



Simultaneous targeting of duplicated genes in *Petunia* protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins

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Abstract

Key message We obtained a complete mutant line of *Petunia* having mutations in both *F3H* genes via Cas9-ribonucleoproteins delivery, which exhibited a pale purplish pink flower color.

The CRISPR-Cas system is now revolutionizing agriculture by allowing researchers to generate various desired mutations in plants at will. In particular, DNA-free genome editing via Cas9-ribonucleoproteins (RNPs) delivery has many advantages in plants; it does not require codon optimization or specific promoters for expression in plant cells; furthermore, it can bypass GMO regulations in some countries. Here, we have performed site-specific mutagenesis in *Petunia* to engineer flower color modifications. We determined that the commercial *Petunia* cultivar ‘Madness Midnight’ has two *F3H* coding genes and designed one guide RNA that targets both *F3H* genes at once. Among 67 T₀ plants regenerated from Cas9-RNP transfected protoplasts, we obtained seven mutant lines that contain mutations in either *F3HA* or *F3HB* gene and one complete mutant line having mutations in both *F3H* genes without any selectable markers. It is noteworthy that only the *f3ha f3hb* exhibited a clearly modified, pale purplish pink flower color (RHS 69D), whereas the others, including the single copy gene knock-out plants, displayed purple violet (RHS 93A) flowers similar to the wild-type *Petunia*. To the best of our knowledge, we demonstrated a precedent of ornamental crop engineering by DNA-free CRISPR method for the first time, which will greatly accelerate a transition from a laboratory to a farmer’s field.

Keywords CRISPR-Cas9 ribonucleoproteins · DNA-free gene editing · One step generation · Protoplast regeneration · *Petunia* × *hybrida*

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Introduction

The CRISPR-Cas system is a versatile and cutting-edge technology that allows researchers to generate various desired mutations in plants at will, enabling the development of plant mutants. In contrast with classical genetic methods that require long-term breeding processes, CRISPR modifies a target trait of interest within a few generations in a site-specific manner (Brooks et al. 2014; Feng et al. 2014; Ma et al. 2016; Pan et al. 2016; Subburaj et al. 2016a; Zhang et al. 2014). The most popular method for CRISPR-mediated genome editing in plants is to use an *Agrobacterium tumefaciens*-mediated transformation system to deliver DNA encoding the components. However, there are issues with this method; plant transformation by *A. tumefaciens* is accompanied with T-DNA integration in the host plant genome, frequently inducing unwanted genetic alterations (Clark and Krysan 2010; Jupe et al. 2019; Nacry et al. 1998) and the constitutive expression of CRISPR-Cas9 complexes

during the life cycle can increase off-target mutations in the genome (Zhang et al. 2018). Alternatively, DNA-free genome editing via the delivery of preassembled Cas9 protein and single guide RNA (sgRNA) ribonucleoproteins (RNPs) has many advantages in plants. This method does not require codon optimization or specific promoters for expression in host cells, so it can be used directly in various crop species (Malnoy et al. 2016). Furthermore, Cas9 RNPs cleave target DNAs immediately after transfection and are rapidly degraded in cells, which drastically reduces the number of unwanted mutations at off-target sites (Kim et al. 2014). In this regard, use of the Cas9 RNP system can bypass genetically modified organism (GMO) regulations in some countries (Subburaj et al. 2016a). To date, we and other research groups have demonstrated genome editing via Cas9-RNPs in *Arabidopsis*, rice, lettuce, tobacco, microalgae, grape, apple, and potato (Andersson et al. 2018; Baek et al. 2016; Lin et al. 2018; Malnoy et al. 2016; Subburaj et al. 2016b; Woo et al. 2015). In a continuation of our previous study in which we established a protocol for gene knock-out in *Petunia* protoplasts via Cas9-RNP delivery (Subburaj et al. 2016b), we have now performed site-specific mutagenesis in *Petunia* to engineer flower color modifications.

Modern Solanaceae *Petunia* hybrids (*Petunia x hybrida*, $2n = 14$) are self-incompatible and taxonomically originated from two parental species of *P. axillaria* (two subspecies: *axillaris*, *parodii*) and *P. integrifolia* (three subspecies: *integrifolia*, *inflata*, *occidentalis*) (Bombarely et al. 2016; Stehmann et al. 2009; Vandenbussche et al. 2016). Flower color is an important trait for ornamental plants like *Petunia* because it has a strong influence on their market value. *Petunia* flower colors are determined by genes that are involved in the flavonoid/anthocyanin biosynthesis pathway, in which flavanone 3'-hydroxylase (F3H) is one of the key enzymes. F3H catalyzes the 3-hydroxylation of (2S)-flavanones such as naringenin to dihydroflavonols, which are important for the synthesis of anthocyanidin and flavonol pigments. Thus, the *F3H* gene is thought to be a potential target in flower color engineering. Previously, many studies have aimed to modify flower color by inhibiting or over-expressing the *F3H* gene through stable genetic transformations in ornamentals of the genera of *Torenia*, *Dianthus* and *Nierembergia* as well as *Petunia* (Suzuki et al. 2000; Tsuda et al. 2004; Ueyama et al. 2006; Zuker et al. 2002). Therefore, in this study, we selected *F3H* as a target for editing by the CRISPR-Cas9 system to change *Petunia* color.

Materials and methods

Plant material

Petunia 'Madness Midnight' seeds were purchased from the PanAmerican seed Company (West Chicago, IL, USA). The surface sterilized seeds treated with 2% NaClO solution for 20 min were germinated and sub-cultured every 4 weeks on Murashige and Skoog (MS) basic medium. In vitro plants were grown in culture environments of 16 h/8 h light and dark photoperiod at 2000 lx and 25 °C. Young, fully expanded leaves of the in vitro-grown plants were selected and cut to 1–2 mm strips for protoplast isolation.

Preparation and isolation of *Petunia* protoplasts

Young leaves (about 12–16) from 3-week-old in vitro grown *P. hybrida* cv. 'Madness Midnight' seedlings were pre-plasmolysed with cell protoplast washing (CPW) solution containing 13% mannitol (w/v), followed by cell wall digestion with 1.2% Viscozyme, 0.6% PectinEX, and 0.6% Celluclast enzyme combination (Novozymes, Bagsvaerd, Denmark), according to our previously published methods (Subburaj et al. 2016b) with minor modifications. After protoplasts were collected by centrifugation at 600 rpm for 5 min, cells were further purified by flotation on 25% sucrose in CPW (w/v) with subsequent centrifugation at 600 rpm for 5 min. The middle layer containing the isolated cells were collected, diluted in suitable CPW 9% Mannitol (W/V) solution. Protoplasts were then counted to determine the yield and their viabilities were determined by staining 50 µl of cells with 1 µl of fluorescein diacetate solution (5 mg/ml in acetone). The cells reacted with FDA solution then observed with green fluorescence microscope to count green cell number for protoplast activity. To optimize the protoplast enzyme treatments and yield, triplicates of both technical and biological samples were carried out.

Validation of protoplast viability and transfection efficiency using a transient GFP expression

To determine the transfection efficiency, a transient GFP expression system was carried to optimize the concentration of PEG and incubation period as described elsewhere in our previous study with minor modifications (Subburaj et al. 2016b). Purified protoplasts were allowed to stabilize at 4 °C for 1 h followed by the resuspension in CPW 9% mannitol (w/v) solution. Then the cells were pelletized (600 rpm for 5 min) and further resuspended in a MMg solution (0.4 M mannitol, 15 mM MgCl₂, 5 mM MES) to adjust the cell density into 10⁶ cells/ml according to previous methods. A 250 µl of protoplast (2.5 × 10⁵ cells/ml) cells were mixed

with 50 µg of plasmid vector pBI221 carrying *green fluorescence protein (GFP)* gene (pBI221::GFP). Then a 270 µl of freshly prepared PEG solution [40% (w/v) PEG4000 (Sigma Aldrich), 0.4 M mannitol, 0.1 M Ca(NO₃)₂] was added and were gently mixed until homogenous appearance. The samples were then incubated at room temperature for 20 min. PEG reaction was stopped by adding 250 µl of CPW 9% Mannitol (w/v) washing solution with an incubation for 10 min. Further, a subsequent addition of CPW washing solution (500 µl, 1 ml, 2 ml, and 3 ml) followed by 10 min incubation, the PEG was completely diluted and finally removed by a final centrifugation at 600 rpm for 5 min. To the protoplast pellets, 1 ml culture media was added and kept for incubation in dark at 25 °C. After 24 h, the transfected cells were observed under the green fluorescence microscope. Based on the number of GFP transformed cells, transfection efficiency was estimated.

Purification of recombinant SpCas9 protein

Plasmid pET28a-SpCas9 was provided by Prof. Jin-Soo Kim. BL21-Pro cells (Ezymomics, CP111) were transformed with pET28a-SpCas9 and grown to mid-log phase (OD₆₀₀ nm of 0.4–0.5) in LB medium containing kanamycin at 37 °C with shaking at 200 rpm. Protein expression was induced with the addition of 0.8 mM IPTG, after which cells were incubated overnight at 18 °C with shaking at 200 rpm. Cell pellets were obtained by centrifugation at 4 °C (3100×g, 20 min). After cells were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole), 2 mg/ml lysozyme and 1 mM PMSF were added. The lysate was incubated for 1 h on ice and sonicated (amplitude 25%, 1 s ON/1 s OFF, pulse 30 times) eight times. The lysate was then cleared by centrifugation at 4 °C (18,000×g, 30 min). The supernatant was filtered through a 0.45-µm syringe filter and then incubated with Ni-NTA agarose for 1 h with rotation at 4 °C. The resin was then washed twice with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole). The bound proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole). The eluted proteins were diluted with storage buffer (150 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 2% sucrose, 20% glycerol). The concentration of the purified protein was determined on an SDS-PAGE gel.

sgRNA preparation

For sgRNA preparation, a partial DNA duplex was used as the template for in vitro RNA transcription. DNA oligonucleotides are listed in Supplementary Table 2. The partial duplex was annealed and then extended with Phusion

polymerase (Thermo Scientific, #F530L) and purified using a PCR cleanup kit (GeneAII, 103-102). sgRNAs were then generated by in vitro transcription at 37 °C overnight, after which the template DNA was cleaved with DNA nuclease I (New England Biolabs, M303L) at 37 °C for 20 min. The RNA product was further purified using an RNA cleanup kit (Qiagen, 74204). The final RNA concentration was measured with a NanoDrop device.

Cas9-RNP transfection

Ribonucleoprotein (RNP) complexes were made by combining Cas9 and sgRNA at a molar ratio of 1:3 during *Petunia* protoplast transfection according to the protocol described previously (Subburaj et al. 2016b; Woo et al. 2015). Briefly, 20 µl of transfection reaction mixture, containing 2 µl of Cas9 protein buffer (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.5), 10 µg of Cas9 protein, 30 µg of sgRNA, and an adequate volume of deionized water, was prepared. This mixture was transfected with resuspended protoplasts along with PEG as described in the above validation of protoplast viability and transfection efficiency via the transient GFP expression assay.

NGS-based targeted deep sequencing and mutation analysis

Genomic DNA was prepared from protoplasts and regenerated plants. DNA segments that encompass Cas9 target sites were amplified with Phusion polymerase using pairs of primers that are listed in Supplementary Table 2. PCR amplicons were subjected to paired-end read sequencing using Illumina MiSeq. Obtained next generation sequencing data were analyzed using Cas-Analyzer (<https://www.rgenome.net/cas-analyzer>). Reads that occurred only once were excluded to remove errors associated with amplification and sequencing. Indels located around the Cas9 cleavage site (3 bp upstream of the protospacer-adjacent motif sequence) were considered to be mutations induced by Cas9.

Petunia protoplast regeneration

RNP-transfected protoplasts were cultured in liquid MS medium supplemented with 6% myo-inositol, 2% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 mg/L 6-benzylaminopurine at 25 °C in darkness with shaking (10 rpm). After 1 day, cell division was initiated, multicellular structure was formed at 3 days after Cas9-RNP transfection. Then colonies were formed after 3–4 weeks. Colonies were transferred to callus-induction MS medium supplemented with 3% sucrose, 0.5 mg/L α-naphthaleneacetic acid (NAA) and 2 mg/L BAP. After 3–4 weeks of culture, calli were transferred to shoot

induction MS medium supplemented with 3% sucrose and 1 mg/L zeatin. The regenerated plantlets with health roots were transplanted to soil for hardening those whole plants.

Estimation of anthocyanin content

Petunia flower petals were collected at 0 and 1 day post anthesis. The total anthocyanin content in petals was estimated according to methods in a previous report (Tan et al. 2013). In brief, total anthocyanins were extracted from flower petals by incubation in acidic (1% HCl [w/v]) methanol for 48 h and quantified by measuring the absorbance at 530 nm. Triplicates of three biological replicates were carried out during this estimation.

Data availability

DNA and amino acid sequence information of *F3HA* and *F3HB* have been deposited in NCBI GenBank under accession number MN727296 (*F3HA*) and MN727297 (*F3HB*). Targeted deep sequencing data used in this study have been deposited in the NCBI Sequence Read Archive database (SRA; <https://www.ncbi.nlm.nih.gov/sra>) under accession number PRJNA591067.

Results

Design of guide RNAs that target two *F3H* genes at once in *Petunia*

We used the commercial *Petunia* cultivar ‘Madness Midnight’ as a model system for examining Cas9-RNP-driven *Petunia* color modification. Whole genome annotation has not been completed for this variety, so we first used Sanger sequencing to examine the *F3H* gene. This analysis revealed that ‘Madness Midnight’ has two *F3H* coding genes; one gene (*F3HA*) originates from *P. axillaris* and the other (*F3HB*) from *P. inflata* (Fig. 1a). The two genes have a high degree of similarity at both the nucleotide (93.3% identity) and amino acid (98.9% identity) levels (Supplementary Figs. 1 and 2). We then designed three sgRNAs that target exon 1 or 2 of both *F3H* genes at once (Fig. 1b) (Bae et al. 2014; Park et al. 2015). To estimate the activity of each sgRNA, we transfected in vitro transcribed sgRNAs with purified SpCas9 proteins, derived from *Streptococcus pyogenes*, into ‘Madness Midnight’ protoplasts via polyethylene glycol (PEG)-mediated transformation and performed high-throughput sequencing (or targeted deep sequencing) of bulk genomic DNA isolated from the protoplasts via a next-generation sequencing (NGS) technology, after 24 h (Fig. 1c). As a

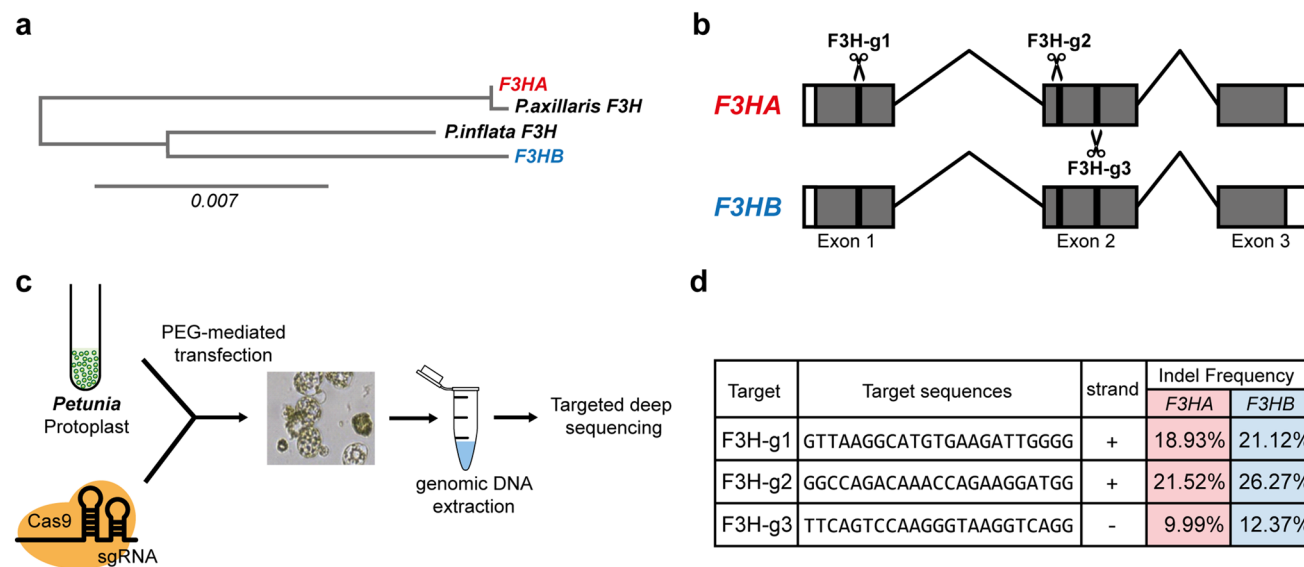


Fig. 1 Simultaneous targeting of duplicated *F3H* genes in *P. hybrida* cv. ‘Madness Midnight’. **a** Phylogeny analysis of *F3H* genes from ‘Madness Midnight’ and the parental wild-type strains. Full nucleotide sequences were aligned with MUSCLE3.8.31 and the phylogeny was analyzed with PhyML3.1/3.0. The tree was prepared using TreeDyn98.3. All of the analysis was accomplished using Phylogeny.fr (<https://www.phylogeny.fr/index.cgi>). **b** Design of sgRNAs simulta-

neously targeting both *F3HA* and *F3HB*. The black bars on the gene diagrams indicate the relative positions of each guide RNA. **c** An experimental scheme of determining indel frequencies after Cas9-RNP transfection in protoplasts. **d** The frequencies of indels near each target region after Cas9-RNP transfection, which were determined by NGS-based targeted deep sequencing

result, we found that the frequency of insertions and deletions (indels) at the targets ranged from 9.99 to 26.27% (Fig. 1d), which were calculated by Cas-Analyzer (Park et al. 2016). The represent mutation types from NGS outcomes are listed in the Supplementary Fig. 3. For further experiments, we chose the target closest to the beginning of *F3H*, F3H-g1, to increase the chances of gene disruption after Cas9-mediated indel mutations.

Successful isolation and regeneration of protoplasts in *Petunia*

To prepare viable and high-quality protoplasts, we employed a modified enzyme combination comprising cellucast (0.6%), PectinEX (0.6%) and Viscozyme (1.2%), and obtained highest yield of $6.90 \pm 0.18 \times 10^6$ cells with an incubation period of 3 h, in which 94.3% of the cells were found to be alive by green colored fluorescence from the FDA test. And plasmid vector (pBI221:GFP) containing GFP were transfected to isolated *Petunia* protoplast cells (2.5×10^5 cells) using a 40% polyethylene glycol (PEG)-mediated transformation, which showed approximately 55% of transfection efficiencies (Supplementary Fig. 4). After Cas9-RNP treatment, we tried to obtain mutant plants through direct protoplast regeneration (Fig. 2a). Briefly, protoplasts transfected with Cas9-RNPs were cultured on colony inducing liquid MS medium. It was found that protoplast cells displayed to divide at or after day 1 of inoculation, and by day 3 the cells appeared to form a mass of multicellular structure. After 3–4 weeks of culture, protoplast derived micro-calli were noticed. When the micro-calli were spread on to the solid callus-inducing medium, friable organogenic calli were appeared after 2–3 weeks. The separated green calli were placed on shooting medium and small protuberances was initially emerged, followed by shoots buds were observed on the surface of the calli after 2–3 weeks. Whole plants with well-developed roots were successfully regenerated in the root-inducing MS medium with 3% sucrose supplementation.

Construction of complete *F3H* knockout mutant line in *Petunia*

Through the regeneration process, we finally obtained 67 regenerated T_0 plants from the F3H-g1 transfected protoplasts, which were derived from 67 independent calli. To investigate the gene mutations in both *F3H* genes, we performed targeted deep sequencing for all regenerated plants. As a result, eight plants (11.9%) showed indel mutations near DNA cleavage sites in *F3H* genes (Fig. 2b); four plants contained homozygous mutations only in the *F3HA* gene, three plants contained homozygous mutations only in the *F3HB* gene, and one plant, named P4C4, contained homozygous mutations in both *F3H* genes. The other plants did not

contain significant mutation frequencies in either gene (Supplementary Table 1). We confirmed the two gene disruptions in P4C4 by Sanger sequencing (Fig. 3a). The 1 bp insertion in the two *F3H* genes in P4C4 would lead to the production of truncated proteins during translation. We checked that the mutations can induce premature termination codons in 78th and 74th positions of *F3HA* and *F3HB* genes, respectively (Supplementary Fig. 5), which eliminate the conserved enzymatic domain found in members of the 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily (pfam03171: <https://pfam.xfam.org/family/PF03171>) (Fig. 3b).

To allow evaluation of the visible flower color phenotype, the regenerated plants were transplanted to soil. Transplants began to bloom approximately 4 weeks later. Using RHS color chart (Royal Horticultural Society, 5th edition), the WT ‘Madness Midnight’ was found to display a purple violet color (RHS 93A). To our surprise, only the P4C4 plant (*f3ha f3hb*) exhibited a clearly modified, pale purplish pink flower color (RHS 69D), whereas the others, including the single gene knock-out plants, displayed purple flowers similar to the wild-type (WT) *Petunia* (Fig. 3c, d). In addition, we quantified the relative total anthocyanin content in flower petals from the WT, single gene mutant, and *f3ha f3hb* mutant plants at 0 and 1 days post anthesis (Fig. 3e). The absorbance values of the *f3ha f3hb* mutant flower petal extracts at 530 nm were significantly lower on both days than those of the WT and single gene mutant *Petunia* petal extracts. This result indicates that *F3H* function was completely knocked out in the mutant *Petunia*, thereby reducing flower petal anthocyanin levels.

To confirm inheritability of the mutations at *f3ha* and *f3hb* genes, those in vitro propagated P4C4 cuttings (T_0) were self-pollinated to obtain T_1 seeds using a bud pollination method to overcome their self-incompatibility. As appeared in Supplementary Fig. 6, all the germinated T_1 plants displayed the same flower colors as pale purplish pink flower in P4C4 line, indicating the stable inheritance of the mutant trait.

Discussion

Following to our previous study reporting a protocol for gene knock-out in *Petunia* protoplasts via Cas9-RNP delivery (Subburaj et al. 2016b), we here performed site-directed mutagenesis to alter two *F3H* genes in *Petunia* using direct delivery of DNA-free Cas9-RNPs. Recent studies showed *Agrobacterium tumefaciens*-mediated plant transformation methods in *Petunia* (Xu et al. 2020; Zhang et al. 2016) but, to the best of our knowledge, DNA-free mutagenesis of *Petunia* in whole procedures including gene knock-out and regeneration is demonstrated in this study for the first time. By targeting the two *F3H* genes with one guide RNA, in

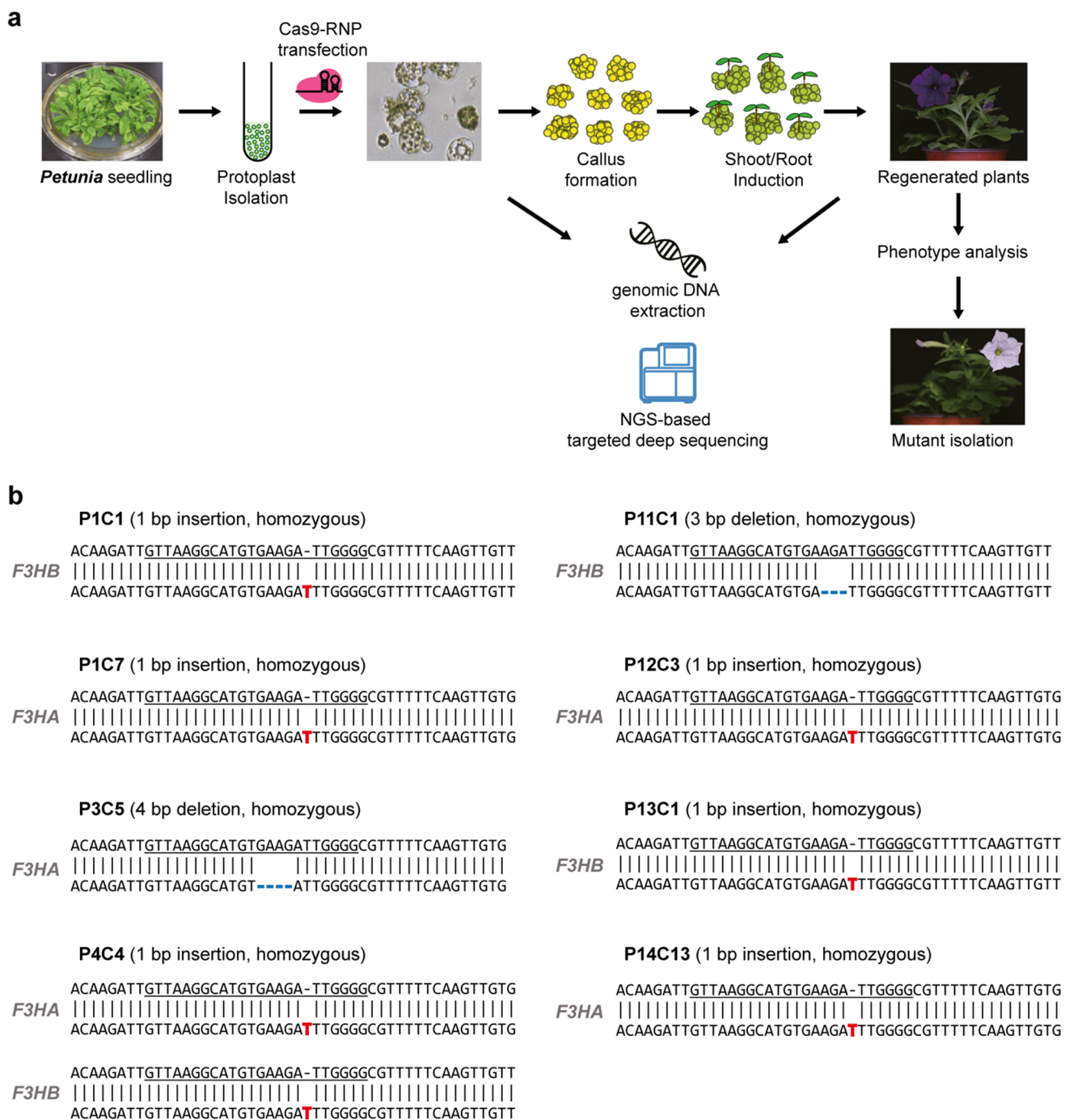


Fig. 2 Process of Cas9-RNP mediated mutant generation in *Petunia*. **a** Schematic procedures showing entire *Petunia* regeneration from protoplasts for targeted mutagenesis of *F3H* via Cas9-RNP. **b** Genotypes of both *F3H* genes in regenerated T_0 plants. Among 67 T_0 plants, eight plants showed mutations in either the *F3HA* or the

F3HB gene, and the P4C4 line contained a frameshift mutation in both genes. Red colored sequences indicate nucleotide insertion, blue bars indicate deletion compared to wild-type sequence, and underbars indicate target sequences of Cas9 (color figure online)

one step we obtained total eight mutant plants (11.9%) in 67 regenerated T_0 plants without any selection processes such as resistance genes. Among the eight mutants, one mutant line (*f3ha f3hb*) ultimately contained homozygous mutations in both *F3H* genes, whereas the others contained mutations

in either *F3HA* or *F3HB* gene. Notably, only the *f3ha f3hb* exhibited a clearly modified, pale purplish pink flower color (RHS 69D), whereas the others displayed purple violet (RHS 93A) flowers similar to the wild-type *Petunia*, indicating that complete knock-out of *F3H* genes is necessary for flower

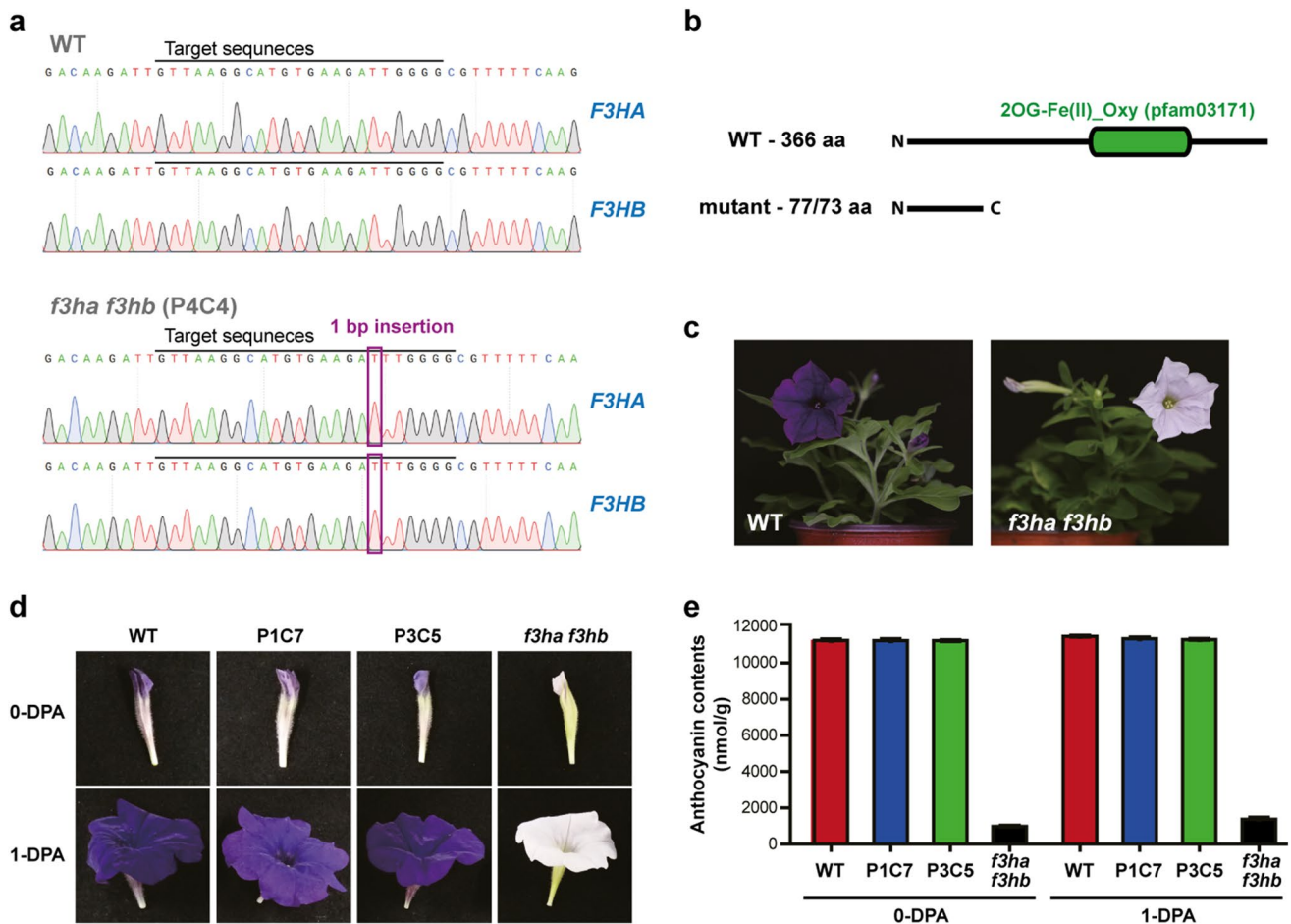


Fig. 3 Isolation of *f3ha f3hb* mutant and its flower traits. **a** *F3HA* and *F3HB* mutations in the *f3ha f3hb* mutant (P4C4). *F3H* sequences of WT and *f3ha f3hb* mutant were confirmed by Sanger sequencing. **b** Domain organization in the F3H protein. The rounded green rectangle indicates the conserved enzymatic domain (2OG-Fe(II)_Oxygenase superfamily; pfam03171) in the WT F3H protein (upper). A predicted mutant protein is also represented (lower; 77 aa for *F3HA* and 73 aa for *F3HB*). A premature termination codon would lead to

color modifications. It is well known that F3H is a key enzyme of anthocyanin biosynthesis pathway and that the suppression of *F3H* gene could induce white and pale shades colors of *Petunia* flower (Tsuda et al. 2004).

Previously, *A. tumefaciens*-mediated *F3H* gene mutations in the model plant *Torenia* (*Torenia fournieri* L.) generated various flower color phenotypes (Nishihara et al. 2018), which were mainly caused by the chimeric (mosaic) genotypes. It is known that the *Agrobacterium tumefaciens*-mediated delivery of CRISPR-Cas9 nucleases enable continuous expression of CRISPR complexes, resulting in various chimeric genotypes in tissues or organisms. In this study, however, we adopted a Cas9 RNP-mediated transformation strategy in *Petunia* and did not any observe chimeric genotypes in each regenerated T_0 plant from NGS outcomes, similar to

a truncated protein that would lack the functional domain. **c** Flower color phenotypes of WT (left) and the *f3ha f3hb* (P4C4, right) mutant. **d** Flower pictures of WT, single gene mutants (P1C7 and P3C5), and the *f3ha f3hb* (P4C4) at 0 and 1 day post anthesis (DPA). **e** Estimation of the anthocyanin content in WT and *f3ha f3hb* mutant flower petals at 0 and 1 DPA. Error bars represent the standard deviation of the mean, and three repeats of the experiment were performed

the previous studies (Woo et al. 2015) using the Cas9 RNP-mediated transformation strategy. As described above, it is supposed that Cas9 RNPs cleave target DNAs immediately after transfection and are rapidly degraded in protoplasts, dominantly generating homozygous or heterozygous genotypes, rather than the chimeric genotypes in each plant.

As seen in this study, orthologous or paralogous genes, which occur widely in plant speciation, should be investigated when the CRISPR-Cas system is applied to target-specific gene editing. Furthermore, our method is a feasible means of creating novel traits in *Petunia*, which can potentially be exempted from GMO regulations because of the absence of any foreign DNA (Waltz 2016); thus, we expect that more extensive applications in ornamental plant breeding will be forthcoming.

Author contribution statement S.B. and G.J.L. conceived this project; T.L., S.S., and J.Y. performed the experiments. J.Y. performed bioinformatics analyses. J.Y., S.S., G.J.L., and S.B. wrote the manuscript with comments of all other authors. S.B. and G.J.L. supervised the research.

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Compliance with ethical standards

Conflict of interest G.J.L., T.L., and S.S. are co-inventors on a patent application related to the methods described in this manuscript.

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