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

Hiroki Komatsu1,1,a, Islam M. Y. Abdellatif1,†, Shaoze Yuan1, Misaki Ono1, Satoko Nonaka1,2, Hiroshi Ezura1,2, Tohru Ariizumi1,2,*; Kenji Miura1,2,**

1Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki 305-8572, Japan; 2Tsukuba-Plant Innovation Research Center, University of Tsukuba, Ibaraki 305-8572, Japan
* E-mail: ariizumi.toru.ge@u.tsukuba.ac.jp; Tel & Fax: +81-29-853-4710
** E-mail: miura.kenji.ga@u.tsukuba.ac.jp; Tel & Fax: +81-29-853-6401

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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.

† These authors contributed equally to this work.

** Present address: Agricultural Resource Development Department, Innovation Division, Kagome Co., Ltd., 17 Nishitomiyama, Nasushiobara, Tochigi 329-2762, Japan

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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 in 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 sgRNA 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 *acetylactate 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 *SIPDS1* 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, *SIPDS1*-F (5’-GATTGTAAGCATCAGTGGTTCAATGGA-3’) and *SIPDS1*-R (5’-AAAATCCATCATGAATTGGTGACGTAATTCTTTCT-3’), were annealed and ligated into the *Bsa*I-digested pZD_AtU6sRNA_HoICas9_NP2TII vector (Watanabe et al. 2017).

A fragment containing U6 promoter and *NbPDS* target sequence was amplified with the primers, pEgPaeF1_AtU6-1F (5’-CAGGCTTCAAGTTAGGCGCCCGCTAG -3’) and AtU6-1_NbPDS-2R (5’-TCACTTCTTTCATGCTGACGATCTACACTTCTCTCTCATCAGAT-3’). Another fragment containing the *NbPDS* target sequence, guide RNA, and U6 terminator was amplified with the primers, *NbPDS*-2- guideRNA_F (5’-GCTGATGAGGAGATGATGATTTTAGAGCGTGGATACTGAAATGCAATGAT-3’) and AtU6end_pEgPaeF1_R (5’-AATCCTAATTGGCGGCCTCGCCGAG-3’). These two fragments were combined with the primers, pEgPaeF1_ AtU6-1F and AtU6end_pEgPaeF1_R. The fragment containing
the U6 promoter, NbPDS target sequence, guide RNA, and U6 terminator was introduced into the ApaI/AscI-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 acetylsyringone, and 10 µM β-mercaptoethanol for transformation of tomato or in MS liquid with 3% sucrose and 100 µM acetylsyringone 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.

![Figure 1](image1.png)

**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.

![Figure 2](image2.png)

**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 adaxial side of leaf discs was placed on the surface of the co-cultivation medium (MS medium with 3% sucrose, 0.3% gelrite, 1 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 cm²) 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). Therefore, 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 SIPDS1 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 TTA ACCTCT CATTG-3′) and PDS-R1 (5′-CCC ATA GGT GTTG ATT GAC TTAC ATC-3′) were used.

Small fragments of N. benthamiana leaf (less than 10 mm²) 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 CCT AGT GAT TTG 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 SIPDS1 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 SIPDS 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 SIPDS1 target site in two genome editing lines e1 and
were performed. The insertion of one nucleotide or the deletion of 8 bases were observed in the line and the deletion of three bases was observed in the 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.
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 T1 seeds were obtained.

The mutations in *NbPDS1* and *NbPDS2* were identified in the T1 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* were identified in the T1 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* were identified in the T1 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* were identified in the T1 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* were identified in the T1 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* were identified in the T1 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* were identified in the T1 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, T1 plants, which possessed a homozygous mutation in one of the NbPDS genes and a heterozygous mutation in another NbPDS gene, were grown and T2 seeds were obtained. These T2 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 HAWAIAN SKIRT ortholog results in facultative parthenocarpic (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 parthenocarpic (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). SIGAD3, a glutamate decarboxylase, which catalyzes the decarboxylation of glutamate to form γ-aminobutyric acid (GABA), contains a C-terminal autoinhibitory domain. The overexpression of SIGAD3 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 SIGAD3 by CRISPR/Cas9 also increases the GABA content in tomato fruits (Nonaka et al. 2017a).

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