

# Targeted genome editing in tetraploid potato through transient TALEN expression by *Agrobacterium* infection

Shuhei Yasumoto<sup>1</sup>, Satoru Sawai<sup>1</sup>, Hyoung Jae Lee<sup>1,2</sup>, Masaharu Mizutani<sup>2</sup>, Kazuki Saito<sup>3</sup>, Naoyuki Umemoto<sup>3</sup>, Toshiya Muranaka<sup>1,\*</sup>

<sup>1</sup>Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan; <sup>2</sup>Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe, Hyogo 657-8501, Japan; <sup>3</sup>RIKEN Center for Sustainable Resource Science, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

\*E-mail: muranaka@bio.eng.osaka-u.ac.jp Tel: +81-6-6879-7423 Fax: +81-6-6879-7426

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**Abstract** Genome editing using site-specific nucleases, such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat–CRISPR-associated protein 9 (CRISPR-Cas9), is a powerful technology for crop breeding. For plant genome editing, the genome-editing reagents are usually expressed in plant cells from stably integrated transgenes within the genome. This requires crossing processes to remove foreign nucleotides from the genome to generate null segregants. However, in highly heterozygous plants such as potato, the progeny lines have different agronomic traits from the parent cultivar and do not necessarily become elite lines. *Agrobacterium* can transfer exogenous genes on T-DNA into plant cells. This has been used both to transform plants stably and to express the genes transiently in plant cells. Here, we infected potato, with *Agrobacterium tumefaciens* harboring TALEN-expression vector targeting *sterol side chain reductase 2 (SSR2)* gene and regenerated shoots without selection. We obtained regenerated lines with disrupted-SSR2 gene and without transgene of the TALEN gene, revealing that their disruption should be caused by transient gene expression. The strategy using transient gene expression by *Agrobacterium* that we call Agrobacterial mutagenesis, developed here should accelerate the use of genome-editing technology to modify heterozygous plant genomes.

**Key words:** genome editing, potato, steroidal glycoalkaloids, TALEN, transient gene expression.

## Introduction

Site-specific nucleases, such as zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated protein 9 (CRISPR-Cas9) systems, recognize specific sequences in the genomic sequence and introduce DNA double-strand breaks (DSBs). DSBs are incorrectly repaired by the endogenous non-homologous end-joining pathway, and targeted mutagenesis is introduced into the target sequence (reviewed in Voytas 2013; Voytas and Gao 2014). Before the development of genome-editing technology using site-specific nucleases, there was no way to disrupt a target gene in the plant genome. The generation of mutant lines for scientific research or agriculture depended completely on random mutations caused by radiation or mutagenic chemicals. It is time-consuming and laborious to select objective mutants (McCallum et al. 2000). By using genome-editing reagents, we can easily generate plants that have objective mutations in the genome. Genome editing in

plants began with the transformation of the expression vector for site-specific nucleases. After introducing targeted mutagenesis in the target gene, the transgenes are removed by genetic segregation to generate null segregants (harboring the targeted mutation without the transgenes) (Wolt et al. 2016). When highly homozygous plants are the subject materials, no problems arise. Vegetatively propagated crops have a highly heterozygous plant genome, such as a tetraploid potato genome. Their genomic information and agricultural traits must be changed completely through segregation and crossing-over. One possible strategy to overcome this problem is transient expression of site-specific nucleases in plant cells without integration of a transgene in the genome. Polyethylene-glycol-mediated protoplast transfection and particle bombardment are usually employed to generate transgenic plants with antibiotic selection. Nocolia et al. (2015), Clasen et al. (2016), and Zhang et al. (2016) used these techniques to deliver nucleotide molecules encoding site-specific nucleases, incubated the transfected plant cells under no selection, and succeeded in generating genome-edited plants without

transgene integration in the genome. *Agrobacterium*-mediated transformation is the method used most widely for generating transgenic plants (Hwang et al. 2017). During the transformation process, T-DNA on a binary vector is processed and transferred into plant cells. Cells that have stable integrated T-DNA can be selected for under different conditions, in the presence of antibiotics or herbicides, for example. *Agrobacterium* is also used for transient expression in plants. Using the *Agrobacterium*-mediated transient expression method for plants, strong gene expression can be observed after cultivation for a few days transiently (Fischer et al. 1999; Kapila et al. 1997). Since there is strong transient gene expression in the infected region, we hypothesized that *Agrobacterium*-mediated transient site-specific nuclease expression in plant cells under non-selection conditions is suitable for plant genome editing. In this study, we infected potato stems with *Agrobacterium tumefaciens* harboring the TALEN-expression vector. All regenerated shoots on medium containing plant hormones without any selection reagents, were checked for gene modification in the target sequence, and evaluated the transgene integration in the genome. We found the shoots contained genome-edited line by *Agrobacterium*-transient expression.

## Materials and methods

### Plant material

Murashige and Skoog (MS) medium containing 3% sucrose solidified with 0.8% agar was used for *in vitro* cultivation of *Solanum tuberosum* cv. Sassy. Shoots were subcultured every 3 to 4 weeks.

### Agrobacterium

The *Agrobacterium tumefaciens* EHA105 strain was transformed with the Platinum Gate TALEN expression vector pYS\_026-SSR2 (Yasumoto et al. 2019). The T-DNA region in this expression vector contained the coding sequence for the TALENs targeting the *sterol side chain reductase 2* (SSR2) gene under the 35S promoter (Figure 1). Transformed *Agrobacterium* clones were selected on YEB medium (1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0.5 g/l MgSO<sub>4</sub> 7H<sub>2</sub>O, pH 7.0) containing 50 mg/l kanamycin and stored in 25% glycerol at -80°C.

### Agrobacterium infection of potato stems

The glycerol stock was inoculated into 2–3 ml YEB containing kanamycin and cultured at 28°C overnight at 180–200 rpm shaking. *Agrobacterium* cells were collected by centrifugation and suspended in the appropriate amount of MS medium containing 3% sucrose. Potato stem segments were prepared by cutting an *in vitro* culture and incubated with *Agrobacterium* solution. After removing the excess solution with sterilized filter paper, the segments were co-cultivated on 3C5ZR plates

(Sheerman and Bevan 1988) for 3 to 7 days. To eliminate bacteria cells, the segments were transferred to 3C5ZR plates containing 250 mg/l carbenicillin every 2 weeks. The shoots that regenerated from the segments were isolated and cultured in plant boxes containing solidified MS medium with 3% sucrose and cefotaxime.

### Heteroduplex mobility assay (HMA)

The leaves were removed from the regenerated shoots and incubated in buffer solution (100 mM Tris-HCl, pH 9.5, 1 M KCl, 10 mM EDTA) at 95°C for 10 min. The supernatant was used as a polymerase chain reaction (PCR) template. The regions surrounding the TALEN target sites were amplified using the primer set pY365/pY366 and KOD FX Neo DNA polymerase (Toyobo, Japan). The PCR products were analyzed on a micro-tip electrophoresis machine (MultiNA; Shimadzu, Japan) or 5% polyacrylamide gels.

### PCR analysis for transgene integration

Shoots that showed extra bands in the HMA assay were subcultured at least three times on MS medium containing 3% sucrose and 250 ppm cefotaxime or carbenicillin, and maintained on MS medium containing 3% sucrose without antibiotics. The genomic DNA was extracted and purified from each regenerated line using a NucleoSpin® Plant II kit (Takara Bio, Japan) or DNazol reagent (Invitrogen, USA). Genomic PCR reactions were performed as follows. Each 20 µl PCR reaction solution contained 10 µl of Hot Start Taq 2× Master Mix (New England Biolabs, USA), 0.4 µl of 10 µM forward and reverse primers, and approximately 10 ng of purified genomic DNA. The genomic DNA from non-transgenic Sassy and plasmid DNA were used for control reactions. Eight primer pairs were used to amplify the corresponding regions [35S promoter\_1 (35SP\_1), 35S promoter\_2 (35SP\_2), TALEN-N-terminal (TAL-N), TALEN-C-terminal (FokI), ribulose-1,5-biphosphate carboxylase small subunit terminator (RbcsT), heat shock protein terminator-nopaline synthase promoter (HSPT-NOSP), neomycin phosphotransferase II (NPTII), and nopaline synthase terminator (NOST)] in T-DNA on the TALEN expression binary vector, and one primer pair was used to amplify the potential off-target site in SSR1, a homologue of SSR2 (Figure 1, Table 1). The PCR reaction mixture was incubated at 95°C for 30 s, followed by 34 cycles of denaturing at 95°C for 15 s, annealing at annealing temperature for 15 s, and extension at 68°C for 30 to 60 s. The PCR reaction mixtures were run on the MultiNA instrument. Table 1 lists the primer sequences used in this study.

### Sequence analysis

The regions surrounding the TALEN target sites were amplified using the primer set pY365/pY366, and Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs) from genomic DNA extracted from genome-edited plants without detectable transgenes. The amplicons were cloned into pJET1.2/blunt Cloning Vector (Thermo Fisher Science, USA), and randomly

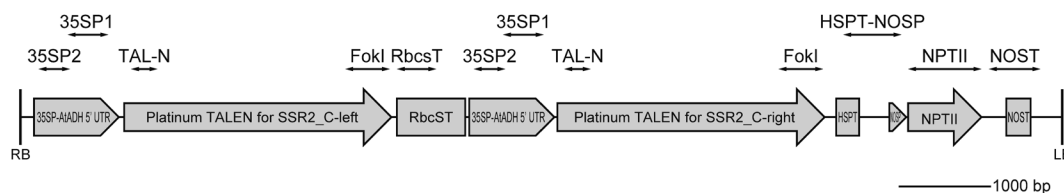


Figure 1. Schematic illustration of the T-DNA region of the Platinum transcription activator-like effector nuclease (TALEN) expression construct. RB and LB, T-DNA right and left borders, respectively; 35SP-AtADH-5'UTR, Cauliflower mosaic virus 35S promoter 5'-untranslated region of *A. thaliana* alcohol dehydrogenase; RbcST, Rubisco small subunit terminator; HSPT, heat shock protein terminator from *Arabidopsis thaliana*; NOSP, nopaline synthase promoter; NPTII, neomycin phosphotransferase II; NOST, nopaline synthase terminator. The double-headed arrows indicate the amplified regions in Figure 2.

Table 1. PCR primers and their applications.

Primer name	Primer sequence (5'→3')	Application	Annealing temp. (°C)	Extension time (s)
pY365/1682/check-for	TGTTCTCTGACACTGTTGTAGCACT	Amplifying the SSR2 target site	55	30
pY366/1683/check-rev	TCGAAGCATAACATACCGGTCATCAT			
1748/StDWF1_C-Forward	TGTTCTCAGACACTGTTGTGTCATA	Amplifying the SSR1 potential off target site	52	30
1749/StDWF1_C-Reverse	TTGAAGCATATCTACCAGTCATGCA			
pY515/35SP-C-1F	AAGACTGGCGAACAGTTCATACAGAG	Detecting TALEN construct (35SP_1)	56	30
pY516/35SP-C-1R	GTCTTGCGAAGGATAGTGGGATTGTG			
pY517/35SP-C-2F	CCCACAGATGGTTAGAGAGGCTTACG	Detecting TALEN construct (35SP_2)	55	30
pY518/35SP-C-2R	CTCTGTATGAACTGTTCCGCCAGTC			
pY519/TALN-C-1F	GAAGGACGCAAGTGGTTGGTCTAG	Detecting TALEN construct (TAL-N)	57	30
pY520/TALN-C_1R	CTCTGGCAACGCCGTGATTATGTG			
pY27/FokI-for	CGCAAGAAATTCAACTCAGGATAGA	Detecting TALEN construct (FokI)	51	30
pY28/FokI-rev	CCGTTATTAATTCCTTCTCACTTCCT			
1658/RbcST-1-seq	TCCCCTTCTGGAATATTCAGC	Detecting TALEN construct (RbcST)	51	30
1659/RbcST-400-rev-seq	CCAAATCTCCAATGGCTATGTC			
pY521/HSPT-C_1F	GGTTGTTGTGTTATGAATTTGTGG	Detecting TALEN construct (HSPT-NOSP)	52	40
pY522/NOSP-C_1R	GGAATTTATGGAACGTCAGTGGAGC			
pY465/NPTII-F2	ATGATTGAACAAGATGGATTGCACGC	Detecting TALEN construct (NPTII)	55	60
pY466/NPTII-R2	TCAGAAGAAGCTCGTCAAGAAGGCG			
pY523/NOSTU-C_1F	TATGAAAGGTTGGGCTTCGGAATCG	Detecting TALEN construct (NOST)	56	40
pY524/NOSTD-C_1R	CGAGATAGGGTTGAGTGTGTTCCA			

selected colonies were used for sequencing analysis.

### Steroidal glycoalkaloid (SGA) measurements of SSR2-edited potato plants

The leaves of in vitro cultured SSR2-edited potato plants were harvested and lyophilized.  $\alpha$ -Solanine and  $\alpha$ -chaconine were extracted and quantified as described previously (Nakayasu et al. 2017).

## Results

### Targeted genome editing in regenerated shoots

SSR2 is the first genome-edited gene in potatoes and the key gene in the steroidal glycoalkaloid (SGA) biosynthetic pathway (Sawai et al. 2014). We infected potato stems with *Agrobacterium* harboring the TALEN expression vector targeting to the gene (Figure 1) and induced the regenerated shoots without selection for transformed cells. The targeted mutagenesis was first screened by HMA with crude template DNA. Of the 371 regenerated shoots produced in four independent experiments, 24 samples showed extra bands that were not detected in the controls, suggesting the presence of

mutations in the TALEN target site (Table 2). To confirm the stability of the mutations, HMA was conducted using purified genomic DNA extracted from the samples after at least three times subculture. As a result, 19 lines still showed the extra-bands, indicating stable mutations of SSR2 in the regenerated shoots (Figure 2).

### No T-DNA insert into the regenerated lines

To assess T-DNA integration in the genome-edited regenerated plants, we performed genomic PCR using eight primer pairs for transgenes (Figure 1). The endogenous SSR1 gene was also amplified from all samples to check the quality and quantity of the template genomic DNA and off-target activity. Of the 19 lines, only six (#5, #21, #28, #4, #84, and #51) showed amplification of all the transgenes tested. Another six lines (#34, #83, #120, #49, and #71) showed partial T-DNA insertion (Figure 2). The remaining seven lines (#26, #24, #117, #118, #75, #79, and #121) did not show amplification of any transgene tested (Figure 2, asterisks). Therefore, these seven lines did not contain transgene and had been genome-edited by transient expression. We did not observe clear changes in genome-editing

Table 2. Genome editing efficiency in regenerated shoots from *Agrobacterium* infected stem explants without selection.

Experiment	No. of infected stem explants	Co-cultivation time with <i>Agrobacterium</i>	No. of stem without <i>Agrobacterium</i> overgrowth	No. of isolated regenerated shoots	No. of HMA positive samples (crude genomic DNA, 1st assay)	No. of HMA positive samples (purified genomic DNA, 2nd assay)	No. of genome edited samples without transgene integration
1	100	3 days	100	67	5	3	1 (1.5%)
2	100	7 days	52	28	4	3	1 (3.6%)
3	200	6 days	200	132	11	7	2 (1.5%)
4	190	3 days	187	144	4	6	3 (2.1%)
Total	590	—	539	371	24 (6.5%)	19 (5.1%)	7 (1.9%)

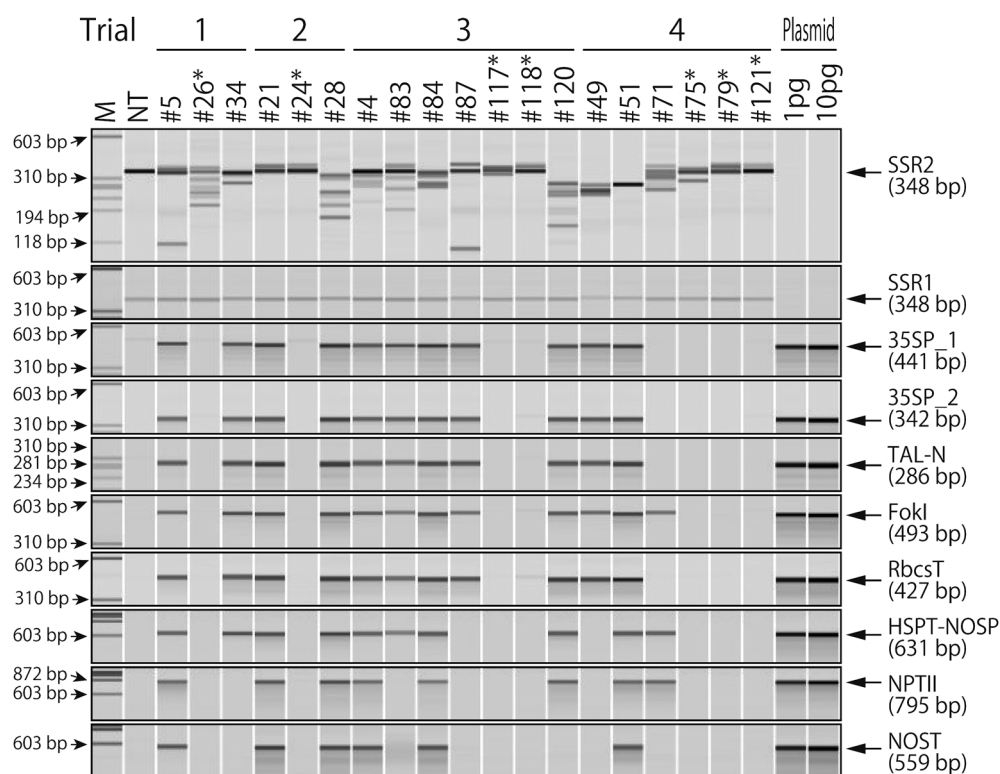


Figure 2. Polymerase chain reaction (PCR) verification of the non-transgenic *sterol side chain reductase 2* (*SSR2*) mutant lines. Purified genomic DNA extracted from regenerated shoots was used as a PCR template. *SSR2* and *SSR1* are the TALEN target and potential off-target, respectively. Eight primer sets were used to amplify the partial T-DNA regions between the left and right border sequences. The amplified regions are shown in Figure 1. The plasmid pYS\_026-*SSR2\_C* was used as a positive control for the transgene amplifications. NT, non-transgenic Sassy. The seven lines did not show amplification of any transgene tested were marked by asterisks.

efficiency among the different times of co-cultivation with *Agrobacterium* (Table 2).

### Targeted mutations and SGA accumulation in regenerated plants

To evaluate the nucleotide sequence of the genome-edited *SSR2* allele, sequences from fifteen or sixteen independent *E. coli* clones were determined. The results indicated that five lines (#26, #24, #118, #79, and #121) had both mutated and intact *SSR2* alleles in their genomes (Figure 3). In lines #75 and #117, three nucleotide deletions (−5, −8, and −51 bp) and three mutations (+1, −2, and −21 bp) were detected respectively (Figure 3). No intact sequence was found among both sequences. As previously reported (Sawai

et al. 2014; Yasumoto et al. 2019), we could not observe any difference in growth among the *SSR2*-knockout lines and the control plants (data not shown). We performed liquid chromatography-mass spectrometry (LC-MS) analysis to evaluate the SGA levels in the regenerated plants. The levels of the predominant SGAs,  $\alpha$ -solanine, and  $\alpha$ -chaconine in the regenerated lines that had no intact *SSR2* sequences (#75 and #117) were much lower than in the non-transgenic sample (Figure 4). The other regenerated lines tested, which had intact *SSR2* without transgene integration, showed varying levels of SGA accumulation. The results suggest the lines #75 and #117 should have complete disrupted-*SSR2* allele.

```

#26
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x4)
tggggcttcttggttcagctgaaa---gcttataccagttgatcaata -4 bp (x4)
-----cagcttataccagttgatcaata +1/-95 bp (x4)
-----
#24
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x9)
tggggcttcttggttcagctgaa-----cttataccagttgatcaata -6 bp (x1)
tggggcttcttggttcagctgaa-----gcttataccagttgatcaata -9 bp (x2)
tggggcttcttggttcagctg-----ataccagttgatcaata -11bp (x4)
#117
tggggcttcttggttcagctgaaa-tcaagcttataccagttgatcaata intact (x0)
tggggcttcttggttcagctgaaaAtcaagcttataccagttgatcaata +1 bp (x6)
tggggcttcttggttcagctgaaa-tc--gcttataccagttgatcaata -2 bp (x5)
tggggcttcttggttcagctg-----CAGTTGATCAATA -21bp (x5)
#118
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x6)
tggggcttcttggttcagctgaaa---gcttataccagttgatcaata -4 bp (x7)
tggggcttcttggttcagctgaaa---gcttataccagttgatcaata -5 bp (x3)
#75
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x0)
tggggcttcttggttcagctgaa-----caagcttataccagttgatcaata -5 bp (x6)
tggggcttcttggttcagctgaa-----gcttataccagttgatcaata -8 bp (x8)
tgggg-----gttataccagttgatcaata -51bp (x2)
#79
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x3)
tggggcttcttggttcagctgaa-----gcttataccagttgatcaata -5 bp (x4)
tggggcttcttggttcagctgaa-----cttataccagttgatcaata -9 bp (x5)
tggggcttcttggttcagctg-----ttataccagttgatcaata -10bp (x3)
#121
tggggcttcttggttcagctgaaatcaagcttataccagttgatcaata intact (x12)
tggggcttcttggttcagctgaaa---ag---ataccagttgatcaata -6 bp (x4)

```

Figure 3. The target *SSR2* sequence in the regenerated lines with no detectable transgenes. Multiple alignment of the *SSR2* target sequence and PCR products amplified from regenerated lines #26, #24, #117, #118, #75, #79, and #121. Gray uppercase letters indicate TALEN-recognition sites. Dashes and black uppercase letters in the sequence alignments indicate nucleotide deletions and insertions, respectively. Deletion sizes are indicated to the right of the alignment.

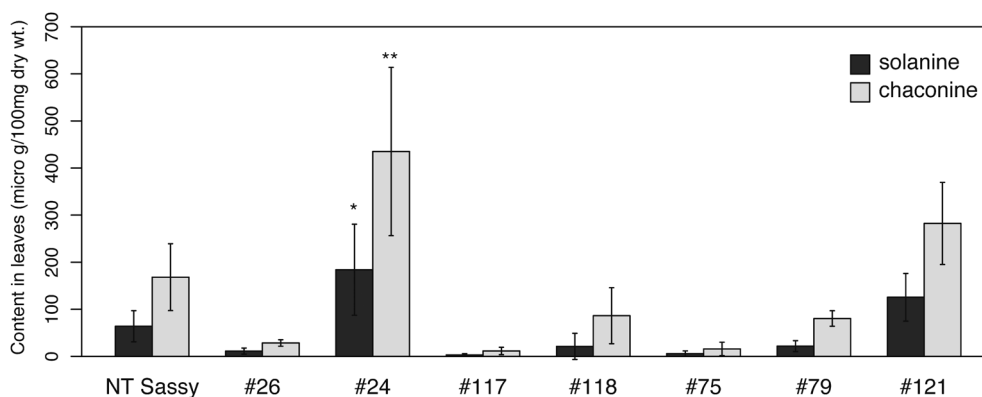


Figure 4. The steroidal glycoalkaloid (SGA) contents of regenerated lines with mutated *SSR2* sequences. The  $\alpha$ -solanine and  $\alpha$ -chaconine levels in non-transformed Sassy and genome-edited lines without detectable transgenes (#26, #24, #117, #118, #75, #79, and #121). The leaves from *in vitro* culture were analyzed for each line. Error bars indicate the standard deviation of three technical replicates. Dunnett's test indicate statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ ).

## Discussion

Genome editing is a powerful tool not only for generating mutants for basic plant science research, but also for crop breeding for agriculture (e.g., Chandrasekaran et al. 2016; Sawai et al. 2014; Yasumoto et al. 2019; Wang et al. 2014). Since it is difficult to deliver genome-editing reagents as RNA or protein into plant cells, genome-editing research in plant science has had to depend almost completely on the generation of transgenic plants expressing site-specific nucleases using stable transformation methods. However, the genome-edited plants produced by stable transformants must be treated as genetically modified organisms (GMOs). The generation of null segregants (harboring the targeted mutation without transgenes) by crossing mutants is one way to overcome this problem (Pacher and Puchta 2017; Wolt et al. 2016). In highly homozygous plants such as rice and soybean, genome-edited plants are usually crossed to generate null segregants (Haun et al. 2014; Li et al. 2012). However, the progenies of highly heterozygous crops, such as potato, have completely different agricultural traits from their parent lines, such

as different yields. In this study, we infected potato stem explants with *Agrobacterium* to transiently express TALEN proteins in potato cells. Unlike conventional plant genome editing research, no antibiotics or herbicides were added to the regeneration medium for selection of transgenic cells. The shoots regenerated from the sample without selection were analyzed by HMA to confirm targeted mutations in the *SSR2* SGA biosynthetic gene; 5.1% (19 lines from 371 regenerated shoots) had stable *SSR2* mutations. We confirmed the presence of T-DNA insertions in the genomes of these lines. Since we could not amplify any of the transgenes tested in seven regenerated lines, this suggested that the introduced T-DNA harboring the TALEN expression cassette was transiently transcribed and translated, and introduced mutations in *SSR2* that were then degraded by endogenous nucleases before the T-DNA was inserted into the genome. The contribution of each *SSR2* allele in SGA biosynthesis is unknown. Therefore, it is possible that the varying levels of SGAs in genome-edited plants with intact *SSR2* sequences is caused by their mutation pattern. It is unknown what caused the #24 SGA content to be higher than that of control. It is possible that *SSR2*

was activated by the mutation. Generally, SGA is rarely low, but occasionally SGA content is increased by the environmental stimuli (such as light and wounding). Since at least the disruption of all *SSR2* alleles is enough for reducing SGA levels in potato, two genome-edited plants (#75 and #117) with no intact *SSR2* sequence showed much lower accumulation levels than that of control. We generated *SSR2* mutants with the desired reduced SGA phenotype and no detectable transgenes.

Previously, we used the same TALEN expression vector to generate genome-edited potato lines by stable transformation. As a result, all the three transgenic potato lines had the targeted mutations in *SSR2* (Yasumoto et al. 2019). The frequencies of genome editing in the present study was lower than that of conventional stable transformation approach. However, we obtained genome edited lines without transgene by transient TALEN expression. The efficiency of transgene-free genome editing in the present study (7 transgene free mutated shoots/371 regenerated shoots, 1.9%) was comparable with that of previous studies using particle bombardment in wheat or protoplast transfection in potato (Clasen et al. 2016; Zhang et al. 2016). We used highly active Platinum TALEN (Sakuma et al. 2013; Yasumoto et al. 2019). It may be difficult to achieve high genome-editing efficiency using other site-specific nucleases. Chen et al. (2018) performed genome editing using *Agrobacterium*-mediated transient CRISPR-Cas9 expression in tobacco, and reported about several percent mutation rate for all regenerated shoots. Their efficiency of transgene-free genome editing was lower than that of our study. They used the *phytoene desaturase* gene as the target gene; this makes it easy to screen the regenerated shoots with targeted mutations based on their albino phenotype. Because the albino phenotype should be due to knockout of all four *PDS* alleles, the genome editing efficiency might be underestimated in that report. In our study, we clearly showed that mutants with invisible phenotypes (e.g., metabolite changes) from *Agrobacterium*-infected samples can be screened simply by PCR with comparable genome-editing efficiency. Many factors would be optimized, including bacterial strain, cell concentration, and co-culture time, to ensure higher efficiency of transgene-free genome editing using *Agrobacterium*.

We used PCR to detect insert T-DNA segments in genome-edited potatoes. Although PCR is highly sensitive, it is difficult to test complete T-DNA region because it depends on the primers used. Recently, a paper on detection of foreign DNA segments in genome-edited rice using high-throughput DNA sequencing has been published (Itoh et al. 2020). We expect that a system that can easily assess transgenes in the genome edited crops will be authorized.

The new varieties generated in this study should have

almost the same genome as the parent cultivar, Sassy, except for the *SSR2* sequences and SGA content, since we did not cross the genome-edited plants to remove the integrated transgenes. The strategy used here will make a substantial contribution to the fine-tuning breeding of vegetative crops, including potato. The strategy is like mutagenesis, so we named *Agrobacterial mutagenesis* to genome editing using transient gene expression by *Agrobacterium*. Since it is difficult to assess SGA levels in many progenies, the levels are usually evaluated late in the breeding cycle. Lenape, a potato variety released in 1968, was withdrawn in 1970 since it had unsafe solanine levels (Akeley et al. 1968; Anonymous 1970). By applying our strategy, a potato line with much SGA level could be improved by disrupting the SGA biosynthesis gene to breed novel cultivars with both unchanged agricultural traits and lower SGA levels. Our strategy could also be applied to modify other important traits of potato breeding, such as cold-induced sweetening by *vacuolar invertase*, enzymatic browning by *polyphenol oxidase*, etc., by knocking out single genes.

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