grown plants.

Having shown that meristems could be generated on seedlings grown aseptically, we next wanted to determine if we could induce genetically modified meristems on soil-grown plants. Transgenic *N. benthamiana* plants that constitutively express Cas9 were pruned to remove all visibly discernible shoot meristems (Fig. 4a). Cut sites were then perfused with *A. tumefaciens* cultures expressing combinations of DRs (Fig. 4b). As before, all DR expression cassettes included a luciferase reporter to monitor transgenesis and the same sgRNA targeting both *PDS* homologs. Sites of perfusion were monitored for shoots, which emerged approximately 12–15 days after inoculation. As was the case for some shoots induced on seedlings, occasionally adverse phenotypes were observed, such as an abundance of leaves or other developmental abnormalities, likely due to expression of the DRs (Supplementary Fig. 6). After 62 days, tissue was harvested from all shoots and assayed for luciferase activity (Fig. 4c). Groups treated with *Wus2* and *ipt*, *ipt* alone or all five DRs, showed luciferase expression in 6–10% of all shoots (Fig. 4b). In contrast, no luciferase positive shoots were obtained using *Wus2* and *STM* or in the mock treated plants. Based on our ability to generate luciferase positive shoots, we concluded that ectopic delivery of DRs can create transgenic meristems and shoots on soil grown plants.

To determine if *de novo* meristems could be induced on agronomically important species, asexually propagated potato and grape cuttings were injected in sterile culture jars with *A. tumefaciens* strains delivering DRs and a luciferase reporter. For both grape and potato, a subset of plants produced bioluminescent shoots (Supplementary Fig. 7, Fig. 8). In the case of grape, bioluminescent shoots at the three-leaf stage were evident as early as 40 days after delivery of the *A. tumefaciens* strains. Affirming results observed using Fast-TrACC on tomato (Supplementary Fig. 3), DRs can induce transgenic shoots on diverse dicot species.

In the *N. benthamiana* experiment (Fig. 5a, Supplementary Fig. 6), a subset of the induced shoots were white, suggesting biallelic inactivation of the two *PDS* homologs. To assess gene editing, genomic DNA was prepared from all tissue harvested for the luciferase expression assays; the sgRNA target site was PCR-amplified for *PDS2* and submitted to next generation sequencing. In total, targeted edits were observed in six tissue samples, and the percentage of sequencing reads with mutations suggested the edits were fixed in a heterozygous or homozygous state (Fig. 5b, Supplementary Fig. 9). From this data we conclude that by using DRs in combination with gene editing reagents, it is possible to generate shoots with targeted gene edits on soil grown plants.

None of the *N. benthamiana* shoots with developmental abnormalities or the *pds* phenotype set seed. Only one of the six shoots with gene edits (carrying a 3 bp deletion in one *PDS* allele) produced viable seed (Supplementary Fig. 9, Supplementary Table 3). To determine if we could obtain additional gene edited shoots, we performed a second experiment in which *Wus2* and *ipt* were delivered either on the same T-DNA or on separate T-DNAs (i.e. a mixed infection with separate strains). Rather than monitoring the total number of shoots produced, we monitored the number of shoots that emerged from each perfusion site. Previous experiments suggested that initial shoots were often not transgenic and, as such, we removed and discarded shoots appearing in the first 20 days. Abundant shoots emerged regardless of whether the DRs were on the same T-DNA or on T-DNAs in different *A. tumefaciens* strains (Fig. 5c). When on the same T-DNA, for example, 46 shoots were recovered from 76 perfusion sites. Of these, 16 shoots had a distorted phenotype, and four were white or had white sectors, indicative of transgene expression and *PDS* targeting, respectively. In contrast, the negative control produced no white shoots; however, some shoots were initially distorted due to trimming but ultimately developed a wild type growth pattern.

One shoot emerged that was chimeric for white and green tissue, but was otherwise phenotypically normal and non-bioluminescent (Fig. 6). From the white tissue, a flower was produced that set seed, which when germinated, produced only white seedlings (Supplementary Fig. 10). White 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 for the vectors delivered by *A. tumefaciens*, as indicated by lack of both luciferase expression and inability to detect the transgene cassette by PCR (Supplementary Fig. 11). Seed and tissue was additionally harvested from the associated green chimeric sector. Germinated seed segregated in an approximately 3:1 ratio for the *pds* phenotype (Fig. 6, Supplementary Fig. 12). The mutations in the seedlings were the same as those observed in the parental green tissue; however, they were distinct from those observed in white sectors. The green shoot that was

produced in the initial experiment was also shown to transmit mutations to progeny seedlings in the absence of a detectable T-DNA (designator 5-14-1-08; Supplementary Table 3). In conclusion, in three independent cases we induced the formation of meristems on soil-grown plants that carried multiple, targeted mutations and that did not harbor the delivered nucleic acid. All modifications were fixed and were transmitted to progeny in a single generation without the use of plant selection or sterile culture methods.

Discussion

Since its inception over 150 years ago, tissue culture has been an important method for plant propagation, and in recent decades, for applying biotechnology, including transgenesis, to advance both basic and applied plant research[9]. Tissue culture is also crucial for success in plant gene editing applications. Reagents such as CRISPR/Cas9 and sgRNAs are delivered to cells in culture to create DNA sequence changes at single nucleotide resolution. Although regeneration of edited or transformed plant cells by tissue culture has been successful in some plant species and genotypes, it can be time consuming and often introduces unintended changes to the genome and epigenome of regenerated plants[17,18]. Consequently, tissue culture is a bottleneck for the production of gene-edited plants and for engineering novel traits to improve crop varieties[9].

Here, we report two approaches by which DRs and gene editing reagents can be effectively combined to create transgenic and gene-edited plants. In the first strategy, a high-throughput method was implemented that produces edited shoots that transmit edits to the next generation. This approach, named Fast-TrACC, is ideal for identifying the optimal combination(s) of DRs for meristem induction. In the second strategy, gene-edited shoots were induced on soil-grown plants, eliminating the need for aseptic culture.

Both approaches are remarkably efficient, requiring no more than five to 15 plants to create multiple gene-edited shoots. The majority of mutations were fixed, suggesting that editing events occurred early in progenitor cells after delivery of the sgRNA. As an added bonus, many gene-edited shoots lacked transgenes, obviating the need to segregate transgenes away in the next generation. We believe that these methods could substantially accelerate the development of plant lines for commercial use.

In addition to experiments in a *N. benthamiana* model, we generated transgenic shoots on tomato, potato and grape in a fraction of the time of it would take using traditional tissue-culture methods. Although *A. tumefaciens* infects diverse plant species, it does have some host restrictions[19]. We anticipate that other delivery methods, including biolistics or nanoparticles, could be used as an alternative to *A. tumefaciens*. In contrast to the *de novo* induction of transgenic and gene-edited meristems, as shown here, others have had some success in creating transgenic plants by delivering transgenes directly to existing meristems, for example, in the monocot wheat[20]. An alternative approach for *in planta* transformation is to deliver DNA to egg cells; however, this method is only robust in *A. thaliana* and its close relatives using floral dip transformation[21]. We anticipate that use of DRs to create gene-edited meristems *de novo* could eventually extend *in planta* transformation to a broad range

of plant species, enabling rapid production of both transgenic and gene-edited plant germplasm.

Online Methods

DNA constructs.

All DNA constructs were assembled using our plant genome engineering toolkit, which provides a suite of promoters and T-DNA vectors as well as Golden Gate cloning strategies to rapidly assemble vectors[22]. The toolkit allows assembly of up to four modular DNA cassettes on a T-DNA destination vector. T-DNA vectors had either one or two developmental regulators expressed from the *35S*, *CmYLCV*, *AtUBQ10* or *nos* promoters (Supplementary Table 1). Some vectors expressed the RNA guided endonuclease, SpCas9, driven by the *35S* promoter and a sgRNA expressed from the *AtU6* promoter. sgRNAs targeted both of the duplicated *N. benthamiana* phytoene desaturase homologs (Niben101Scf14708g00023.1, designated *PDS1*; Niben101Scf01283g02002.1, designated *PDS2*) (Supplementary Table 4)[14]. A luciferase reporter, driven by either the *35S* or *CmYLCV* promoter made it possible to visually confirm construct delivery to plant cells. All constructs were cloned into a T-DNA backbone that produces geminiviral replicons[22]. The replicons are derived from Bean Yellow Dwarf Virus (BeYDV) and replicate upon delivery to plant cells[23]. Replication increases copy number and consequently leads to high levels of gene expression. Additionally, replicon vectors have the potential to replicate regardless of whether they integrate into the genome, enabling transient expression of developmental regulators. Plasmids in Supplementary Table 1 are available at Addgene along with their corresponding DNA sequences.

Fast-TrACC.

Fast-TrACC is a modified version of the AGROBEST protocol, which involves treating *Agrobacterium tumefaciens* cultures (GV3101) for three days prior to a two day co-culture with newly germinated seedlings[11]. The first step is to grow the cultures overnight (12 hrs, 28°C). Next, to increase expression of *vir* genes, cells are harvested by centrifugation and suspended to an OD_{600} of 0.3 in AB:MES salts (17.2 mM K_2HPO_4, 8.3 mM NaH_2PO_4, 18.7 mM NH_4Cl, 2 mM KCl, 1.25 mM $MgSO_4$, 100 μM $CaCl_2$, 10 μM $FeSO_4$, 50 mM MES, 2% glucose (w/v), 200 μM acetosyringone, pH 5.5) and then grown overnight. Prior to incubating with seedlings, the culture is again centrifuged and resuspended to OD_{600} 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 (1/2 MS salt supplemented with 0.5% sucrose (w/v), pH 5.5).

Seeds are sterilized using 70% ethanol for 1 min and 50% bleach (v/v) for 5 min. They are then rinsed 5 times with sterile water. Seeds are transferred to 6-well plates (~5 seeds per well in 2 mL ½ MS) and subsequently germinated and maintained in growth chambers for 2–3 days at 24°C under a 16hr/8hr light/dark cycle. *A. tumefaciens* is added and the seedlings are incubated for two days before being washed with 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*.

Seedlings are analyzed for delivery of the T-DNA constructs using a luciferase reporter. Luciferin (5μL of 50 mM stock into 2 mL of ½ MS) is added to the liquid culture with the seedlings to bring the concentration to 125 μM. The plate of seedlings is then lightly shaken for five minutes to ensure proper mixing of the luciferin. Long-exposure imaging (5.5 min exposure using a UVP BioImaging Systems EpiChemi3 Darkroom) is then performed to capture the luminescence.

Seedlings showing luciferase expression are monitored for the development of *de novo* meristems. Callus-like "bumps" begin to appear, typically on cotyledons in *N. benthamiana* and on hypocotyls in tomatoes, roughly 12 days after removal of *A. tumefaciens* (Supplementary Fig. 1b-d). Over approximately the next 10–14 days, the bumps continue to grow and either remain in an undifferentiated, callus-like state or begin to form differentiated tissues. Initially, leaf-like structures emerge (Supplementary Fig. 1e-f) and eventually shoot-like structures (Supplementary Fig. 1g-h). The shoot-like growths are excised and transferred to rooting media (1/2 MS, 0.8% agar (w/v), 3% sucrose (w/v), 0.5 mg/L indole butyric acid (IBA), 100 μM timentin). Roots typically form after about a week, but there is considerable variability. After about 12 days on rooting media, enough of a root system has typically developed for transfer to soil. Humidity is elevated by covering the plants on soil with a clear plastic water bottle with the bottom removed. After three days the cap is loosened but left on; after another two days the cap is removed. Finally after another two days the bottle is removed and the plant can be grown in a growth chamber (16 hr days, 22°C).

Induction of meristems on soil-grown plants.

N. benthamiana plants harboring a 35S:Cas9 transgene were grown to maturity (63–66 days)[13]. All plants were culled for all visible shoot meristems, leaving 2–3 nodes and supporting leaves. Plants were immediately inoculated with *A. tumefaciens* cultures at the wound sites using syringes and 31G needles. The *A. tumefaciens* cultures were grown overnight (12 hrs, 28°C) in growth medium (10 mM MES, pH 5.6, 20 uM acetosyringone, 50 mg/L kanamycin, 50 mg/L gentamycin), pelleted at 5,000 rpm for 10 min, suspended in infiltration media (10 mM MES, 150 uM acetosyringone, 10 mM $MgCl_2$) and adjusted to an OD_{600} of 0.2–0.3. Cultures were then incubated at room temperature for 2–4 hrs prior to inoculation.

Plants were observed for shooting at cut sites 38–48 days post inoculation (p.i.). Each injection site with newly formed tissues or meristems was counted as a single event. Shoots were scored for the appearance of white tissue, indicative of loss of *PDS* function, and/or abnormal morphology. Tissue samples were harvested and imaged for bioluminescence as an indicator of transgene presence and expression. DNA was extracted and assessed for mutations at the sgRNA target sites (see below). For the experiment shown in Fig. 5c, all meristems occurring within 20 days of inoculation were culled from all plants.

Grape plants (*Vitis vinifera*, Pixie Pinot Meunier Purple) were asexually propagated on sterile growth media (per liter: 2.41 g of Lloyd & McCown woody plant basal medium with vitamins; PhytoTechnology Laboratories, LLC; 5.7 μM indole-3-butyric acid; 4.4 μM 6-benzylaminopurine; 1.4 μM gibberellic acid; 0.1 g myo-inositol; 2% sucrose; 0.05% casein

hydrolysate; 0.3% activated charcoal; 0.7% agar; 2 ml of Plant Preservative Mixture, Plant Cell Technology; pH 5.76). Existing meristems were removed and inoculated with *A. tumefaciens* strains as described above. Forty days after inoculation, leaf discs were taken from leaves of newly formed shoots. All leaf discs from an individual plant were pooled and imaged for luciferase activity as described above.

Potato plants (*Solanum tuberosum*, Ranger Russet) were sterilely propagated on 1x MS media (3% sucrose, 0.75% plant agar, pH 5.6–5.7) two weeks prior to inoculation with *A. tumefaciens*. Existing meristems were removed leaving 0–1 nodes and 0–1 supporting leaves. Plants were immediately inoculated with *A. tumefaciens* cultures at the wound site using syringes with 31G needles as described above. At approximately 100 days after inoculation, shoots that emerged were harvested and imaged for luciferase activity as described above.

DNA analyses.

DNA was extracted from all collected tissues by CTAB[24]. The target sites for *PDS* were then amplified and either gel or column purified. Primers for amplifying *PDS* targets for next generation or Sanger sequencing are listed in Supplementary Table 4. For amplicons subjected to Sanger sequencing, resulting peak chromatograms were analyzed by TIDE[25] or ICE Analysis (Synthego Performance Analysis, ICE Analysis. 2019. v2.0. Synthego). For amplicons subjected to Illumina sequencing, all primers contained 4bp barcodes in the forward and reverse directions, as well as Illumina adapters (Supplementary Table 4). Fifteen to 20 amplicons were pooled and sequenced using GENEWIZ Amplicon-EZ services. Each pool was demultiplexed for unique forward and reverse adapters using ea-utils[26]. Mutations were assessed for each demultiplexed sample using Cas-Analyzer[27]. Minority read sequences represented less than 10 times were considered background. Samples found to have >30% modified reads at the sgRNA target site, as compared to reference, were considered edited. Samples found to have a single unique sequence modification for >30% and <60% of all reads (with the remainder of sequences being mostly WT) for a given sample were considered to be heterozygous for the observed mutation at that homolog. Samples with a single unique sequence for >90% of reads were considered to be homozygous for a given mutation. Edited samples with <30% of reads consisting of a single mutation were considered unfixed, chimeric, mutations. Reads between 60% and 90% for a single unique sequence were not observed.

Statistics.

No statistical methods were used to predetermine sample size. Samples were blindly processed without designators during collection, sequencing and assessment of editing.

Data availability statement

High-throughput sequencing data have been deposited in the NCBI Sequence Read Archive database under the BioProject accession number PRJNA575069. Sanger DNA sequence data is provide as a Supplementary Data Set. Constructs expressing DRs and gene editing reagents are available from Addgene (plasmids 127210 – 127230, 133312 – 133315).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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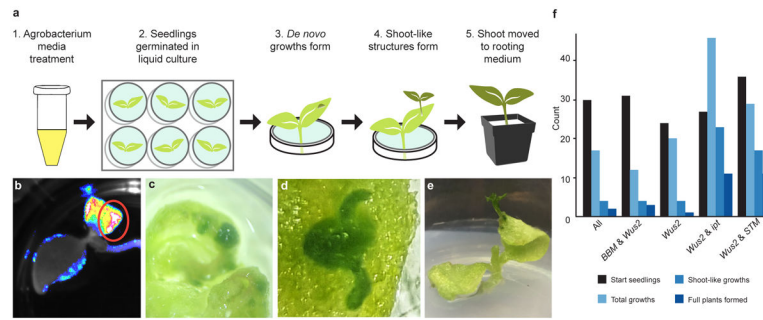
Figure 1. Ectopic delivery of developmental regulators to seedlings induces meristems.
a, The first step in Fast-TrACC is the optimization of *A. tumefaciens* cultures for gene transfer (1). Seedlings are germinated in 6-well plates and co-cultured with the optimized *A. tumefaciens* strains (2). After approximately two weeks, dark green growths begin to form (3) that ultimately produce shoot-like structures (4). The shoot-like growths are then induced to form roots. **b**, Fast-TrACC is effective in delivering transgenes to seedlings, as evidenced by luciferase expression. **c**, When DRs, such as *Wus2* and *STM* (Supplementary Fig. 1), are delivered by Fast-TrACC, globular growths form at sites of high transgene delivery. **d-e**, Some growths turn into meristem-like structures with defined tissues such as leaflets and stems. The growth depicted in **d** was formed on the seedling shown in **b** at the site marked by the orange circle. **f**, To determine combinations of DRs most effective in creating *de novo* meristems, pools of *A. tumefaciens* strains, each with a single DR, were co-delivered to seedlings (Supplementary Table 2). Five combinations produced *de novo* meristems and subsequently plants.
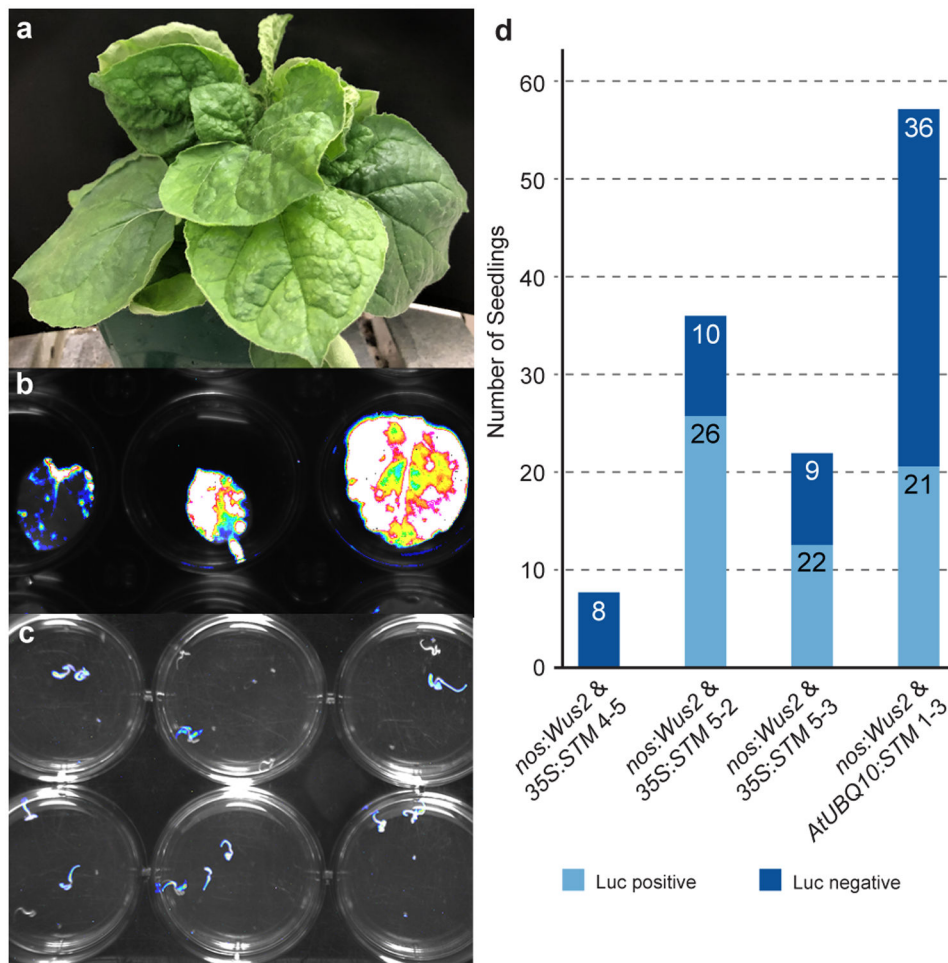
Figure 2. Transgenic shoots transmit transgenes to progeny.
a, A plant (5-3) is shown that was generated by *de novo* meristem induction. Some plants showed distorted, wrinkled leaves, likely due to persistent expression of DRs (see Supplementary Fig. 2). **b**, Many plants, including 5-3, are transgenic, as indicated by luciferase expression in leaves. **c**, Luciferase transgenes are transmitted to progeny, as indicated by luciferase positive seedlings derived from 5-3. The images in panels **a-c** are representative of those obtained from three, independently transformed plants. **d**, To determine the frequency at which the transgene was transmitted to the next generation, seedlings from one luciferase negative plant (4-5) and three luciferase positive plants (5-2, 5-3, 1-3) were germinated and assessed for luminescence.
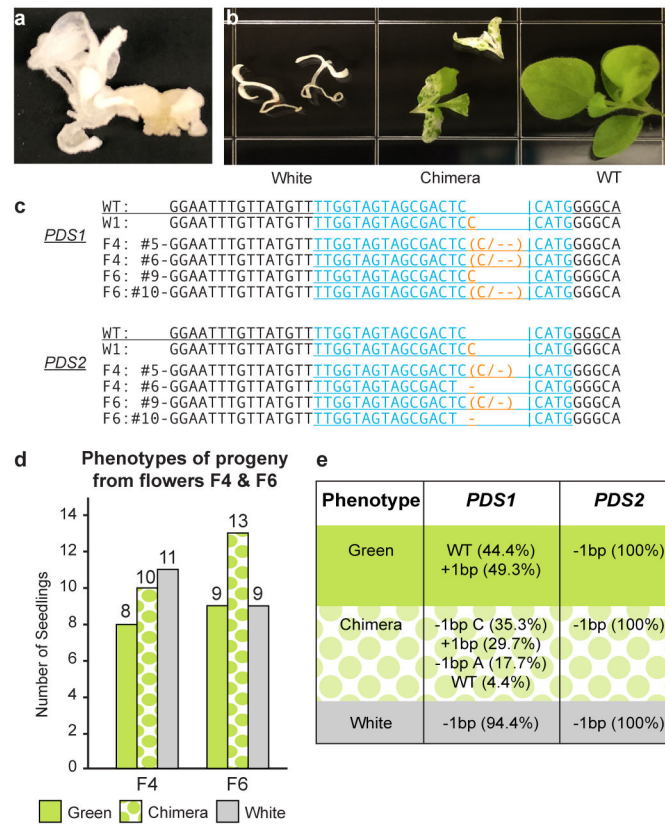
Figure 3. Gene-edited shoots transmit mutations to progeny.

a, DRs were delivered to seedlings that constitutively express Cas9 (Fig. 1f); the T-DNAs also expresses a sgRNA that targets both homologs of phytoene desaturase (*PDS1* and *PDS2*). Approximately 15% of the shoots were white, suggesting biallelic inactivating mutations in *PDS1* and *PDS2*; white shoots could not be grown into full plants. **b and d,** Some green plants (e.g. 1-7) were chimeric for edits at both *PDS* loci. Progeny from flowers F4 and F6 of plant 1–7 had three distinct phenotypes: green, green and white chimeras and white. **c,** DNA was prepared from the white shoot in **a** as well as white progeny from flowers F4 (seedlings 5 and 6) and F6 (seedlings 9 and 10). In all cases, frameshift mutations were observed in both alleles of both *PDS* loci. Shown in blue is the sgRNA target sequence; the predicted Cas9 cut site is represented by a vertical line. Mutations are in orange; mutations in parentheses denote sequences found at each allele; mutations not in parentheses denote identical biallelic mutations. **e,** In contrast to green and white seedlings, chimeric seedlings retain the transgene that expresses the sgRNA (Supplementary Fig. 5). Chimerism is therefore likely due to continued mutagenesis at *PDS1* (note that *PDS2* is a biallelic single bp deletion); this is supported by new alleles appearing in the chimeras whereas alleles are fixed in the green and white seedlings (see also Supplementary data set).
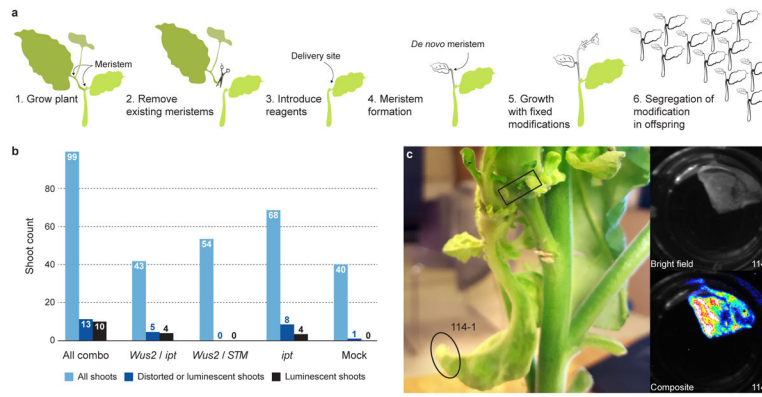
Figure 4. Induction of transgenic shoots on soil-grown plants.
a, Method to create shoots that transmit genetic modifications to the next generation. Plants are grown until apical and axillary meristems are clearly differentiated (1). Meristems are removed (2), and DRs and gene editing reagents are delivered by *A. tumefaciens* (3). Over time, *de novo* gene edited shoots form (4), and editing events are transmitted to the next generation (5,6). **b**, Shoots induced by different combinations of DRs. Phenotypes scored included distorted morphology (likely induced by DRs) and luminescence. All combo = *Wus2*, *STM*, *BBM*, *MP*, *ipt*. **c**, Luciferase activity identifies transgenic shoots. An example of a morphologically distorted shoot is shown. The box denotes the site of *A. tumefaciens* delivery; the circle identifies the tissue that was harvested (sample 114-1). To the left are bright field and composite images showing luciferase activity in the harvested sample.
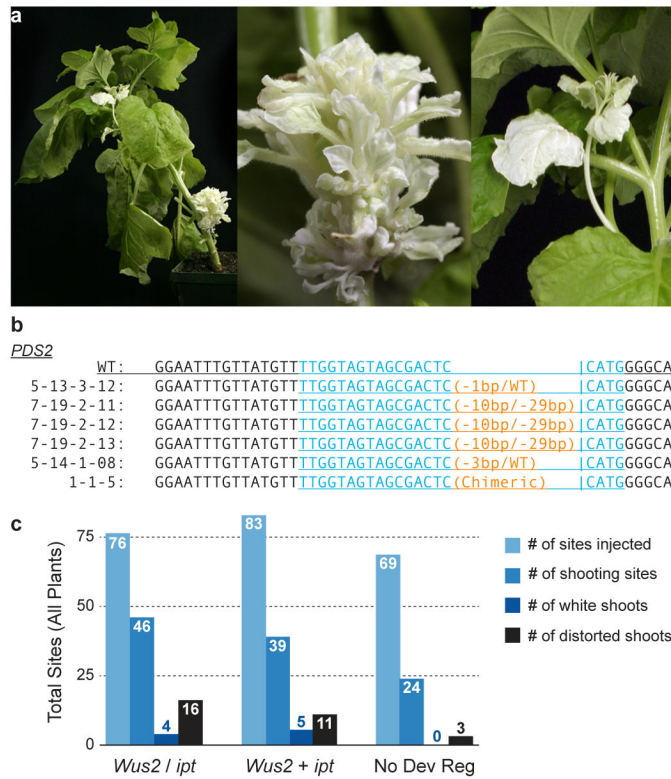
Figure 5. Induction of gene edited shoots on soil-grown plants.
a, Left panel, representative image of a plant with newly formed shoots displaying photobleaching at two delivery sites. Magnified images to the left show that the upper shoot is morphologically wild type; the lower shoot displays developmental abnormalities. The two white shoots shown are representative of the nine white shoots obtained in the experiment described in panel **c**. **b,** DNA sequence of gene edits in induced shoots. Blue bases denote the sgRNA target site. Mutations are in orange; parentheses denote sequences found at each allele of *PDS2*. **c,** Gene edited shoots induced by *Wus2* and *ipt*. Shoot phenotypes scored include distorted morphology and photobleaching.
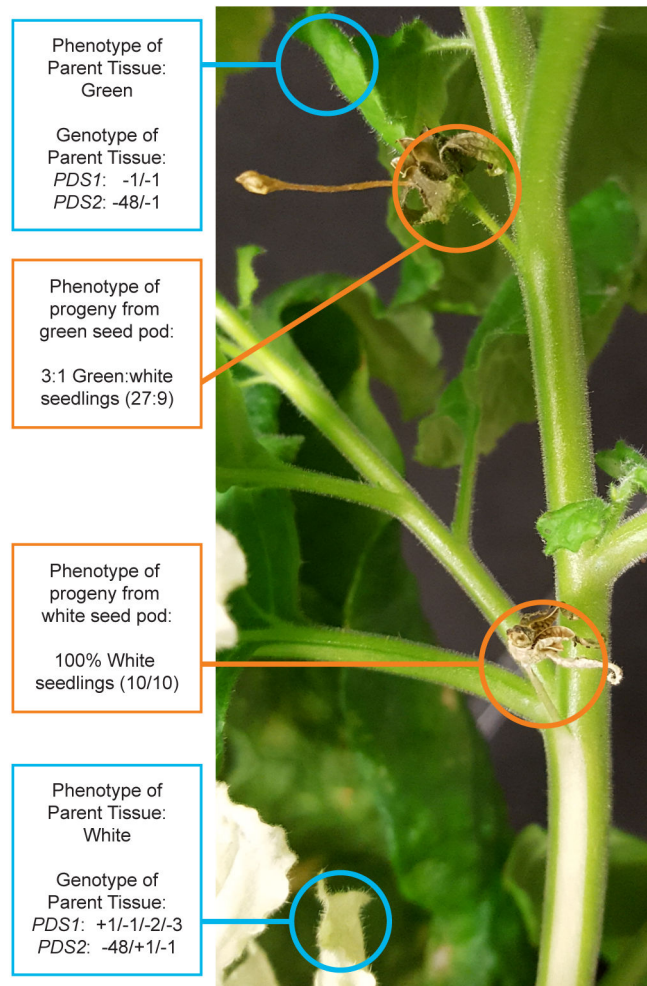
Figure 6. Transmission of gene edits to progeny.

A morphologically wild type shoot that is chimeric for green and photobleached tissue, both of which produced viable flowers and seed (orange circles). To the left are the phenotypes and genotypes of the parental green and white tissues (blue circles). Note the −48bp deletion is in-frame and maintains *PDS* activity. The phenotypes of progeny from the green and white seed pods are indicated; genotypes are presented in Supplementary Fig. 10 and 12).