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

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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.

*Agrobacteria* 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. Nicola 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. *Agrobacteria* 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 *sterol side chain reductase 2* (Figure 1, Table 1). The PCR reaction mixture was run on the MultiNA instrument. Table 1 lists the primer sequences used in this study.

**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 3CSZR 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 2X 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 SSRI, 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 2X 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
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. α-Solanine and α-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 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...
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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, α-solanine, and α-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.

**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 *Agrobacterium* | No. of stem without *Agrobacterium* 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.

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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 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 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 previous studies using particle bombardment in wheat or protoplast transfection in potato (Clasen et al. 2016; Zhang et al. 2016).

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