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Original Paper

Genome editing in *PDS* genes of tomatoes by non-selection method and of *Nicotiana benthamiana* by one single guide RNA to edit two orthologs

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Abstract The CRISPR/Cas9 system is widely used for targeted mutagenesis in many organisms including plants. For application of this system, tissue culture methods need to be established. In this study, detailed methods for introduction of mutations in tomato and *Nicotiana benthamiana* plants using the CRISPR/Cas9 system are described. The methods include tissue culture protocols for tomato and *N. benthamiana*. We also demonstrate the methodology to generate Cas9-free genome edited tomato plants and use of one single guide RNA (sgRNA) to edit two orthologs in *N. benthamiana*. The examples of editing the *PHYTOENE DESATURASE* (*PDS*) genes in these plants are also provided. The Cas9-free tomato line was obtained when tomato plants were cultured on a non-selective medium after transformation with the CRISPR/Cas9 system. Two orthologs of *PDS* in *N. benthamiana* were mutated using a sgRNA, because these orthologs contain the same nucleotide sequences with PAM motif. These mutations were inherited to the next generation. The mutations in the *PDS* genes resulted in an albino phenotype in tomato and *N. benthamiana* plants. These results demonstrate that the non-selective method is one of the ways to obtain Cas9-free genome editing in tomato plants and that the two orthologs can be edited by one sgRNA in *N. benthamiana*.

Key words: albino, CRISPR/Cas9, genome editing.

Introduction

Development of sequence-specific nuclease-based technologies, including zinc finger nucleases (ZFNs) (Kim et al. 1996), transcription activator-like effector nucleases (TALENs) (Bogdanove and Voytas 2011), and clustered regulatory interspaced short palindromic repeat (CRISPR)-associated protein system (CRISPR/Cas9) (Doudna and Charpentier 2014), has enabled site-specific modification of genomes. Genome editing techniques were first applied to bacteria and mammalian cell lines, but were rapidly used for modifying plant genomes. In several plant species, including tomatoes and tobaccos, CRISPR/Cas9-mediated gene editing is a useful tool for introducing mutations in genes of interest. This precise and straightforward strategy to edit genes of interest is widely available for investigation of gene function and for production of different varieties (Yamamoto et al. 2018b).

Tomato and tobacco are members of the *Solanaceae* family. Tomato, *Solanum lycopersicum*, is the top ranked vegetable grown over the world and about 180 metric tons of tomatoes are produced every year. The major goals of tomato breeding are diversified, such as high productivity, biotic and abiotic stress tolerance, and high nutritive value of the fruit. Tomato is also a model species for basic and applied research in fields, such as genetics, fruit development, and disease resistance, because tomato plants have a short life cycle, are easy to cross and self-pollinate, and are transformed with a high success

Abbreviations: ALS, acetolactate synthase; BAP, 6-benzylaminopurine; CRISPR, clustered regulatory interspaced short palindromic repeat; GABA, γ-aminobutyric acid; NAA, 1-naphthaleneacetic acid; PAM, protospacer adjacent motif; PDS, phytoene desaturase; sgRNA, single guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

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rate. To produce a desired tomato cultivar, management of tomato genetic resources and diversification of traits are required. Our group produced mutant lines of tomato cv. Micro-Tom as genetic resources (Saito et al. 2011; Shikata et al. 2016). Furthermore, a lot of genetic and genomic resources and several databases are available. Recently, genome-editing techniques have been applied to tomato. These advances have contributed to the promotion of tomato research, especially in the area of plant physiology (Shimatani et al. 2017; Rothan et al. 2019).

Nicotiana benthamiana is a wild relative of tobacco, a member of the *Solanaceae* family. Because of high amenability to *Agrobacterium*-mediated transient expression of transgenes, this species is mainly used for production of recombinant proteins, including antibodies, virus-like particles, other pharmaceutical proteins (Donini and Marusic 2019; Kopertekh and Schiemann 2019; Yamada et al. 2020), and natural products such as alkaloids, lignans, betalains, and terpenoids (Reed and Osbourn 2018), and for identification of protein function (Miura et al. 2020). Using this species, high yield of recombinant proteins can be obtained within 3–10 days by transient expression using 'deconstructed' viral vector system, such as the magnICON (Marillonnet et al. 2005) and the Tsukuba system (Yamamoto et al. 2018a). By using these systems, protein yield is about 4–5 mg GFP/g fresh weight, which is comparable to the yield obtained using other heterologous expression systems, such as *E. coli* and baculovirus. Because *N*-glycan, with core of α(1,3)-fucose and β(1,2)-xylose, in the plant-derived glycoproteins are absent in mammalian-derived glycoproteins, *N. benthamiana* plants lacking two *FucT* genes and two *XylT* genes, which encode α(1,3)-fucosyltransferases and β(1,2)-xylosyltransferases, respectively, were produced by genome editing (Li et al. 2016). Plant-specific modifications sometimes negatively affect the quality of pharmaceutical proteins. Thus, the genome editing techniques are required for engineering *N. benthamiana* to produce more suitable plants for production of pharmaceutical proteins.

The methodology is important to produce plants amenable to genome editing. To produce transgene-free genome editing plants, generally, transformants harboring the *Cas9* gene and, then, sgRNA are produced and the transgenes are removed by segregation. Sometimes, it takes time to remove the transgenes by crossing with wild type plants. If the transgene-free plants are produced in the T_0 generations, it shortens time to produce genome editing plants. Another modification is required for plant genome editing method. When two genes are edited, two or more guide RNAs are produced for targeting these genes. The production of multiplex sgRNAs is now improved, but it still takes time. Because of redundancy, two or more isozymes are knocked out to obtain the desired phenotype. Some genes encoding isozymes contains the same sequences in 20 bases. When using only one sgRNAs to knock out both genes, it is easy to prepare the plasmid.

Recently, transgene-free genome editing has been performed in several plants (Metje-Sprink et al. 2019). Preassembled Cas9 protein and guide RNA were introduced into protoplasts of Arabidopsis, tobacco, lettuce, and rice and up to 46% of regenerated plants contained targeted mutagenesis (Woo et al. 2015). By using particle bombardment to immature wheat embryos, CRISPR/Cas9 DNA or RNA was delivered. After growth of callus without selection, from 1.0 to 9.5% of regenerated plants contained mutation (Zhang et al. 2016). Tomato and potato ACETOLACTATE SYNTHASE (*ALS*) genes were edited by CRISPR/Cas9 with cytidine base editors by using *Agrobacterium*-mediated transient expression. And 12.9% and 10% edited tomato and potato, respectively, without transgene in the first generation were obtained (Veillet et al. 2019). In wheat, 5.2% of plants carried the mutation in *TaGASR7* by biolistic delivery of gold particles coated with plasmid expressing CRISPR/Cas9 (Hamada et al. 2018).

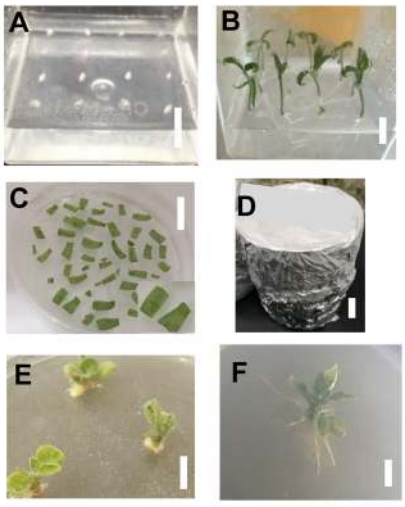
In this study, detailed methods for introduction of mutations in tomato and *N. benthamiana* plants using the CRISPR/Cas9 system targeting *SlPDS1* or *NbPDS1* and *NbPDS2*, respectively, are described. For genome editing in tomatoes, the transgene-free genome edited plants were obtained. For genome editing in *N. benthamiana*, two genes were simultaneously mutated by one sgRNA.

Materials and methods

Construction of plasmids for genome editing

To introduce the mutation in tomato *PDS1* gene, DNA oligonucleotides, SlPDS1-F (5′- GAT TGT AAC GAT CGA TTG CAA TGG A-3′) and SlPDS1-R (5′-AAA CTC CAT TGC AAT CGA TCG TTA C-3′), were annealed and ligated into the *Bsa*I-digested pZD_AtU6gRNA_HolCas9_NPTII vector (Watanabe et al. 2017).

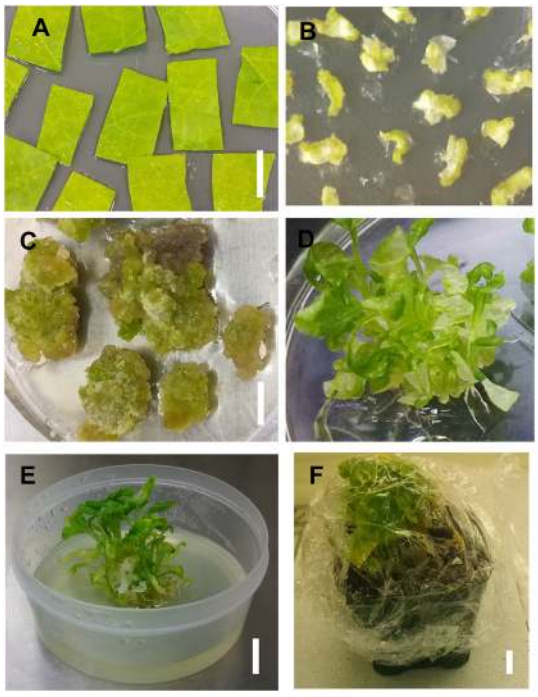
A fragment containing U6 promoter and *NbPDS* target sequence was amplified with the primers, pEgPaef1_AtU6-1F (5′-CCAAGCTCCAATTAGGGCCCCGCTAG -3′) and AtU6-1_NbPDS-2R (5′-TCA TCA TCT TTC CAT GCA GCA ATC ACT ACT TCG TCT CTA ACC AT-3′). Another fragment containing the *NbPDS* target sequence, guide RNA, and U6 terminator was amplified with the primers, NbPDS-2_guideRNA_F (5′-GCTGCATGGAAAGATGATGAGTTTTAG AGCTAGAAATAGCAAGT-3′) and AtU6end_pEgPaef1_R (5′-AAT CCT AAT GGC GCG CCT TCG CGC AG-3′). These two fragments were combined with the primers, pEgPaef1_AtU6-1F and AtU6end_pEgPaef1_R. The fragment containing



Media for genome editing of tomato with non-selective media

MS medium	MS, 1.5% sucrose, 0.3% gelrite
Co-cultivation	MS, 3% sucrose, 40μM acetosyringone, 1.5 mg/L *trans*-zeatin, 0.3% gelrite
Callus induction	MS, 3% sucrose, 1.5 mg/L *trans*-zeatin, 375 mg/L augmentin, 0.3% gelrite
Shoot elongation	MS, 3% sucrose, 1 mg/L *trans*-zeatin, 375 mg/L augmentin, 0.3% gelrite
Root formation	MS, 1.5% sucrose, 375 mg/L augmentin, 0.3% gelrite

Figure 1. Steps in the *Agrobacterium*-mediated transformation of tomato plants. A) Sowing of tomato seeds in Murashige and Skoog's (MS) medium. B) The appropriate stage of tomato plant growth that was suitable for transformation. C) The growth of explants or leaf discs in co-cultivation medium after incubation with *Agrobacterium*. D) The incubation process in the dark for leaf discs in the co-cultivation medium. E) The stage of plant growth in shoot induction medium. F) The stages of plant growth in the root induction medium. Bars=1 cm. The outline of the method is 1) preparation of cotyledon leaves, 2) incubation of leaf discs with *Agrobacterium*, 3) incubation of leaf discs on the medium without kanamycin, and 4) growth of regenerated plants.



Media for genome editing of *N. benthamiana*

Co-cultivation	MS, 3% sucrose, 1mg/L BAP, 0.1 mg/L NAA, 0.8% agar
Callus induction (at least 3 times)	MS, 3% sucrose, 1 mg/L BAP, 0.1 mg/L NAA, 400 mg/L cefotaxime, 10mg/L hygromycin, 0.3% gelrite
Shoot elongation	MS, 3% sucrose, 400 mg/L cefotaxime, 10mg/L hygromycin, 0.3% gelrite
Root formation	1/2 MS, 0.5% sucrose, 400 mg/L cefotaxime, 5 mg/L hygromycin, 0.25% gelrite

Figure 2. Steps in *Agrobacterium*-mediated transformation of *Nicotiana benthamiana*. A) Leaf discs were prepared after sterilization of leaves. B, C) The calli appeared and were grown. D) After the formation of shoots, the calli were removed from the shoots. E) Roots were regenerated from the shoots. F) The plants were transferred to soil and covered with plastic wraps. Bars=1 cm. The outline of the method is 1) preparation of leaf discs, 2) incubation of leaf discs with *Agrobacterium*, 3) incubation of leaf discs on the callus formation media, 4) repeat incubation on the callus formation media at least three times, and 5) growth of regenerated plants.

the U6 promoter, *NbPDS* target sequence, guide RNA, and U6 terminator was introduced into the *Apa*I/*Asc*I-digested pEgPaef1 (Osakabe et al. 2016) with an In-Fusion reaction (TaKaRa Bio).

Preparation of Agrobacterium containing the vector for genome editing

The plasmids was transformed to *Agrobacterium* GV2260 or GV3101 by electroporation. *Agrobacterium* strain GV2260 or GV3101 was used for transformation to tomato or *N. benthamiana*, respectively. *Agrobacterium* was incubated on LB agar medium containing 50 mg/l kanamycin at 28°C. The colony was picked and incubated in LB liquid medium. Then, *Agrobacterium* cells were collected by centrifugation and suspended in MS liquid medium with 3% sucrose, 100 μM acetosyringone, and 10 μM β-mercaptoethanol for transformation of tomato or in MS liquid with 3% sucrose and 100 μM acetosyringone for transformation of *N. benthamiana*.

Agrobacterium-mediated transformation to tomato cv. Micro-Tom

The transformation was performed according to the previously described protocol (Sun et al. 2006). The culture room was set up at 25°C under long-day light condition (16 h light/8 h dark). Briefly, for getting cotyledon leaves, tomato seeds were sterilized in 3% sodium hypochlorite for 10 min and cultivated on MS medium with 1.5% sucrose and 0.3% gelrite (Figure 1A). After the formation of cotyledon leaves or initial formation of true leaves (Figure 1B), the edge of the cotyledon was cut and soaked in the *Agrobacterium* solution for 10 min. The *Agrobacterium* solution was removed with sterile filter paper.

The adaxial side of leaf discs was placed on the surface of the co-cultivation medium (MS medium with 3% sucrose, 0.3% gelrite, 40 μM acetosyringone, and 1.5 mg/l *trans*-zeatin) (Figure 1C), and incubated for 3–4 days covered with aluminum foil (Figure 1D). Thereafter, the abaxial side of leaf discs was placed on the surface of the callus induction medium (MS medium with 3% sucrose, 0.3% gelrite, 1.5 mg/l *trans*-zeatin, and 375 mg/l augmentin). The leaf discs were transferred onto the fresh callus induction medium every 7–14 days until the formation of shoots (Figure 1E). The shoots were then grown on the shoot elongation medium (MS medium with 3% sucrose, 0.3% gelrite, 1 mg/l *trans*-zeatin, and 375 mg/l augmentin) for 7–14 days until they elongated to a length of 1–2 cm. The shoots were transferred onto the root formation medium (1/2 MS media with 1.5% sucrose, 0.3% gelrite, and 375 mg/l augmentin) to enhance the root formation (Figure 1F). The plants were transferred to soil after the rooted plants attained a height of more than 5 cm and were covered with plastic wraps. After acclimation to the soil conditions, small holes were made in the plastic wraps and the plants were allowed to grow. After flowering, the seeds were harvested from each plant.

Transformation to Nicotiana benthamiana mediated by Agrobacterium

The culture room was set up at 25°C under long-day light condition (16 h light/8 h dark). Leaves from 3- to 4-week-old plants were harvested and rinsed with 70% ethanol. Then, the leaves were soaked in 0.5% sodium hypochlorite for 15 min and washed with sterilized water 5 times. Leaf segments (approximately 1 cm^2) were cut out with a scalpel. The leaf discs were soaked in *Agrobacterium* solution for 5 min. After removing the bacterial culture with sterile filter papers, leaf discs were placed onto co-cultivation plates (MS medium with 3% sucrose, 0.8% agar, 1 mg/l 6-benzylaminopurine (BAP), and 0.1 mg/l 1-naphthaleneacetic acid (NAA)). Usually, 10 leaf discs were placed on one plate. The lower epidermis was on the agar surface and these leaf discs were incubated for 2–3 days (Figure 2A).

The leaf discs were transferred onto the callus induction medium (MS medium with 3% sucrose, 0.3% gelrite, 1 mg/l BAP, and 0.1 mg/l NAA, 400 mg/l cefotaxime, and 10 mg/l hygromycin) every 10–14 days. After incubating the leaf discs on the regeneration medium at 25°C, callus appeared (Figure 2B). Callus was transferred onto the callus formation medium at least three times and allowed to grow (Figure 2C). The shoots were regenerated on the shoot elongation medium (MS medium with 3% sucrose, 0.3% gelrite, 400 mg/l cefotaxime, and 10 mg/l hygromycin). After appearance of the shoots, calli were removed from shoots (Figure 2D) and the shoots were transferred onto the rooting medium (1/2 MS medium with 0.5% sucrose, 0.25% gelrite, 400 mg/l cefotaxime, and 5 mg/l hygromycin) (Figure 2E). Thereafter, the plants with roots were incubated until the height of the shoot was more than 5 cm and then transferred to soil and covered with plastic wraps (Figure 2F). After acclimation to the soil conditions, small holes were made in the plastic wraps and the plants were grown further. After flowering, seeds were harvested from each plant.

PCR amplification of tomato and N. benthamiana gene

Tomato genome was prepared by using Maxwell Plant DNA kit (Promega). To amplify the *NPTII* or *SlPDS1* gene, the primers, NPTII-F (5′-ATG ATT GAA CAA GAT GGA TTG CAC-3′) and NPTII-R (5′-TCA GAA GAA CTC GTC AAG AAG GCG-3′), or PDS-F1 (5′-GTA AGT TTG ACC TCT CAT TG-3′) and PDS-R1 (5′-CCC ATA GGT GTG ATT GAC TTA TC-3′) were used.

Small fragments of *N. benthamiana* leaf (less than 10 mm^2) were ground in a buffer (100 mM Tris-HCl, pH 9.5, 1 M KCl, 10 mM EDTA) with a pestle and incubated at 95°C for 5 min. After centrifugation, the supernatant was used as a template for PCR amplification with KOD FX Neo (Toyobo). To amplify the *NbPDS1* (Niben101Scf01283Ctg022) or *NbPDS2* (Niben101Scf14708Ctg003) gene, the primers, NbPDS1-check2F (5′-TTT TAA ACT GAG TCA ATT TTA ACC G-3′) and NbPDS1-check2R (5′-TAT GAG TCA CCA TAC GAG TTA GCA G-3′), or NbPDS2-check2F (5′-ACA GCA TAT TAG GTA TAT GGA AAG TAT-3′) and NbPDS2-check2R (5′-AGA GTA TTA ATG GTC AAT GGA CTA ATC-3′) were used, respectively.

Results

Generation of PDS knockout tomato plants

The genome of tomato contains a single *PDS* gene. PDS is a phytoene desaturase and plays an important role in the carotenoid biosynthesis pathway (Giuliano et al. 1993). Knockdown of *PDS* by virus-induced gene silencing and its knockout using CRISPR/Cas9 produced albino tomato plants (Liu et al. 2002; Pan et al. 2016). In this study, a 20-bp sequence, 5′-TAA CGA TCG ATT GCA ATG GA-3′, with protospacer adjacent motif (PAM) was selected as a single guide RNA (sgRNA) complementary site for targeting the *SlPDS1* gene (Figure 3A). Cas9 with sgRNA expression cassettes were transformed into tomato cv. Micro-Tom plants by the *Agrobacterium*-mediated transformation method. And the leaf discs were incubated on the media without any selection antibiotics. In agreement with the previous report (Pan et al. 2016), the chimeric albino phenotype was observed in some of the regenerated tomato shoots (Figure 3B). In one of the regenerated tomato plants, line *e1*, the *NPTII* fragment was not amplified by PCR (Figure 3C), suggesting that the transgene was not integrated in this line. The DNA fragment containing a sgRNA target site in the *SlPDS* gene was amplified. Longer and shorter bands were detected in the lines *e2* and *g1*, respectively (Figure 3C), suggesting that large insertion and deletion (about 100 bp according to the gel image) occurred because of genome editing. The sequence analysis of the *SlPDS1* target site in two genome editing lines *e1* and

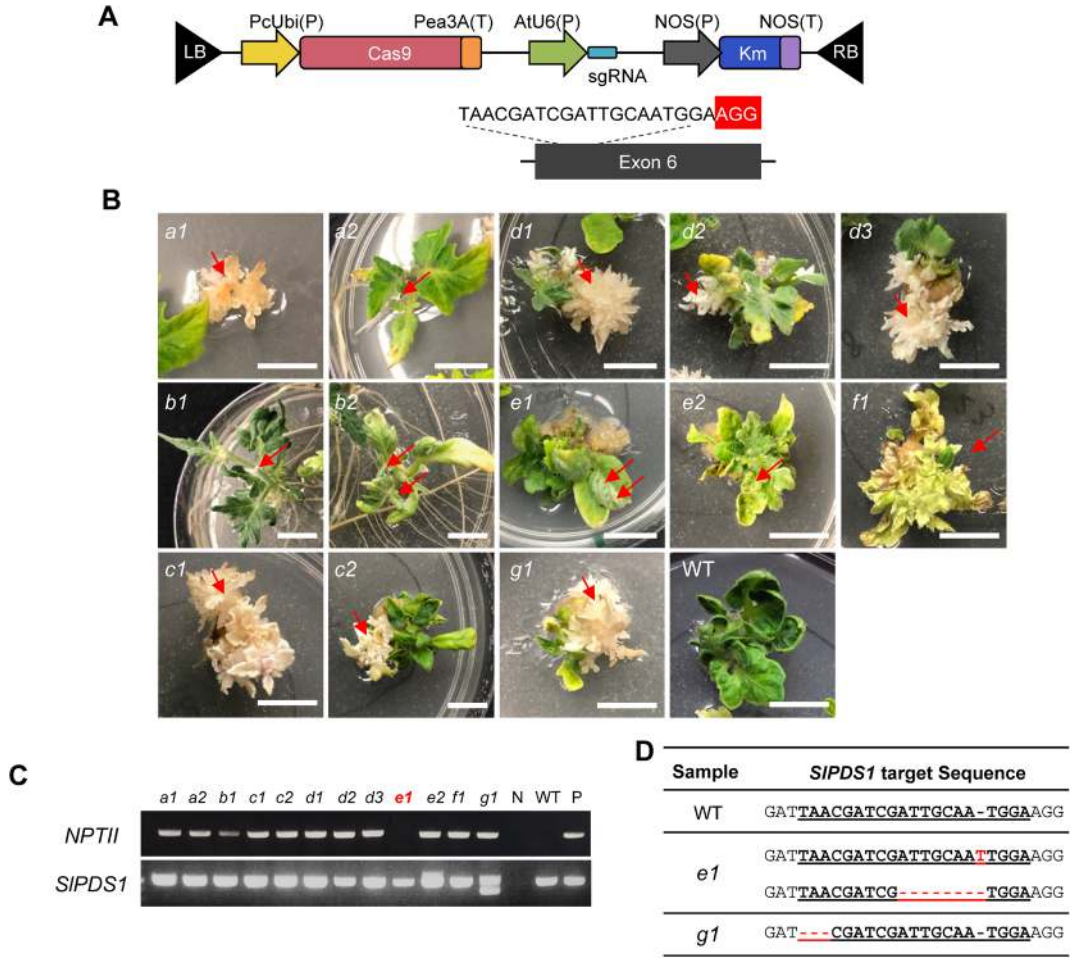


Figure 3. Disruption of *SlPDS* and isolation of T_0 null segregant plants using the non-selection method. (A) Vector map and sequence targeting *SlPDS*. PcUbi(P), parsley ubiquitin promoter; Cas9, Arabidopsis-codon optimized spCas9; Pea3A(T), Pea3A terminator; AtU6(P), Arabidopsis U6 promoter; NOS(P), NOS promoter; Km, kanamycin resistance gene; NOS(T), NOS terminator. The target sequence was designed in exon 6 of *SlPDS1*. Red highlighted sequence is the PAM motif. (B) Regeneration of genome edited plants obtained after selection without antibiotics. The different alphabet in each picture represents an independent *SlPDS*-edited plant derived from the different callus. The number after the alphabets represent different regenerated plants from the same callus. Red arrows indicate white leaf segments caused by the disruption of *SlPDS1*. Size bar=1 cm. (C) Detection of *NPTII* and *SlPDS1* gene by PCR. The line *e1* did not contain the *NTPII* gene, suggesting that the *e1* line is the transgene-free line. N, no template was added in the PCR mixture. WT, genome from WT Micro-Tom was used as positive control to detect *SlPDS1*. P, positive control for the detection of both *NPTII* and *SlPDS1*. (D) Sequencing analysis of *SlPDS1* target site in genome editing lines. DNA editing patterns for two transgenic lines *e1* and *g1* are shown. The target sequence by CRISPR/Cas9 is underlined. The insertion and deletion within the target site specified by sgRNA were evident in line *e1* and *g1*, respectively. WT target sequence is also shown as comparison.

g1 were performed. The insertion of one nucleotide or the deletion of 8 bases were observed in the *e1* line and the deletion of three bases was observed in the *g1* line (Figure 3D). Among regenerated plants on the non-selective media, 12 plants exhibited albino phenotype. One of 12 plants harbored the targeted mutation in *SlPDS1*, thus the efficiency to obtain the transgene-free and genome edited tomato was approximately 8%.

Generation of PDS knockout N. benthamiana plants

Two *PDS* genes are present in the genome of *N. benthamiana*. Previous reports have shown that knockout of the *NbPDS* gene can be done by CRISPR/Cas9 (Li et al. 2013; Nekrasov et al. 2013). However, whether the mutation in *NbPDS* was inherited to the next generation was not examined in these studies. We evaluated the hereditary transfer of the mutation in *NbPDS* edited by CRISPR/Cas9 and also evaluated whether a sgRNA could disrupt both the *NbPDS* genes. Two *NbPDS* gene sequences were aligned. The sequence, 5′-GCT GCA TGG AAA GAT GAT GA TGG-3′ (underlined sequences indicates the PAM sequence), was found in both the *PDS* genes (Figure 4A). This sequence was introduced to generate sgRNA for the targeting of *NbPDS*. The vector for expressing the plant codon-optimized Cas9 (fcoCas9) (Osakabe et al. 2016) and *NbPDS*-target sgRNA was transformed into *N. benthamiana* leaf discs using the *Agrobacterium* GV3101 strain. The callus was obtained from leaf discs

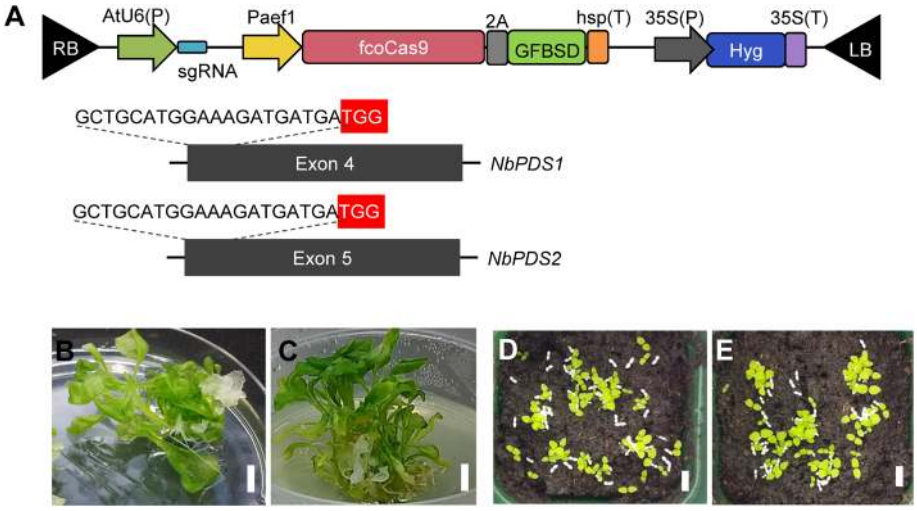


Figure 4. Phenotype of *NbPDS*-edited transgenic *Nicotiana benthamiana* plants. (A) Vector map and sequence targeting *NbPDS*. AtU6(P), Arabidopsis U6 promoter; Paef1, promoter of Arabidopsis elongation factor 1; fcoCas9, fungal and plant codon-optimized Cas9; 2A, 2A peptide; GFBSD, GFP and the blasticidin resistance fusion gene; hsp(T), heat shock protein terminator; 35S(P), cauliflower mosaic virus 35S promoter; Hyg, hygromycin resistance gene; 35S(T), cauliflower mosaic virus 35S terminator. The target sequence was designed in exon 4 of *NbPDS1* and in exon 5 of *NbPDS2*. Red highlighted sequence is the PAM motif. Chimeric albino phenotype was observed in T_0 transgenic *N. benthamiana* lines #12 (B) and #16 (C). The T_2 seeds were obtained from T_1 plants, which possessed the homozygous mutation in one of the *PDS* genes and the heterozygous mutation in another *PDS* gene. The T_2 seeds were sown in soil and grown for 7 days. The pictures are of representative T_2 lines from #12 (D) and #16 (E).

Figure 5. *NbPDS* sequences in the T_1 generation of transgenic *Nicotiana benthamiana* lines #12 and #16. The mutation caused by genome editing was inherited to the next generation. TGG indicates the PAM sequence.

and was transferred onto the callus formation medium at least three times. Thereafter, shoots and roots were regenerated. At this time, chimeric albino leaves caused by the *NbPDS* mutation were observed in the healthy transgenic plants (Figure 4B, C). The DNA from 20 transgenic leaves was extracted and DNA fragments were amplified and sequenced. The lines #12 and #16 had chimeric mutations in both *NbPDS1* and *NbPDS2*. The plant #12 and #16 were transferred to the soil and T_1 seeds were obtained.

The mutations in *NbPDS1* and *NbPDS2* were identified in the T_1 plants of lines #12 and #16. In the line #12, six nucleotides in *NbPDS1* and 22 nucleotides in *NbPDS2* were deleted (Figure 5). In line #16, one nucleotide was inserted in *NbPDS1* and 11 were deleted in *NbPDS2* (Figure 5). A stop codon was generated because of frame shift near the site of mutation, except in the case of mutation in *NbPDS1* in line #12. Probably, two amino acids were important for the function of *NbPDS1*. As a result of homozygous mutations in both *NbPDS1* and

NbPDS2, the plants were unable to grow, because of whitening of tissues. Thus, T_1 plants, which possessed a homozygous mutation in one of the *NbPDS* genes and a heterozygous mutation in another *NbPDS* gene, were grown and T_2 seeds were obtained. These T_2 seeds were sown in soil and incubated for 7 days. Approximately 30% of the seedlings were white in lines #12 (Figure 4D) and #16 (Figure 4E). These results indicate that plant codon-optimized Cas9 (fcoCas9) can be used for genome editing in *N. benthamiana* and a single target sequence can disrupt both *NbPDS1* and *NbPDS2*. Among 20 hygromycin-resistant plants, two plants contained both mutation in *NbPDS1* and *NbPDS2*, thus, the efficiency to obtain the double mutation by one sgRNA in *N. benthamiana* was 10%.

Discussion

In this study, we describe detailed protocols for the genome editing in tomato and *N. benthamiana*. As examples, we have shown the knockout of *PDS* genes in these plants using the CRISPR/Cas9 system, which resulted in the albino phenotype and caused failure of plant growth.

Stable genomic integration of CRISPR/Cas9 components through *Agrobacterium*-mediated transformation, as shown in this study, is the most widely used approach in dicotyledonous plants. But elimination of foreign DNA is required and is sometimes difficult in vegetatively-propagated plants. Recently, *Agrobacterium*-mediated transient expression of CRISPR/Cas9 cytidine base editor was achieved in tomato and potato plants. Using this method, transgene-free tomato and potato plants were obtained in the first generation (Veillet et al. 2019). When cellular concentrations of sgRNA were increased by transient expression using *Tobacco mosaic virus*-derived vector (TRBO), the percentages of indels averaged about 70% within 7 days of inoculation in *N. benthamiana* overexpressing Cas9. Furthermore, multiplexed sgRNAs were delivered into plants using the TRBO system (Cody et al. 2017). Previously, we created a new transient protein expression system, termed the Tsukuba system, using the pBYR2HS vector (Yamamoto et al. 2018a). The system utilizes a combination of geminivirus replication and a double terminator with HSP and Ext terminators, resulting in approximately 4 mg/g fresh weight of protein expression in *N. benthamiana* (Yamamoto et al. 2018a). We demonstrated that this system is effective not only for *N. benthamiana* but also for several kinds of plants, including tomato, eggplant, pepper, lettuce, melon, soybean, and common bean (Suzaki et al. 2019; Yamamoto et al. 2018a). Furthermore, this system along with gabT (Nonaka et al. 2017b) enhances the protein expression levels in tomato fruits (Hoshikawa et al. 2019). The introduction of mutation is dependent on the amount of Cas9 and sgRNA. Thus, the Tsukuba system may be useful for *Agrobacterium*-mediated transient expression of Cas9 and sgRNA for producing genome editing plants.

The *Solanaceae* plants include eggplant, potato, tomatillo, and pepper as well as tomato. The transformation protocols are established for these plants (Van Eck 2018). Although there are several articles demonstrating the transformation of pepper, it is difficult to transform DNA into the pepper plants. Furthermore, there are several plants for which transformation protocols are not established. For these plants, other approaches are required to obtain genome-edited plants besides stable genomic integration of the CRISPR/Cas9 components through *Agrobacterium*-mediated transformation.

In addition to the technology of genome editing, information about target genes is more important for production of new cultivars. Genetic resources are among the tools to obtain information about target genes. From tomato mutant lines, several mutations, which cause phenotypes, have been identified. The loss-of-function of receptor-like kinase in tomato induces parthenocarpic fruit set, with impairment of male fertility (Takei et al. 2019). Disruption of the tomato *HAWAIIAN SKIRT* ortholog results in facultative parthenocarpy (Damayanti et al. 2019). These mutations have been identified in tomato cv. Micro-Tom and can be used to introduce other phenotypes in other cultivars with genome editing.

Analysis of gene function is also important. The reduction of *IAA9* expression by antisense method causes the induction of parthenocarpy (Wang et al. 2005), indicating that IAA9 is a key mediator of fruit set. The *iaa9* mutant generated by CRISPR/Cas9 also exhibits seedless fruit (Ueta et al. 2017). SlGAD3, a glutamate decarboxylase, which catalyzes the decarboxylation of glutamate to form γ-aminobutyric acid (GABA), contains a C-terminal autoinhibitory domain. The overexpression of *SlGAD3* without an autoinhibitory domain enhances the GABA levels in tomato fruits by 11- to 18-fold (Takayama et al. 2017). Thus, removal of the autoinhibitory domain in SlGAD3 by CRISPR/Cas9 also increases the GABA content in tomato fruits (Nonaka et al. 2017a). The aberrant forms of *ARF8* (auxin response factor) expressed in tomato resulted in the stimulation of parthenocarpy (Goetz et al. 2007). The knockdown of *SmARF8* by RNAi enhanced the parthenocarpy in eggplants (Du et al. 2016). These information are more important for producing plants with a desired trait and can be shared among the *Solanaceae*.

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References

Bogdanove AJ, Voytas DF (2011) TAL effectors: Customizable proteins for DNA targeting. *Science* 333: 1843–1846

Cody WB, Scholthof HB, Mirkov TE (2017) Multiplexed gene editing and protein overexpression using a tobacco mosaic virus viral vector. *Plant Physiol* 175: 23–35

Damayanti F, Lombardo F, Masuda J-i, Shinozaki Y, Ichino T, Hoshikawa K, Okabe Y, Wang N, Fukuda N, Ariizumi T, et al. (2019) Functional disruption of the tomato putative ortholog of *HAWAIIAN SKIRT* results in facultative parthenocarpy, reduced fertility and leaf morphological defects. *Front Plant Sci* 10: 1234

Donini M, Marusic C (2019) Current state-of-the-art in plant-based antibody production systems. *Biotechnol Lett* 41: 335–346

Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346: 1258096

Du L, Bao C, Hu T, Zhu Q, Hu H, He Q, Mao W (2016) SmARF8, a transcription factor involved in parthenocarpy in eggplant. *Mol Genet Genomics* 291: 93–105

Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* 5: 379–387

Goetz M, Hooper LC, Johnson SD, Rodrigues JCM, Vivian-Smith A, Koltunow AM (2007) Expression of aberrant forms of *AUXIN RESPONSE FACTOR8* stimulates parthenocarpy in Arabidopsis and tomato. *Plant Physiol* 145: 351–366

Hamada H, Liu Y, Nagira Y, Miki R, Taoka N, Imai R (2018) Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. *Sci Rep* 8: 14422

Hoshikawa K, Fujita S, Renhu N, Ezura K, Yamamoto T, Nonaka S, Ezura H, Miura K (2019) Efficient transient protein expression in tomato cultivars and wild species using agroinfiltration-mediated high expression system. *Plant Cell Rep* 38: 75–84

Kim YG, Cha J, Chandrasegaran S (1996) Hybrid restriction enzymes: Zinc finger fusions to *Fok* I cleavage domain. *Proc Natl Acad Sci USA* 93: 1156–1160

Kopertekh L, Schiemann J (2019) Transient production of recombinant pharmaceutical proteins in plants: Evolution and perspectives. *Curr Med Chem* 26: 365–380

Li J, Stoddard TJ, Demorest ZL, Lavoie PO, Luo S, Clasen BM, Cedrone F, Ray EE, Coffman AP, Daulhac A, et al. (2016) Multiplexed, targeted gene editing in *Nicotiana benthamiana* for glyco-engineering and monoclonal antibody production. *Plant Biotechnol J* 14: 533–542

Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat Biotechnol* 31: 688–691

Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *Plant J* 31: 777–786

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat Biotechnol* 23: 718–723

Metje-Sprink J, Menz J, Modrzejewski D, Sprink T (2019) DNA-free genome editing: Past, present and future. *Front Plant Sci* 9: 1957

Miura K, Renhu N, Suzaki T (2020) The PHD finger of Arabdiopsis SIZ1 recognizes trimethylated histone H3K4 mediating SIZ1 function and abiotic stress response. *Commun Biol* 3: 23

Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31: 691–693

Nonaka S, Arai C, Takayama M, Matsukura C, Ezura H (2017a) Efficient increase of γ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis. *Sci Rep* 7: 7057

Nonaka S, Someya T, Zhou S, Takayama M, Nakamura K, Ezura H (2017b) An agrobacterium tumefaciens strain with γ-aminobutyric acid transaminase activity shows an enhanced genetic transformation ability in plants. *Sci Rep* 7: 42649

Osakabe Y, Watanabe T, Sugano SS, Ueta R, Ishihara R, Shinozaki K, Osakabe K (2016) Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. *Sci Rep* 6: 26685

Pan C, Ye L, Qin L, Liu X, He Y, Wang J, Chen L, Lu G (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci Rep* 6: 24765

Reed J, Osbourn A (2018) Engineering terpenoid production through transient expression in *Nicotiana benthamiana. Plant Cell Rep* 37: 1431–1441

Rothan C, Diouf I, Causse M (2019) Trait discovery and editing in tomato. *Plant J* 97: 73–90

Saito T, Ariizumi T, Okabe Y, Asamizu E, Hiwasa-Tanase K, Fukuda N, Mizoguchi T, Yamazaki Y, Aoki K, Ezura H (2011) TOMATOMA: A novel tomato mutant database distributing Micro-Tom mutant collections. *Plant Cell Physiol* 52: 283–296

Shikata M, Hoshikawa K, Ariizumi T, Fukuda N, Yamazaki Y, Ezura H (2016) TOMATOMA update: Phenotypic and metabolite information in the Micro-Tom mutant resource. *Plant Cell Physiol* 57: e11

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, et al. (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nat Biotechnol* 35: 441–443

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiol* 47: 426–431

Suzaki T, Tsuda M, Ezura H, Day B, Miura K (2019) Agroinfiltration-based efficient transient protein expression in leguminous plants. *Plant Biotechnol* 36: 119–123

Takayama M, Matsukura C, Ariizumi T, Ezura H (2017) Activating glutamate decarboxylase activity by removing the autoinhibitory domain leads to hyper gamma-aminobutyric acid (GABA) accumulation in tomato fruit. *Plant Cell Rep* 36: 103–116

Takei H, Shinozaki Y, Yano R, Kashojiya S, Hernould M, Chevalier C, Ezura H, Ariizumi T (2019) Loss-of-function of a

tomato receptor-like kinase impairs male fertility and induces parthenocarpic fruit set. *Front Plant Sci* 10: 403

Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R, Ezura H, Osakabe Y, Osakabe K (2017) Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Sci Rep* 7: 507

Van Eck J (2018) Genome editing and plant transformation of solanaceous food crops. *Curr Opin Biotechnol* 49: 35–41

Veillet F, Perrot L, Chauvin L, Kermarrec MP, Guyon-Debast A, Chauvin JE, Nogue F, Mazier M (2019) Transgene-free genome editing in tomato and potato plants using *Agrobacterium*-mediated delivery of a CRISPR/Cas9 cytidine base editor. *Int J Mol Sci* 20: 402

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latche A, Pech JC, Bouzayen M (2005) The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *Plant Cell* 17: 2676–2692

Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M (2017) CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory Ipomoea (Pharbitis) nil. *Sci Rep* 7: 10028

Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat Biotechnol* 33: 1162–1164

Yamada Y, Kidoguchi M, Yata A, Nakamura T, Yoshida H, Kato Y, Masuko H, Hizawa N, Fujieda S, Noguchi E, et al. (2020) High-yield production of the major birch pollen allergen Bet v 1 with allergen immunogenicity in Nicotiana benthamiana. *Front Plant Sci* 11: 344

Yamamoto T, Hoshikawa K, Ezura K, Okazawa R, Fujita S, Takaoka M, Mason HS, Ezura H, Miura K (2018a) Improvement of the transient expression system for production of recombinant proteins in plants. *Sci Rep* 8: 4755

Yamamoto T, Kashojiya S, Kamimura S, Kameyama T, Ariizumi T, Ezura H, Miura K (2018b) Application and development of genome editing technologies to the Solanaceae plants. *Plant Physiol Biochem* 131: 37–46

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu JL, Gao C (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat Commun* 7: 12617