Molecules and Cells

RPS5A Promoter-Driven Cas9 Produces Heritable Virus-Induced Genome Editing in *Nicotiana attenuata*

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The virus-induced genome editing (VIGE) system aims to induce targeted mutations in seeds without requiring any tissue culture. Here, we show that tobacco rattle virus (TRV) harboring guide RNA (gRNA) edits germ cells in a wild tobacco, *Nicotiana attenuata*, that expresses *Streptococcus pyogenes* Cas9 (SpCas9). We first generated *N. attenuata* transgenic plants expressing SpCas9 under the control of 35S promoter and infected rosette leaves with TRV carrying gRNA. Gene-edited seeds were not found in the progeny of the infected *N. attenuata*. Next, the *N. attenuata* ribosomal protein S5 A (RPS5A) promoter fused to SpCas9 was employed to induce the heritable gene editing with TRV. The RPS5A promoter-driven SpCas9 successfully produced monoallelic mutations at three target genes in *N. attenuata* seeds with TRV-delivered guide RNA. These monoallelic mutations were found in 2%-6% seeds among M_1 progenies. This editing method provides an alternative way to increase the heritable editing efficacy of VIGE.

Keywords: CRISPR/Cas9, heritable plant genome editing, ribosomal protein S 5A promoter, tobacco rattle virus, virus-induced genome editing

INTRODUCTION

"Omics" technologies allow us to identify a list of genes responsible for specific traits in plants (Alonso and Ecker, 2006). To characterize gene function accurately, loss- or gain-of-function mutants of interest are required. The CRISPR (clustered regularly interspaced short palindromic repeats) system is extremely useful for this reverse genetic approach, because it can be used to generate indel (insertion and deletion) mutations or nucleotide substitutions at the target loci in any plant species with high accuracy and efficiency (Zhu et al., 2020). The most widely used CRISPR system is derived from *Streptococcus pyogenes;* this system consists of CRISPR-associated protein 9 (SpCas9) and a single-stranded guide RNA (gRNA) (Jinek et al., 2012). For plant genome editing, SpCas9 and gRNA are expressed in cells, and a whole plant is regenerated from the edited callus. These processes, which are called transformation and regeneration, often take from several months to over a year (Ahn et al., 2013; Altpeter et al., 2016). In addition, because tissue culture media and growth conditions need to be optimized depending on genotype, even for the same species, establishing a standard tissue culture method for one species is difficult (Altpeter et al., 2016).

To generate gene-edited plants without tissue culture, plant viral vectors have been engineered; these vectors express SpCas9 proteins and/or gRNA in plants (Ali et al., 2015; Ariga et al., 2020; Ellison et al., 2020; Lei et al., 2021; Li et al., 2021; Wang et al., 2017; Zhang et al., 2020). This system is called the virus-induced genome editing (VIGE) system (Oh et

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al., 2021a). The delivery capacity of viral vectors is negatively correlated with the size of the cargo involved. Although sonchus yellow net rhabdovirus can deliver a large cargo, SpCas9 (~4.2 kbp) with gRNA (~100 bp), into the systemic tissues (Ma et al., 2020), the movement of commonly used viruses, such as tobacco rattle virus (TRV) (Ali et al., 2018), tobacco mosaic virus (Cody et al., 2017), and geminivirus (Yin et al., 2015), is hampered by the size of SpCas9.

Rather than focus on the delivery of both SpCas9 and gRNA, scientists have focused on the delivery of gRNA into SpCas9-expressing meristematic cells. This approach results in the production of gene-edited seeds (Ellison et al., 2020; Lei et al., 2021; Li et al., 2021). Guide RNA fused with the mobile *Flowering Locus T* (*FT*) mRNA sequence enabled the acquisition of heritable mutants with a mutation frequency of 65%-100% from the TRV-infected *Nicotiana benthamiana* (Ellison et al., 2020), which expresses a SpCas9 gene under the control of the 35S*PPDK* promoter (the 35S promoter fused to the maize C4PPDK basal promoter; Baltes et al., 2015; Li et al., 2013; Yoo et al., 2007). FT-fused gRNA induced the heritable mutation with a frequency of 4%-9% from the cotton leaf crumple virus-infected *Arabidopsis* (Lei et al., 2021), which expressed SpCas9 under the control of YAO and 35S promoter. A barley stripe mosaic virus-derived gRNA also generated heritable mutations (13%-100%) in the transgenic wheat, which expresses SpCas9 under the control of maize ubiquitin promoter (Li et al., 2021). However, whether the promoter used for SpCas9 expression is critical to induce heritable gene editing with virus-delivered gRNA remains unknown.

In this study, we developed a TRV-based VIGE system for the ecological model plant *Nicotiana attenuata*. Previous *Arabidopsis* studies showed that the expression of SpCas9 in shoot apical meristem or germ cells is critical for heritable gene editing (Mao et al., 2016; Yan et al., 2015). Ribosomal protein S5 A (RPS5A) promoter strongly confers expression of transgenes in meristematic cells and germlines (Tsutsui and Higashiyama, 2017). Therefore, we hypothesized that the efficacy of the VIGE system would be enhanced when SpCas9 proteins were expressed under the control of the RPS5A promoter in *N. attenuata*.

MATERIALS AND METHODS

Plant materials

The wild-type *N. attenuata* Utah seeds were a kind gift from the Department of Molecular Ecology at the Max Plank Institute for Chemical Ecology. Seeds were germinated following protocol as previously described (Krügel et al., 2002). All M_1 and F_1 seeds were pooled from three infected plants and randomly germinated. The plants were grown in a plant growth chamber under a 16/8 h light/dark photoperiod at 25°C with ± 2°C. To generate SpCas9-expressing transgenic plants, the pHAtC (35S:Cas9; accession No. KU213971; Kim et al., 2016) and pRPS5A:Cas9 binary vectors were transformed into the *Agrobacterium tumefaciens* strain LBA4404 by the thaw-freeze method. The hypocotyl tissues were used for transformation and tissue culture as described previously (Kang et al., 2020).

Vector construction and guide RNA design

The *N. attenuata* RPS5A promoter was amplified from the genomic DNA of *N. attenuata* with the RPS5A-F and RPS5A-R primers. The coding sequence of SpCas9 was synthesized by Macrogen (Korea) and amplified with Cas9-F and Cas9-R primers. Heat shock protein terminator was amplified from the pKI1.1R vector (Tsutsui and Higashiyama, 2017) with HSP-F and HSP-R primers. These amplified polymerase chain reaction products were then recombined into pHAtC (Kim et al., 2016) to acquire pRPS5A:Cas9 vector (Supplementary Table S1) using an In-Fusion HD Cloning Kit (Takara, Japan). The U6 sequences were removed from pHAtC and pRPS5A:Cas9 vectors with SacI (NEB, USA).

The TRV1 and TRV2 vectors were a gift from Sir David Baulcombe (University Cambridge). The subgenomic promoter of pea early-browning virus was used to express *N. attenuata phytoene desaturase* (NaPDS)-targeted gRNA (TRV2:gPDS), *N. attenuata chalcone synthase 2* (NaCHAL2)-targeted gRNA (TRV2:gCHAL2), and *N. attenuata chalcone synthase 3* (NaCHAL3)-targeted gRNA (TRV2:gCHAL3) (Supplementary Table S2). The subgenomic promoter of TRV was used to express NaPDS-targeted gRNA (T:gPDS and TH:gPDS) and TH:gPDS fused with *Arabidopsis FT* mRNA sequence (TH:gPDS-FT) (Supplementary Table S2). The coding sequence of *FT*, which was described in Ellison et al. (2020), was synthesized by Macrogen. The target sequence of *NaPDS* (A4A49_02763), *NaCHAL2* (A4A49_34074), and *NaCHAL3* (A4A49_39367) was designed using Cas-Designer program implemented in CRISPR RGEN Tools (http://www.rgenome.net/) (Bae et al., 2014; Park et al., 2015). Primers used in this study were synthesized by Macrogen. Genome sequence information for *N. attenuata* is available in the *N. attenuata* Data Hub (http://nadh.ice.mpg.de/NaDH/). All primer sequences are summarized in Supplementary Table S3.

Agroinfiltration

N. attenuata infiltration was performed as previously described (Senthil-Kumar and Mysore, 2014) with minor modifications. Briefly, the TRV1 and TRV2 derivatives were introduced into *Agrobacterium tumefaciens* strain AGL1. The AGL1 containing TRV2 derivatives, was mixed with AGL1 carrying TRV1 in a ratio of 1:1 before inoculation; the mixed solution was co-infiltrated into the leaves of 4- to 5-week-old SpCas9-expressing *N. attenuata*. Three plants were used for each agroinfiltration. After agroinfiltration, *N. attenuata* plants were maintained under a 48 h dark at 22°C and then transferred into the growth chamber (a 16/8 h light/dark photoperiod at 25°C).

Targeted deep sequencing

Targeted deep sequencing was performed as previously described (Oh et al., 2020). Briefly, the genomic DNA was extracted from leaves, petals, anthers, and ovaries using HiGene genomic DNA prep kit (BIOFACT, Korea). All primer sequences are summarized in Supplementary Table S3. High-throughput sequencing was performed in the KAIST BIOCORE center (http://biocore.kaist.ac.kr/) with Illumina Miniseq (Illumina, USA). The indel frequency and patterns were analyzed using the Cas-Analyzer tool, which is available at CRISPR RGEN

Tools (Park et al., 2017). The indel frequency (%) was calculated by dividing the number of sequencing reads containing mutations by the number of total sequencing reads.

RESULTS

No gene-edited plants were obtained from infected 35S:Cas9 plants

To develop a VIGE system for *N. attenuata*, we first generated transgenic *N. attenuata* expressing SpCas9 under the control of the 35S promoter (35S:Cas9) (Oh et al., 2021b). A gRNA was designed to target the *N. attenuata phytoene desaturase* (*NaPDS*) gene and cloned into the TRV2 vector under the subgenomic promoter of the pea early-browning virus (TRV2:gPDS) (Fig. 1). We infected 35S:Cas9 leaves with TRV2:gPDS and then collected M_1 seeds from the infected plants. If germ cells were mutated by the SpCas9-gRNA complex and viruses were not detected in the next generation, we would expect three distinct genotypes in the seeds (M_1 generation): wild-type allele, monoallelic mutation (one wild-type allele and one mutated allele), and biallelic mutation. To screen gene-edited seeds, we pooled equal amounts of leaf tissue from M_1 seedlings and performed targeted deep sequencing across the target site (Fig. 1). If one monoallelic mutant was found in pooled 20 M_1 seedlings, the mutation frequency would be expected to be near 2.5% (1 monoallelic mutation/40 total alleles = 2.5%). We then examined the mutation frequencies in 174 M_1 progenies from TRV2:gPDS-infected 35S:Cas9 (Supplementary Fig. S1). Although the mutation was observed in the infected leaves (19.7% ± 2.7%, Oh et al., 2021b), we found no gene-edited seeds in the pooled M_1 seedlings of TRV2:gPDS-infected 35S:Cas9 (Supplementary Fig. S1, Oh et al., 2021b).

We next manipulated gRNA expression or movement to induce heritable gene editing in seeds. Firstly, we tested whether a NaPDS-targeted gRNA expressed by the TRV subgenomic promoter (T:gPDS) performed better than TRV2:gPDS. Among 223 M_1 progenies from T:gPDS-infected 35S:Cas9, we could not obtain gene-edited plants in the pooled M_1 seedlings (Supplementary Fig. S2). Next, we inserted a hammerhead ribozyme sequence between the subgenomic promoter of TRV2 and NaPDS-targeted gRNA (TH:gPDS), because we found that TH:gPDS induced more indel mutations in the infected leaves than did T:gPDS (Oh et al., 2021b). A total of 123 M_1 progenies from TH:gPDS-infected 35S:Cas9 contained no mutations in the target site (Supplementary Fig. S3). To increase the movement of gRNA into germ cells, a NaPDS-targeted gRNA was fused with *Arabidopsis FT* mRNA and cloned into the TRV2 vector (TH:gPDS-FT) (Ellison et al., 2020). TH:gPDS-FT generated a similar mutation frequency compared with TH:gPDS in the infected leaves (Supplementary Fig. S4A). No mutated seeds were found from TH:gPDS-FT-infected plants (Supplementary Fig. S4B). These results suggest that the 35S promoter-driven SpCas9 was not able to produce gene-edited seeds with TRV-delivered gRNA in *N. attenuata*.

Heritable virus-induced gene editing in pRPS5A:Cas9 plants

We hypothesized that the germline expression of SpCas9 would be critical to increase the editing efficiency of plant viruses in germ cells. For *Arabidopsis* germline mutation, the RPS5A-driven SpCas9 performed much better than the 35S-driven SpCas9 (Tsutsui and Higashiyama, 2017). In addition, the adenine base editor (ABE) is known to produce the edited T_1 plants of *Arabidopsis* when the RPS5A promoter is used to express ABE rather than the 35S and the YAO promoter (Kang et al., 2018). Although the YAO promoter was also developed for editing germ cells in *Arabidopsis* (Yan et al., 2015), the YAO promoter did not perform better than the 35S promoter for virus-mediated germline editing in *Arabidopsis* (Lei et al., 2021). Therefore, we generated the transgenic *N. attenuata* plants expressing SpCas9 driven by the *N. attenuata* RPS5A promoter (pRPS5A:Cas9).

To explore the effects of the RPS5A promoter-driving SpCas9, we infected pRPS5A:Cas9 leaves with TRV2:gPDS. The mutation frequency was 6.4% ± 0.2% in the pRPS5A:Cas9 leaves infected with TRV2:gPDS (Fig. 2A). The major mutation patterns were T and A insertions in the infected leaves (Fig. 2B). Next, several hundred seeds were collected to investigate the heritable editing ability of TRV2:gPDS in pRPS5A:Cas9. A total of 206 M_1 progenies were divided into 11 groups for pooling genomic DNA (Fig. 2C). The targeted deep sequencing analysis showed that in all three groups,

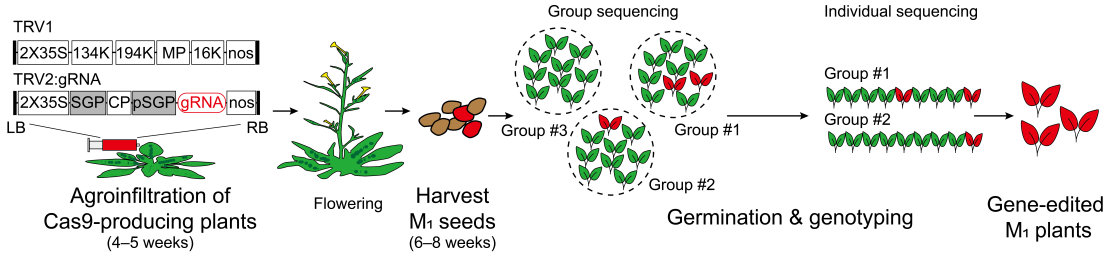
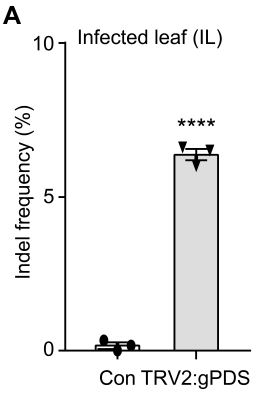


Fig. 1. Procedure of TRV-induced heritable gene editing in *N. attenuata*. *A. tumefaciens* containing TRV2 derivatives was mixed with *Agrobacterium* carrying TRV1 in a ratio of 1:1. The mixed solution was co-infiltrated into the leaves of 4- to 5-week-old SpCas9-producing *N. attenuata*. M_1 seeds were collected from TRV-infected plants and germinated. Equal amounts of leaf tissues from M_1 seedlings (n = 11-26) were pooled for performing targeted deep sequencing. The editing frequencies in individual plants of groups harboring a mutant were examined. It takes three to four months to obtain gene-edited M_1 seeds. nos, nopaline synthase terminator; SGP, TRV subgenomic promoter; CP, coat protein; pSGP, pea early-browning virus subgenomic promoter; LB, left border; RB, right border.



B

gRNA targeting NaPDS PAM

WT TCAAGTTGGTCATGTGACAA AGG

Total indel 6.5%

IL1 TCAAGTTGGTCATGTGTCAA 3.3%

TCAAGTTGGTCATGTGACAA 2.3%

Total indel 6.0%

IL2 TCAAGTTGGTCATGTGTCAA 2.7%

TCAAGTTGGTCATGTGACAA 1.9%

Total indel 6.6%

IL3 TCAAGTTGGTCATGTGTCAA 3.0%

TCAAGTTGGTCATGTGACAA 2.5%

C M1 seedlings from TRV2:gPDS-infected pRPS5A:Cas9

Group #	Seedling #	Total reads	Indels	Frequency (%)	Mutation patterns
1	20	35981	14	0.0	-
2	16	42293	48	0.1	-
3	17	40143	869	2.2	G ins (840)
4	18	40085	391	1.0	-
5	17	44736	65	0.1	-
6	20	44364	48	0.1	-
7	23	44352	29	0.1	-
8	23	45569	36	0.1	-
9	15	41151	2397	5.9	A ins (1580) T ins (753)
10	16	49475	2275	4.6	C ins (2134)
11	21	47874	34	0.1	-

D M1 from TRV2:gPDS-infected pRPS5A:Cas9

Group #		Total reads	Indels	Frequency (%)
3	1	21373	10529	49.3
	2	22191	24	0.1
	3	12759	26	0.2
	4	22378	36	0.2
	5	21897	99	0.5
	6	24026	54	0.2
	7	24387	80	0.3
	8	25578	43	0.2
	9	21790	24	0.1
	10	24790	19	0.1
	11	9437	4	0.0
	12	22336	4	0.0
	13	17156	16	0.1
	14	23335	36	0.2
	15	26363	44	0.2
	16	26247	30	0.1
	17	25117	20	0.1
9	1	26010	20	0.1
	2	28380	25	0.1
	3	24332	9	0.0
	4	24317	6	0.0
	5	28030	26	0.1
	6	28381	14	0.0
	7	27306	13088	47.9
9	8	24737	12125	49.0
	9	24051	11998	49.9
	10	13093	61	0.5
	11	19496	40	0.2
	12	17781	31	0.2
	13	18837	46	0.2
	14	23842	17	0.1
	15	20603	58	0.3
10	1	22698	63	0.3
	2	15242	8	0.1
	3	38848	107	0.3
	4	24835	11	0.0
	5	23139	8	0.0
	6	19783	280	1.4
	7	19986	6	0.0
	8	20411	10	0.0
	9	22917	17	0.1
	10	28185	25	0.1
	11	25603	13543	52.9
	12	25749	7	0.0
	13	22421	19	0.1
	14	27407	27	0.1
	15	29686	6	0.0
	16	9034	11	0.1

E

gRNA targeting NaPDS PAM

WT TCAAGTTGGTCATGTGACAA AGG

M1 3-1 TCAAGTTGGTCATGTGGCAA 49%

TCAAGTTGGTCATGTG - CAA 51% (WT)

9-7 TCAAGTTGGTCATGTGTCAA 48%

TCAAGTTGGTCATGTG - CAA 52% (WT)

9-8 TCAAGTTGGTCATGTGACAA 49%

TCAAGTTGGTCATGTG - CAA 51% (WT)

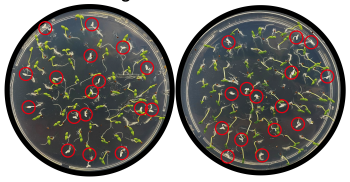
9-9 TCAAGTTGGTCATGTGACAA 50%

TCAAGTTGGTCATGTG - CAA 50% (WT)

10-11 TCAAGTTGGTCATGTGCCAA 53%

TCAAGTTGGTCATGTG - CAA 47% (WT)

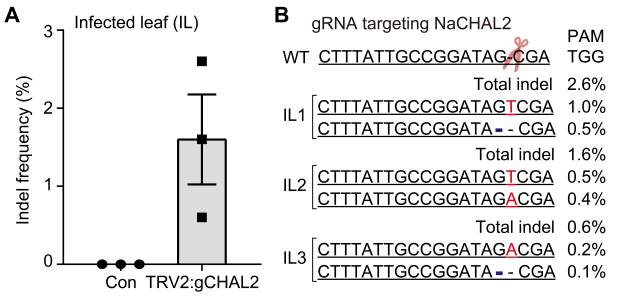
F M2 seedlings from M1-3-1



Wild type	Albino
113	32
3.5	1

Fig. 2. Heritable virus-induced gene editing in TRV2:gPDS-infected pRPS5A:Cas9 plants. (A) Indel frequency (%) in pRPS5A:Cas9 leaves infected with TRV2:gPDS. Error bars represent the SEM of three replicates (two-tailed Student's *t*-test; $****P < 0.0001$). (B) Mutation patterns and frequency (%) observed in the infected leaves. Wild-type (WT) sequences of *NaPDS* are shown with spacer sequence (underlined) and PAM. Insertion mutations are presented in red. Total indel frequency (%) was calculated by dividing the number of reads containing indel mutation by the number of total sequencing reads. (C) Mutation patterns and frequency (%) observed in pooled M_1 seedlings of TRV2:gPDS-infected pRPS5A:Cas9 plants. Groups with mutations were highlighted in gray. The mutation pattern is given followed by the number of its reads in parentheses. (D) Mutation frequency of individual plants of three groups: group #3, #9, and #10. The mutated M_1 seedlings are highlighted in gray. (E) Monoallelic mutations in M_1 seedlings. (F) Green and white phenotypes of M_2 seedlings from an M_1-3-1 plant. The red circles indicate the albino phenotype of NaPDS mutants. Con, control; TRV2:gPDS, NaPDS-targeted guide RNA expressed under the control of pea early-browning virus subgenomic promoter.

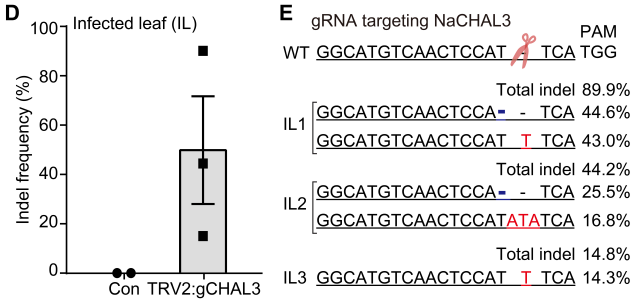
the editing frequency was more than 2%: group #3, 2.2% (n = 17); group #9, 5.9% (n = 15); and group #10, 4.6% (n = 16) (Fig. 2C). We then examined the editing frequencies in individual plants of three groups: one monoallelic mutant in group #3, three monoallelic mutants in group #9, and one monoallelic mutant in group #10 were identified (Fig. 2D). All five mutants had monoallelic mutations and no biallelic mutation (Fig. 2E). The mutation patterns of five M_1 mutants were single nucleotide insertions; M_1-3-1, M_1-9-7, M_1-9-8, M_1-9-9, and M_1-10-11 had a G, T, A, A, and C insertion, respectively, at the cleavage site (Fig. 2E). The total mutation frequency of TRV2:gPDS was putatively about 2.4% (5 mutants/206 total progenies = 2.4%) in pRPS5A:Cas9 (Fig. 2D). Next, we examined the heritability of mutations found in an



C

M1 from TRV2:gCHAL2-infected pRPS5A:Cas9

Total reads	Indels	Frequency (%)	Mutation patterns	Total reads	Indels	Frequency (%)	Mutation patterns
32882	0	0.0	-	27640	0	0.0	-
32225	0	0.0	-	31476	0	0.0	-
36503	0	0.0	-	31054	15095	48.6	G del (15095)
34339	0	0.0	-	30636	13	0.0	-
31320	0	0.0	-	28253	0	0.0	-
34259	0	0.0	-	34342	0	0.0	-
35385	0	0.0	-	31019	0	0.0	-
35912	0	0.0	-	30295	3	0.0	-



F

M1 from TRV2:gCHAL3-infected pRPS5A:Cas9

Total reads	Indels	Frequency (%)	Mutation patterns	Total reads	Indels	Frequency (%)	Mutation patterns
58165	143	0.2	-	49583	734	1.5	-
63835	202	0.3	-	61308	94	0.2	-
63999	19	0.0	-	78868	217	0.3	-
54634	393	0.7	-	65711	11	0.0	-
50772	30403	59.9	10 del (29919)	66720	10	0.0	-
68183	79	0.1	-	57928	270	0.5	-
53287	6	0.0	-	62886	165	0.3	-
58063	369	0.6	-	58060	648	1.1	-
51257	450	0.9	-	51964	8	0.0	-
57145	368	0.6	-	53559	202	0.4	-
54384	4	0.0	-	59000	98	0.2	-

Fig. 3. Heritable virus-induced gene editing in pRPS5A:Cas9 plants infected with TRV2:gCHAL2 and TRV2:gCHAL3. (A and D) Indel frequency (%) of pRPS5A:Cas9 leaves infected with TRV2:gCHAL2 (A) and TRV2:gCHAL3 (D). Error bars represent the SEM of three replicates. (B and E) Mutation patterns and frequency (%) observed in the infected leaves. Wild-type (WT) sequences of *NaCHAL2* (B) and *NaCHAL3* (E) are shown with spacer sequence (underlined) and PAM. Insertion and deletion mutations are presented in red and blue, respectively. Total indel frequency (%) was calculated by dividing the number of reads containing indel mutations by the number of total sequencing reads. (C and F) Mutation pattern and frequency (%) observed in M_1 seedlings of pRPS5A:Cas9 plants infected with TRV2:gCHAL2 (C) and TRV2:gCHAL3 (F). The M_1 seedling with mutation is highlighted in gray. The mutation pattern is given followed by the number of its reads in parentheses. Con, control; NaCHAL, *N. attenuata* chalcone synthase; TRV2:gCHAL2/3, NaCHAL2/3-targeting guide RNA expressed under the control of pea early-browning virus subgenomic promoter.

M_1-3-1 plant to the next generation. The 145 M_2 seeds from a M_1-3-1 plant were germinated, and the obvious albino phenotype of seedlings was observed to occur at a ratio of 3.5:1 (113 green: 32 white seedlings) (Fig. 2F), which was consistent with the segregation ratio of the monoallelic mutation.

In addition, we targeted the *chalcone synthase 2* (*NaCHAL2*) and *chalcone synthase 3* (*NaCHAL3*) gene of *N. attenuata* to demonstrate the feasibility of our VIGE system. A gRNA targeting *NaCHAL2* and *NaCHAL3* was cloned into TRV2:gCHAL2 and TRV2:gCHAL3, respectively. We infected pRPS5A:Cas9 leaves with TRV2:gCHAL2 and TRV2:gCHAL3. The mutation frequency of TRV2:gCHAL2 and TRV2:gCHAL3 was 1.6% ± 0.6% and 49.6% ± 21.9% in the infected leaf, respectively (Figs. 3A and 3D). The major mutation patterns were single nucleotide insertions and deletions at each cleavage site in the infected leaves (Figs. 3B and 3E). We examined the mutation frequencies in individual M_1 plants collected from the infected pRPS5A:Cas9 and identified one monoallelic M_1 mutant of *NaCHAL2* (G deletion) (Fig. 3C, Supplementary Fig. S5A), and one monoallelic M_1 mutant of *NaCHAL3* (ten nucleotide deletion) (Fig. 3F, Supplementary Fig. S5B).

Male and female germ cells were edited in pRPS5A:Cas9 plants

We further examined the editing frequencies in floral organs to determine which organs were responsible for heritable mutations in pRPS5A:Cas9 plants. The editing frequencies in floral tissues of pRPS5A:Cas9 plants infected with TRV2:gPDS were 4.7% ± 0.4% in petals (Fig. 4A), 5.2% ± 1.2% in anthers (Fig. 4B), and 3.3% ± 0.3% in ovaries (Fig. 4C). The major mutation patterns were single nucleotide insertions at the cleavage site in floral tissues (Figs. 4D-4F) as shown in infected leaves (Fig. 2B). These results suggest that a few microsporocytes or megasporocytes are edited by TRV2:gPDS. To clarify which germ cells are edited, we pollinated wild-type *N. attenuata* flowers with the pollen from TRV2:gPDS-infected pRPS5A:Cas9 (Cross A, Fig. 5A), and we also pollinated TRV2:gPDS-infected pRPS5A:Cas9 flowers with the pollen from wild-type plants (Cross B, Fig. 5B). We removed all anthers before hand pollination. A total of 109 F_1 progenies from Cross A were divided into 5 groups (Fig. 5A). We found that group #1 of F_1 seedlings contained mutations with a frequency of 1.2% (n = 24) (Fig. 5A). The editing frequencies in individual plants of group #1 were examined; one mutant harboring an A insertion was obtained (Fig. 5C). Next, 35 F_1 progenies from Cross B were divided into 2 groups (Fig. 5B). The mutation frequencies of group #1 and group #2 were 3.4% (n = 17) and 2.7% (n = 18), respectively (Fig. 5B). The editing frequencies in individual plants of group #1 were examined: two monoallelic mutants were identified (Fig. 5D). Both mutants had a T insertion at the cleavage site (Fig. 5D). These results suggest that both female and male germ

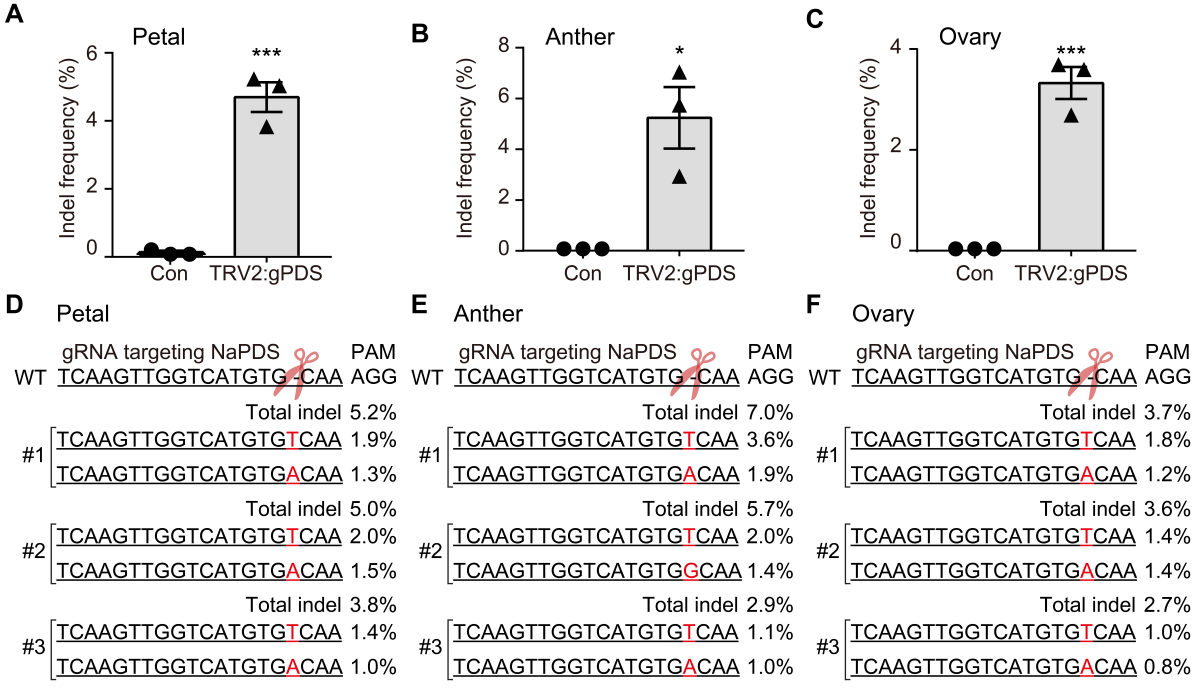
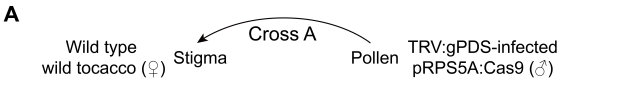
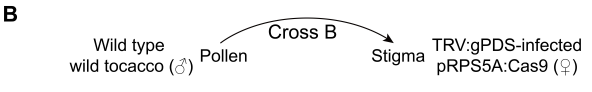


Fig. 4. Gene-editing efficacy of TRV2:gPDS-infected pRPS5A:Cas9 flowers. (A-C) Indel frequency (%, mean ± SEM) of pRPS5A:Cas9 infected with TRV2:gPDS in the petals (A), anthers (B), and ovaries (C) (two-tailed Student's *t*-test; $***P < 0.001$, $*P < 0.05$). (D-F) Mutation patterns and frequency (%) observed in petals (D), anthers (E), and ovaries (F). Wild-type (WT) sequences of NaPDS are shown with spacer sequence (underlined) and PAM. Insertion mutations are shown in red. Total indel % was calculated by dividing the number of reads containing indel mutations by the number of total sequencing reads. Con, control; TRV2:gPDS, NaPDS-targeted guide RNA expressed under the control of pea early-browning virus subgenomic promoter.



Group #	Seedling #	Total reads	Indels	Frequency (%)	Mutation patterns
1	24	12653	162	1.2	A ins (152)
2	24	38579	6	0.0	
3	24	36346	15	0.0	
4	24	39667	26	0.1	
5	13	11179	9	0.0	



Group #	Seedling #	Total reads	Indels	Frequency (%)	Mutation patterns
1	17	21932	738	3.4	T ins (736)
2	18	30329	816	2.7	T ins (816)

C F1 from Cross A: WT (♀) x VIGE (♂) Group #1

Total reads	Indels	Frequency (%)	Mutation patterns	Total reads	Indels	Frequency (%)	Mutation patterns
23547	3	0.0	-	25170	2	0.0	-
27675	10	0.0	-	27117	25	0.1	-
28683	6	0.0	-	27432	20	0.1	-
37212	3	0.0	-	32934	5	0.0	-
26942	4	0.0	-	27959	4	0.0	-
26437	4	0.0	-	30238	2	0.0	-
31359	3	0.0	-	29031	5	0.0	-
29148	3	0.0	-	28676	65	0.2	-
25678	12580	49.0	A ins (12553)	22670	20	0.1	-
28136	32	0.1	-	29582	3	0.0	-
28237	5	0.0	-	32980	42	0.1	-
30717	29	0.1	-	27424	97	0.4	-

D F1 from Cross B: VIGE (♀) x WT (♂) Group #1

Total reads	Indels	Frequency (%)	Mutation patterns	Total reads	Indels	Frequency (%)	Mutation patterns
22167	95	0.4	-	28707	13929	48.5	T ins (13742)
27269	59	0.2	-	29068	13	0.0	-
25184	3	0.0	-	29567	5	0.0	-
26419	2	0.0	-	25282	3	0.0	-
24451	11	0.0	-	23048	4	0.0	-
27506	0	0.0	-	29215	7	0.0	-
29036	30	0.1	-	27534	12366	44.9	T ins (12167)
29180	4	0.0	-	29077	2	0.0	-
				31193	755	2.4	-

Fig. 5. Pollen and egg cells are edited in TRV2:gPDS-infected pRPS5A:Cas9 flowers. (A and B) Cross A: Flowers of wild-type *N. attenuata* were hand-pollinated with pollens from TRV2:gPDS-infected pRPS5A:Cas9. Cross B: Flowers of TRV2:gPDS-infected pRPS5A:Cas9 were hand-pollinated with pollens from wild-type *N. attenuata*. Mutation patterns and frequency (%) observed in pooled F_1 seedlings from Cross A (A) and Cross B (B). Groups with mutations are highlighted in gray. The mutation pattern is given followed by the number of its reads in parentheses. Indel frequency (%) was calculated by dividing the number of reads containing indel mutations by the number of total sequencing reads. (C and D) Mutation patterns and frequency of F_1 seedlings from Cross A (C) and Cross B (D). F_1 seedlings with mutations are highlighted in gray.

cells were edited by TRV-delivered gRNA in the pRPS5A:Cas9 plant.

DISCUSSION

Mei et al. (2019) successfully edited the target genes by delivering gRNA with foxtail mosaic virus in SpCas9-expressing maize and *Setaria viridis* under the control of a maize ubiquitin promoter, and *N. benthamiana* expressing SpCas9 with a double 35S promoter. Uranga et al. (2021) also delivered gRNA with potato virus X into *N. benthamiana* expressing SpCas9 under the control of the 35S promoter. However, they failed to obtain gene-edited seeds from virus-infected plants. Recently, Ellison et al. (2020) found that gRNA fused to *FT* sequence dramatically increased the efficacy of germline editing in *N. benthamiana*. Despite our attempts to develop a VIGE system for wild-type species in the genus *Nicotiana*, we failed to obtain gene-edited progenies from 35S:Cas9 plants infected by gRNA fused with *FT* sequence (Supplementary Fig. S4). This result suggests that the *FT* mRNA movement might have occurred only in *N. benthamiana*, not in *N. attenuata*. Another possible explanation is that the level of SpCas9 proteins driven by the 35S promoter was insufficient to induce the mutation in *N. attenuata*, because the 35S promoter activity in germ cells is species-specific. For instance, GUS activity derived by 35S promoter was not detected in *Arabidopsis* pollen (Wilkinson et al., 1997) but was detected in *Nicotiana tabacum* pollen (Patro et al., 2012; Wilkinson et al., 1997; Zheng et al 2007). In cotton, the 35S promoter was not active during the early stage of embryogenesis (Sunilkumar et al., 2002). Here we showed that the promoter for expressing SpCas9 is crucial for heritable genome editing. To develop a VIGE system for a wide range of plants, species- and tissue-specific promoters should be examined carefully.

Previous studies reported the mutation efficacy of VIGE system: 13%-100% seeds from infected wheat (Li et al., 2021), 65%-100% seeds from infected *N. benthamiana* (Ellison et al., 2020), and 4%-9% seeds from infected *Arabidopsis* (Lei et al., 2021). The efficiency of VIGE in *N. attenuata* (2.4%-6.3%) was similar to its efficiency in *Arabidopsis* and much lower than its efficiency in wheat and *N. benthamiana*. In wheat, the editing efficiency in anther cells including pollen and pollen sacs was more than 75%. This result suggests that a virus infects the shoot apical meristem or floral meristem in wheat, which in turn produces mutated stamens and/or carpels. Recently, Wu et al. (2020) reported that WUSCHEL inhibits the spread of cucumber mosaic virus and TRV in the shoot meristematic region of *Arabidopsis*. Therefore, TRV would not express gRNA in meristematic cells of *N. attenuata*. TRV may infect microsporocytes or megasporocytes and mutate a few of them, which explains the mutation efficacy of M_1 seeds (2.4% for TRV2:gPDS, 6.3% for TRV2:gCHAL2, and 4.5% for TRV2:gCHAL3 in pRPS5A:Cas9) that we observed. Virus infection and replication are also host-plant dependent. For instance, *N. benthamiana* has a natural mutation at RNA-dependent RNA polymerase (RdRP), which enhances the susceptibility of plants to virus infection (Yang et al., 2004). That might be the reason for high heritable mutation efficacy in virus-infected *N. benthamiana*. Transient silencing of RdRPs may help to enhance the spreading of gRNA in a plant.

The mutation patterns induced by TRV2:gPDS were exclusively single nucleotide insertions in leaves, petals, anthers, ovaries, and M_1 plants (Figs. 2, 4, and 5). TRV2:gCHAL2 and TRV2:gCHAL3 mainly induced single nucleotide insertions and deletions in infected leaves (Fig. 3). These results suggest that mutation patterns are associated with the context of gRNA or target DNA sequences. We did not detect chimeric mutations in M_1 edited seeds, which suggests that VIGE occurs in the ovaries and pollen and/or the progenitor cells of ovules and anthers in *N. attenuata*. Although both egg and pollen cells were edited in TRV-infected pRPS5A:Cas9 (Figs. 4 and 5), we could not obtain biallelic M_1 seeds from infected plants. A low mutation frequency in each haploid cell might be responsible.

The transformation and regeneration procedure of *N. attenuata* takes more than six months to generate T_0 plants (Kang et al., 2020). In contrast, our TRV-derived VIGE system takes an average of three to four months to obtain monoallelic M_1 seeds without sterilized equipment, culture media, and intensive labor (Fig. 1). Thus, the time and effort required to obtain gene-edited plants are dramatically reduced. However, it is worthwhile mentioning that at least one transgenic plant should be generated using a traditional transformation method when applying this technique to other plants. In this study, we developed a TRV-derived VIGE system for producing heritable mutants in *N. attenuata* expressing SpCas9-driven by the RPS5A promoter. The mutation frequencies of our VIGE system were about 2%-6%, which are similar to the frequency of *Arabidopsis* transformation (1%-5%). Just as the floral dip method enables scientists to explore gene function in *Arabidopsis*, our VIGE system allows us to evaluate the fitness consequences of altering target gene sequences in non-model plant species.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

S.G.K. conceived the research. Y.O. and S.G.K. designed the experiments. Y.O. performed the experiments. Y.O. and S.G.K. wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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