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Journal
The Plant Journal, 122(1)

ISSN
0960-7412

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Publication Date
2025-04-01

DOI
10.1111/tpj.70115

the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2025) **122,** e70115 doi: 10.1111/tpj.70115

TECHNICAL ADVANCE

Heritable virus-induced germline editing in tomato

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Received 15 January 2025; revised 7 February 2025; accepted 10 March 2025.
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SUMMARY

Here, we report the successful implementation of heritable virus-induced genome editing (VIGE) in tomato (*Solanum lycopersicum*). We generated three transgenic tomato lines expressing *Streptococcus pyogenes Cas9* (SpCas9) under the control of Cauliflower mosaic virus 35S (35S), *S. lycopersicum* ribosomal protein S5A (*Sl*RPS5A), or *S. lycopersicum* YAO promoters (*Sl*YAO). These three lines were tested for somatic and heritable editing using the tobacco rattle virus (TRV)-based system carrying guide RNAs (gRNAs) fused with mobile RNA sequences. TRV with gRNA targeted to *Phytoene desaturase* (*SlPDS*) and *Downy mildew resistance 6* (*SlDMR6*) genes fused to mobile RNA sequences showed significant somatic editing efficiency in all three tomato lines expressing SpCas9. However, the progenies from the *Sl*YAO promoter-driven SpCas9 tomato infected with TRV with gRNA targeted to *SlDMR6* fused to the mobile RNA sequence resulted in monoallelic mutations with a frequency of 3%. Optimization of environmental conditions, such as reduced light intensity, significantly increased heritable editing frequencies, from 0% to 86% at the *SlPDS* and from 3% to 100% at the *SlDMR6*, including biallelic mutations. These findings underscore the use of appropriate promoters to express Cas nucleases and optimized environmental conditions to enhance heritable genome editing efficiency in tomato using VIGE. Furthermore, our method enables the generation of mutants without additional tissue culture or transformation once a SpCas9-expressing tomato line is established.

Keywords: heritable plant genome editing, tobacco rattle virus-induced genome editing, tomato, YAO promoter.

INTRODUCTION

Genome editing is promising for agricultural biotechnology, offering precise and targeted modifications that can enhance crop traits, improve disease resistance, and boost yield. Due to its efficiency and versatility, the CRISPR/Cas9 system has emerged as the most widely adopted among the various genome editing tools (Jinek et al., 2012). However, generating gene-edited plants using CRISPR/Cas9 is challenging due to the complexities of delivering gene-editing components into germline cells, the time-consuming process of transformation and regeneration, and the need to optimize tissue culture methods for different genotypes (Altpeter et al., 2016).

To address these challenges, plant viruses, such as the tobacco rattle virus (TRV), have been developed as vectors to deliver guide RNAs (gRNAs) into germline cells of plants expressing *Streptococcus pyogenes Cas9* (SpCas9) that produce gene-edited progenies (Ellison et al., 2020; Lee et al., 2024; Nagalakshmi et al., 2022; Oh et al., 2021; Oh & Kim, 2021; Lei et al., 2021). The TRV system, notable for its wide host range —including tomato (Senthil-Kumar & Mysore, 2014)—has induced heritable mutations in model species Arabidopsis (Liu et al., 2022; Nagalakshmi et al., 2022) and *Nicotiana* (Ellison et al., 2020; Oh & Kim, 2021) expressing SpCas9. In *Nicotiana benthamiana* and Arabidopsis, SpCas9 expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter could yield heritable mutations with a frequency of 65%–100% by delivering gRNA fused with mobile RNA sequences such as mutant Arabidopsis

Flowering Locus T (mFT) or tRNA-like sequences (TLS) using TRV (Ellison et al., 2020; Nagalakshmi et al., 2022). In *Nicotiana attenuata*, the delivery of gRNAs using the TRV system into plants expressing SpCas9 under the control of the *N. attenuata* ribosomal protein S5A (*Na*RPS5A) promoter resulted in a heritable mutation frequency of 2%–6% (Oh & Kim, 2021). Additionally, a cotton leaf crumple virus vector-based delivery of gRNAs fused to Arabidopsis FT into Arabidopsis expressing SpCas9 under the control of the CaMV 35S or YAO promoter induced heritable mutation at 4%–9% (Lei et al., 2021). In tomato, the delivery of gRNA fused with Arabidopsis mFT through TRV into SpCas9 expressed under the CaMV 35S promoter failed to induce heritable editing. However, trimming the apical or axillary meristem of TRV-gRNA-mFT infected plants generated heritable editing (Liu et al., 2024). Another study while our manuscript was under review showed that transgenic tomato expressing SpCas9 under the control of the *Solanum lycopersicum* ubiquitin 10 promoter (*Sl*UBI10) could increase the heritable mutation frequencies when gRNA targeted to *S. lycopersicum Phytoene desaturase* (*SlPDS*) gene fused with the mutant *S. lycopersicum Single Flower Truss* (*Sl*mFT) delivered through TRV (Lee et al., 2024). However, a systematic analysis of the efficiency of TRV-based delivery of gRNAs on inducing heritable mutation in tomato expressing SpCas9 under different promoters has not been explored.

Here, we tested the efficiency of somatic and heritable editing using a TRV-based delivery system in tomato expressing SpCas9 under the control of CaMV 35S, *Sl*RPS5A, and *S. lycopersicum* YAO (*Sl*YAO) promoters. We found that SpCas9 expressed under the control of the *Sl*YAO promoter yields heritable editing. Furthermore, we found that environmental conditions, such as reduced light intensity, significantly improve heritable gene editing in tomatoes.

RESULTS

Previous studies have highlighted the importance of the promoter used to express SpCas9 in plant genome editing (Lee et al., 2024; Oh & Kim, 2021; Tsutsui & Higashiyama, 2017; Yan et al., 2015). To evaluate the efficacy of different promoters in heritable virus-induced editing, we generated transgenic tomato lines with SpCas9 expressed under the control of the CaMV 35S, *Sl*RPS5A, or *S. lycopersicum* YAO (*Sl*YAO) promoters (Figure 1a). To deliver guide RNAs (gRNAs) into these transgenic tomato lines, we employed a TRV-based gRNA expression system. A gRNA specific to the *SlPDS* gene fused with Arabidopsis mFT (Ellison et al., 2020) was engineered into a TRV2 vector (TRV-g*SlPDS*-mFT), which was then introduced together with the TRV1 into the transgenic tomato lines expressing SpCas9 via agroinfiltration (Figure 1b). Following agroinfiltration, plants were monitored for the

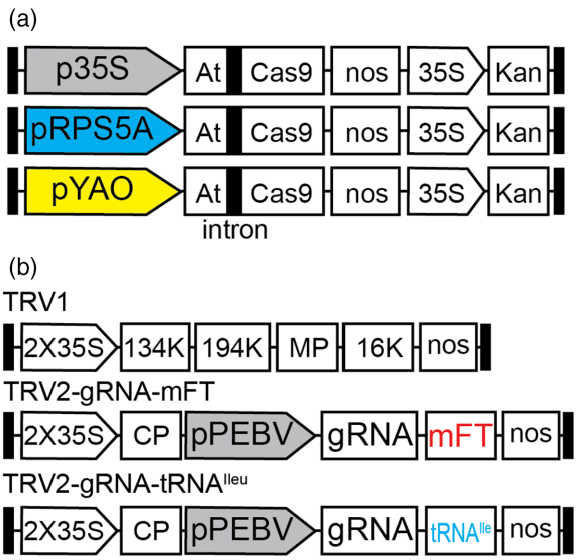

Figure 1. Vectors used for heritable virus-induced genome editing in tomato.
(a) Schematic representation of vectors used to generate SpCas9-expressing tomato. The vectors contain different promoters (p35S, p*Sl*RPS5A, or p*Sl*YAO) driving the expression of the *A. thaliana* codon-optimized SpCas9 with intron, followed by a nopaline synthase (nos) terminator and kanamycin resistance gene as a selection marker (Kan).
(b) Schematic representation of the TRV1 and TRV2 vectors used to deliver guide RNA (gRNA) into the SpCas9-expressing tomato. The constructs include 2X35S promoter, viral components (134 K, 194 K, MP, 16 K, CP), and the subgenomic promoter from pea early browning virus (pPEBV), followed by gRNA fused with mobile mutant Arabidopsis *Flowering T Locus* (mFT) (red) or isoleucine tRNA (blue) sequences.

characteristic photobleaching phenotype associated with *SlPDS* gene disruption. DNA was extracted from the photobleached leaves and subjected to PCR and sequencing of the region flanking the *SlPDS* target site to measure somatic editing frequency. The plants infected with TRV-g*SlPDS*-mFT exhibited high somatic mutation efficiencies in systemic leaves: 46.0 ± 6.0% in p35S::SpCas9, 41.4 ± 4.5% in p*Sl*RPS5A::SpCas9, and 32.3 ± 5.4% in p*Sl*YAO::SpCas9 plants (Figure 2; Figure S1). The editing efficiency was similar among p35S, p*Sl*RPS5A, and p*Sl*YAO transgenic plants, indicating that all three promoters and the TRV system effectively facilitate gene editing in somatic cells.

Notably, we identified a photobleached fruit from one of two TRV-g*SlPDS*-mFT-infected p*Sl*YAO::SpCas9 plants (pYAO-#2), whereas no photobleached fruit was observed in the p35S::SpCas9 and p*Sl*RPS5A::SpCas9 plants under normal growth conditions (Figure 3a, right). The ICE analysis revealed that the editing efficiency at the photobleached pericarp from pYAO-#2 was 67% (Figure 3a, right). To investigate the heritable mutations, we germinated 28 seeds from the photobleached fruit of pYAO-#2, 307 seeds from p35S::SpCas9, and 115 seeds from

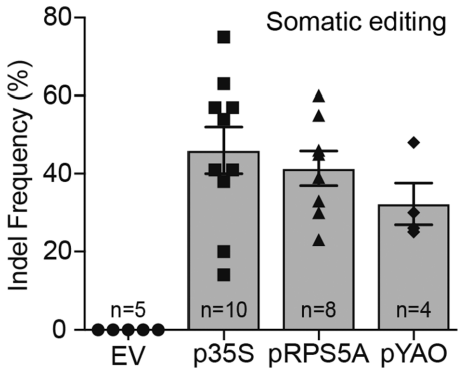

Figure 2. Gene-editing efficiency in the systemic leaf of TRV-g*SlPDS*-mFT-infected p*SlYAO*::SpCas9 tomato.
Indel frequency (%, mean ± SEM) observed in the systemic photobleached leaves of p*SlYAO*::SpCas9 tomato infected with TRV-g*SlPDS*-mFT. Error bars represent the standard error of the mean (SEM) of multiple replicates. Individual data points correspond to measurements taken from different plants, with sample sizes of $n = 10$, $n = 8$, and $n = 4$ for p35S::SpCas9, p*SlRPS5A*::SpCas9, and p*SlYAO*::SpCas9, respectively. No editing was observed in the empty vector (EV) control ($n = 5$).

p*SlRPS5A*::SpCas9 plants. Despite the successful infection and expression of the TRV-g*SlPDS*-mFT, as confirmed by the photobleaching phenotype in the leaves and pericarp, none of the M_1 seedlings harvested from these plants exhibited the photobleaching phenotype. Sequencing results also revealed no indels at the *SlPDS* target site, indicating that heritable editing did not occur (Figure 3c). Although no gene-edited M_1 seedlings were identified in the next generation, the presence of the photobleaching phenotype and mutation in the fruit of the pYAO-#2 plant indicates that, with further optimization, achieving heritable editing in pSlYAO::SpCas9-expressing plants is more likely compared to p35S::SpCas9 and p*SlRPS5A*::SpCas9 plants.

Previous studies emphasized the importance of environmental factors—light intensity, temperature, and humidity—on virus-induced gene silencing and post-transcriptional gene silencing (Bhattarai et al., 2007; Caplan & Dinesh-Kumar, 2006; Nethra et al., 2006; Patil & Fauquet, 2015). Therefore, we assessed the effect of environmental factors on the induction of heritable genome editing in virus-infected plants. To better understand the impact of environmental factors, we measured light intensity (photon flux density, PFD), temperature (°C), and relative humidity (RH, %) at different time points of the day in both normal and shaded areas. The measured PFD ranged from 592 to 1912 μmol m^{-2} s^{-1} (average 1164 ± 311 μmol m^{-2} s^{-1}) in the normal area and from 282 to 1053 μmol m^{-2} s^{-1} (average 626 ± 198 μmol m^{-2} s^{-1}) in the shaded area. Light intensity in the shaded area was reduced by 27–59% compared to the normal environment (Figure S2A). There was no significant difference in temperature between the conditions, except from 6 AM to 12 PM, when the temperature in the shaded area was 1–3°C lower than in the normal environment (Figure S2B). Relative humidity showed no significant difference between the conditions (Figure S2C). To determine whether the light intensity induces heritable genome editing in virus-infected plants, we placed plants infected with TRV-g*SlPDS*-mFT in the shaded area, including five p35S::SpCas9 plants, four pSlRPS5A::SpCas9 plants, and two pSlYAO::SpCas9 plants.

Interestingly, all p*SlYAO*::SpCas9 plants (#1 and #4) grown in the shaded area produced fruits with the *SlPDS*-knockout phenotype, whereas no photobleached fruit was observed in the p35S::SpCas9 and p*SlRPS5A*::SpCas9 plants under the same conditions (Figure 3a). This suggests that reduced light might enhance the overall effectiveness of gene editing or improve virus movement within the reproductive organs in plants expressing SpCas9 under the control of the *SlYAO* promoter (Figure 3a). We then isolated genomic DNA from the photobleached pericarp and performed PCR to determine the mutation frequency. The results showed a high somatic mutation frequency across all photobleached fruits, ranging from 67% to 88% (Figure 3a).

We then investigated the editing frequency in individual M_1 seedlings from the photobleached fruits to examine the heritability of the targeted gene editing in the next generation. Notably, albino M_1 seedlings were observed in seeds harvested from two photobleached fruits from the p*SlYAO*-#4 plant grown in the shaded area, indicating successful heritable genome editing (Figure 3b). Analysis of seven M_1 seedlings from the first fruit revealed four biallelic mutants (57%), two monoallelic mutants (29%), and one wild type, resulting in 86% gene-edited progenies. The second fruit yielded 26 M_1 seedlings, with eight identified as biallelic mutants (31%), ten as monoallelic mutants (38%), and eight as wild type, leading to a total of 69% gene-edited progenies (Figure 3d). We further assessed the heritability of mutations in an M_1 progeny carrying a monoallelic mutation from the first fruit of p*SlYAO*-#4 to the next generation. A total of 110 M_2 seeds from this progeny were germinated, revealing an apparent albino phenotype in the M_2 seedlings, with a ratio of 4.2:1 (89 green to 21 white seedlings) (Figure S3). In p*SlYAO*-#1, two photobleached fruits produced no albino M_1 seedlings despite these fruits also displaying the photobleached phenotype. We analyzed 50 M_1 seedlings from the first fruit of p*SlYAO*-#1 and found one monoallelic mutant (2%), while the remaining were wild type. Examination of 62 M_1 seedlings from the second fruit revealed no gene edits (Figure 3d). The results showed that the gene edits were heritable in the p*SlYAO*::SpCas9 plants, as the progeny exhibited both the photobleaching phenotype and corresponding indels at the *SlPDS* target site (Figure 3d). The frequency of obtaining gene-edited progenies ranged from 2% to 86%

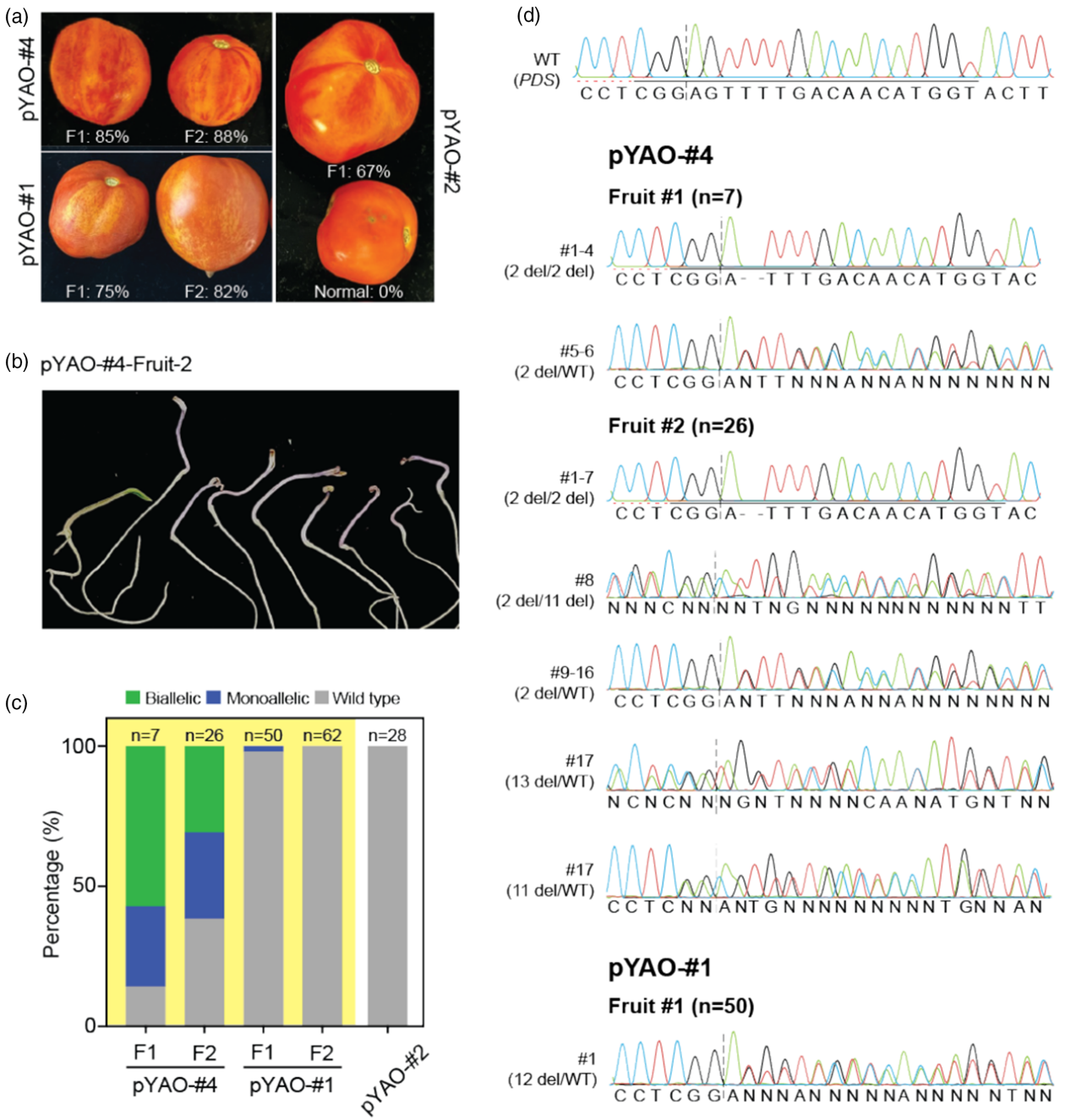

Figure 3. Heritable virus-induced genome editing in p*SlY*AO::SpCas9 tomato infected with TRV-g*SlPDS*-mFT.
(a) Representative image of photobleached fruits from p*SlY*AO::SpCas9 tomato infected with TRV-g*Sl*PDS-mFT. A portion of the pericarp was collected, and the indel frequency (%) was calculated using the Synthego ICE analysis.
(b) Representative image of photobleached M_1 seedlings from the second photobleached fruit harvested from p*SlY*AO-#4.
(c) The percentage of M_1 seedlings with biallelic (green) and monoallelic (blue) mutations and wild-type sequence (gray) is shown. The total number of M_1 seedlings analyzed for each fruit (F) is at the top of each bar. p*SlY*AO-#1 and #4 were grown under shaded conditions (yellow), and p*SlY*AO-#2 was grown under normal conditions.
(d) The sequencing result of PCR products confirmed the mutation patterns observed in the M_1 seedlings with either biallelic or monoallelic mutations. Wild-type sequences of *SlPDS* are shown with spacer sequence (underlined) and PAM (red dash).

across three fruits, showing variability among individual plants. Despite this variation, the consistent production of gene-edited offspring underscores the robustness and effectiveness of the approach.

In *N. benthamiana* and Arabidopsis expressing p35S::SpCas9, delivery of gRNAs fused to the isoleucine tRNA using the TRV system has been shown to enhance heritable editing efficiency significantly (Ellison et al., 2020;

Nagalakshmi et al., 2022). To determine whether adding isoleucine tRNA could augment editing efficiency in tomato, we generated TRV with g*SlPDS* fused to the isoleucine tRNA (TRV-g*SlPDS*-tRNA^{Ileu}) (Figure 1b). We then determined the somatic mutation efficiency of the TRV-g*SlPDS*-tRNA^{Ileu} in systemic leaves, achieving 53%–67% in p35S::SpCas9, 46%–66% in p*Sl*RPS5A::SpCas9, and 51%–80% in p*Sl*YAO::SpCas9 plants (Figure S4). The plants were grown in a growth chamber under optimized conditions (24°C, 300 μmol m^{-2} s^{-1}, 70% humidity), like the shaded area in the greenhouse. Notably, all virus-infected transgenic plants produced several photobleached fruits under these conditions (Figure S5A). High somatic mutation frequencies across the photobleached fruits in the skin and pericarp were observed, with values ranging from 61% to 67% in p35S::SpCas9, 38% to 67% in p*Sl*RPS5A::SpCas9, and 71% to 87% in p*Sl*YAO::SpCas9 fruit skin and 23% to 48% in p35S::SpCas9, 32% to 35% in p*Sl*RPS5A::SpCas9, and 68% to 73% in p*Sl*YAO::SpCas9 fruit pericarp (Figure S5B). Despite producing photobleached fruits, none of the plants yielded gene-edited M_1 seedlings. These results indicate that TRV with gRNA fused to isoleucine tRNA failed to induce heritable editing in tomato under the conditions tested.

To ensure whether the gene-edited M_1 progenies were free of the virus used for editing, RT-PCR was performed using primers specific to the viral RNA genome on the albino M_1 seedlings identified from TRV-g*SlPDS*-mFT-infected-pYAO-#4, which included four seedlings from the first fruit and eight from the second fruit. No viral RNA was detected in these 12 albino M_1 seedlings, confirming that the virus was absent in the next-generation edited plants (Figure S6). These results ensure that the observed gene edits were stably inherited and not the result of residual viral activity, thereby validating the effectiveness and safety of the heritable gene-editing process in producing virus-free edited progeny.

To further confirm the feasibility of the approach, we targeted gRNA specific to the *S. lycopersicum Downy mildew resistance 6* (*SlDMR6*) gene, which is associated with key agronomic traits, including resistance to biotic stress (Thomazella et al., 2021). Given that editing of *SlDMR6* does not induce a visual phenotype, we randomly collected systemic leaves to measure mutation frequency. The results showed a mutation frequency of 66.7 ± 17.8% in p*Sl*YAO::SpCas9 plants (Figure 4a). A total of six p*Sl*YAO::SpCas9 plants were included in the study: three plants (#1–3) were grown in the normal area and three (#4–6) in the shaded area. To assess the heritable transmission, we pooled seeds from over ten fruits harvested from virus-infected p*Sl*YAO::SpCas9 plants to ensure a sufficient sample size. We first examined 82, 123, and 36 M_1 seedlings collected from p*Sl*YAO-#1, #2, and #3 plants, respectively, all grown under normal conditions. In the p*Sl*YAO-#3 plant, we found one monoallelic mutant among 36 M_1 seedlings (3%) (Figure S7). However, no gene-edited M_1 seedlings were found in p*Sl*YAO-#1 and #2, consistent with previous observations that high light intensity may inhibit heritable genome editing. To determine whether shaded conditions could improve the efficiency of heritable genome editing, we next evaluated plants grown in the shaded areas. We randomly selected four fruits from p*Sl*YAO-#4, five from p*Sl*YAO-#5, and six from p*Sl*YAO-#6. Interestingly, high somatic mutation frequencies across the fruits were observed, with values ranging from 0% to 24% in p*Sl*YAO-#4, 0–99% in p*Sl*YAO-#6, and 0–95% in p*Sl*YAO-#6 pericarp (Figure S8). We then germinated M_1 seeds harvested from the five fruits with mutations and six without detectable mutations in their pericarp (Figure 4b). Among 13 M_1 seedlings from the first fruit (F1) of p*Sl*YAO-#4, four had biallelic mutations (31%), seven displayed monoallelic mutations (54%), and two were identified as wild type, resulting in 85% gene-edited progenies. Interestingly, all 56 M_1 seedlings from the four fruits with high mutation frequencies in their pericarp (92–99%; Figure S8) were biallelic mutants (100%). These results indicate that heritable transmission of gene edits was successfully achieved (Figure 4). In contrast, we did not obtain any gene-edited seedlings from six fruits harvested from p*Sl*YAO-#4 and #5, which lacked detectable mutations in their pericarp (Figure 4b). These results suggest that the mutations in the pericarp may act as an indicator of potential heritable mutations in virus-infected p*Sl*YAO::SpCas9 plants. Moreover, shaded growth conditions enhance gene editing efficiency and the likelihood of heritable mutations. This evidence supports further studies into how light affects VIGE, aiming to refine environmental conditions to maximize efficient, heritable genome editing in crops.

We also determined the somatic mutation efficiency of the TRV-g*SlDMR6*-mFT in systemic leaves, achieving 50.3 ± 11.3% in p35S::SpCas9 and 74.5 ± 11.9% in p*Sl*RPS5A::SpCas9 plants (Figure S9A). These plants were grown in the greenhouse under either normal or shaded conditions. Notably, two virus-infected plants produced several fruits with mutations under shaded areas (Figure S9B). High somatic mutation frequencies across the fruits in the pericarp were observed, with values ranging from 0 to 97% in p35S::SpCas9 and 0–34% in p*Sl*RPS5A::SpCas9 (Figure S9B). Despite producing fruits with somatic mutations, none of these plants yielded gene-edited M_1 seedlings (Figure S9C). These results are consistent with our observations that the *Sl*YAO promoter is critical in driving SpCas9 expression to enable heritable gene editing in tomato.

DISCUSSION

Our findings indicate that under both normal and shaded conditions tested, p*Sl*YAO::SpCas9 plants infected with TRV

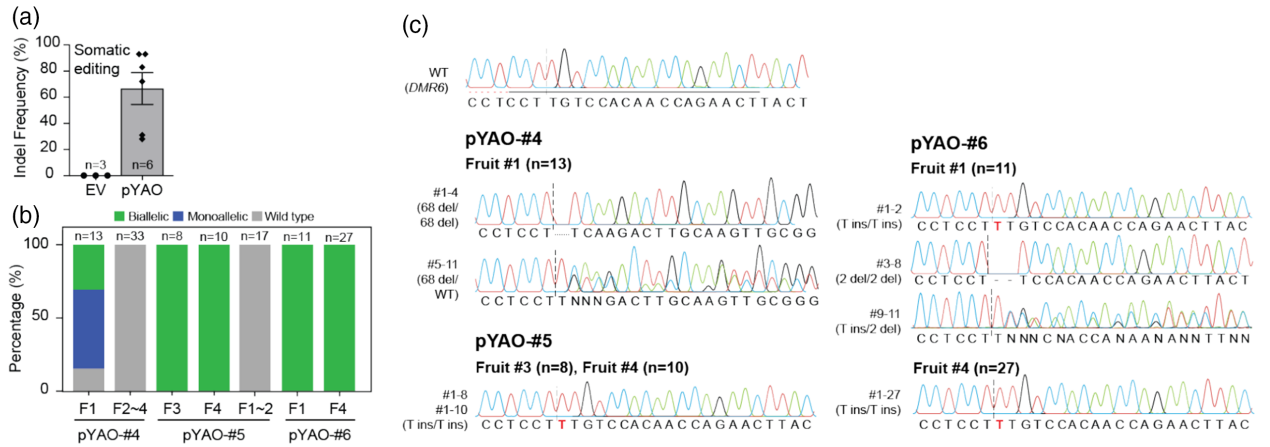

Figure 4. Heritable virus-induced genome editing in p*SlY*AO::SpCas9 tomato infected with TRV-g*SlDMR6*-mFT.
(a) Indel frequency (%, mean ± SEM) observed in the systemic leaves of p*SlY*AO tomato infected with TRV-g*SlDMR6*-mFT. Error bars represent the standard error of the mean (SEM) of multiple replicates. Individual data points correspond to measurements taken from p*SlY*AO::SpCas9 plants, with sample sizes $n = 6$. No editing was observed in the EV control ($n = 3$).
(b) The percentage of M_1 seedlings with biallelic (green), monoallelic (blue), and wild-type (gray) mutations is shown. The total number of M_1 seedlings analyzed for each fruit (F) is at the top of each bar.
(c) The sequencing result of PCR products confirmed the mutation patterns observed in the M_1 seedlings with either biallelic or monoallelic mutations. WT sequences of *SlDMR6* are shown with the spacer sequence (underlined) and PAM (red dash).

produce gene-edited progeny from fruits with somatic edits (Figures 3 and 4), whereas p35S::SpCas9 and pRPS5A::SpCas9 plants did not (Figure S9). This indicates that, among the promoters tested, only p*SlY*AO-driven SpCas9 enables heritable mutations in tomato, likely due to the strong expression of YAO in actively dividing cells and tissues such as the shoot apical meristem, embryo sac, embryo, endosperm, and pollen (Yan et al., 2015). This tissue specificity is crucial because effective heritable gene editing requires functional SpCas9 expression in germline cells.

We found that p*SlY*AO::SpCas9 plants grown under lower light intensity (282–1053 μmol m^{-2} s^{-1}) exhibited significantly higher heritable editing efficiency, with editing rates ranging from 69% to 100% (Figures 3 and 4). In contrast, p*SlY*AO::SpCas9 plants exposed to normal light intensity (592–1912 μmol m^{-2} s^{-1}) showed reduced heritable editing efficiency (0%–3%) (Figure S9). These results suggest that high light intensity might inhibit the movement of the virus into germline cells. Therefore, a lower light intensity of 282–1053 μmol m^{-2} s^{-1} is optimal for effective TRV-mediated heritable gene editing under our experimental conditions. This indicates that light management is crucial for achieving reliable heritable edits. In greenhouse conditions, using shade cloths or light filters may help reduce light exposure to the ideal range, preventing excessive exposure (>1000 μmol m^{-2} s^{-1}), which could impair viral editing efficiency and heritability. However, under lower light intensity conditions, we were unable to obtain M_1 progeny when p*SlY*AO::SpCas9 plants were infected with TRV-g*SlPDS*-tRNA^{Ileu} (Figure S5) in a controlled growth chamber (300 μmol m^{-2} s^{-1}). This lack of germline editing may indicate that, under the tested conditions, isoleucine tRNA may not deliver sufficient gRNA to germline cells in tomato, limiting its effectiveness for heritable gene editing.

Not all fruits from infected-p*SlY*AO::SpCas9 plants produced gene-edited M_1 seedlings, which may stem from two possibilities. First, SpCas9 activity may be temperature-sensitive, potentially decreasing at lower temperatures (LeBlanc et al., 2018). Using temperature-tolerant Cas12a (Schindele et al., 2023; Schindele & Puchta, 2020) driven by the *SlY*AO promoter may increase the overall efficiency of heritable mutations. Second, *meristem exclusion*, where viral movement is restricted in meristematic regions (Wu et al., 2020), may limit virus access to reproductive tissues. This restriction could reduce the infection rates; however, once reproductive tissues are infected, the chances of obtaining gene-edited progeny are high, ranging from 61% to 100% (Figures 3 and 4).

Besides our findings described here, only in one instance has virus-mediated heritable editing using tomato lines expressing SpCas9 under different promoters been reported (Kang et al., 2025; Lee et al., 2024; Lee et al., 2024a; Liu et al., 2024; Uranga et al., 2024; Wang et al., 2024). Liu et al. (2024) reported that the tomato line expressing SpCas9 under the CaMV 35S promoter failed to yield heritable mutation when gRNA fused to Aradidopsis mFT was delivered using TRV. Instead, they achieved heritable editing by generating *de novo* shoots from edited somatic cells by trimming TRV-infected plants or delivering gRNA through TRV to axillary meristems (Liu et al., 2024). Similarly, gRNA delivered using Potato virus X (PVX) did not yield heritable mutations in SpCas9-expressing tomato under the control of the CaMV 35S promoter (Kang

et al., 2025; Uranga et al., 2024). A gRNA delivered using TRV was also ineffective in tomato lines expressing SpCas9 under the maize or Arabidopsis ubiquitin 10 (UBI10) promoter and the CaMV 35S promoter (Kang et al., 2025; Lee et al., 2024a; Wang et al., 2024). Interestingly, while our manuscript was under review, Lee et al. (2024) reported that the delivery of gRNA targeted to *SlPDS* fused to mutant *S. lycopersicum Single Flower Truss* (*Sl*mFT) into a transgenic Saladette tomato line expressing SpCas9 under the control of *S. lycopersicum UBI10* (SlUBI10) promoter showed a photobleaching phenotype and induced 15%–100% heritable mutation frequencies. However, they failed to observe a photobleaching phenotype when gRNA targeted to *SlPDS* fused to Arabidopsis mFT was delivered using TRV (Lee et al., 2024). In contrast, our findings show that the delivery of gRNA fused to Arabidopsis mFT using TRV into the M82 tomato cultivar expressing SpCas9 under the *Sl*YAO promoter induces a photobleaching phenotype in leaves and fruits. Furthermore, we observed heritable mutations with efficiencies ranging from 2% to 100% when infected with TRV carrying gRNA fused to Arabidopsis mFT (Figures 3 and 4). These findings suggest that the *Sl*YAO promoter might be more effective than the *Sl*Ubi10 promoter in driving SpCas9 expression. Therefore, the promoter used for expressing SpCas9 appears crucial in achieving heritable editing using viral vector-based gene editing in tomato.

CONCLUSION

In conclusion, our study demonstrates the successful heritable virus-induced genome editing in tomato, with the *Sl*YAO promoter playing a critical role in driving SpCas9 expression. The findings also emphasize the importance of environmental factors, particularly light intensity, in optimizing heritable mutations. The findings described here provide a foundation for future studies to refine heritable virus-induced genome editing techniques in other crops. These techniques have the potential to significantly impact agricultural biotechnology by enabling the development of improved crop varieties through precise and heritable genetic modifications.

MATERIALS AND METHODS

Plant materials

Tomato seeds (cultivar M82) were germinated on moistened filter paper in petri dishes and planted in the soil. The plants were grown in a Conviron growth chamber under a 14/10 h light/dark photoperiod at 23 ± 2°C. To generate transgenic SpCas9-expressing tomato lines, the pCGS710, p*Sl*RPS5A, and p*Sl*YAO binary vectors were transformed into the *Agrobacterium tumefaciens* strain AGL1 by the thaw–freeze method. *A. tumefaciens* carrying the corresponding binary vectors was transformed into the M82 cultivar as previously described (Gupta & Van Eck, 2016) with minor modifications at the Plant Genomics and Transformation Facility at Innovative Genomics Institute (IGI Berkeley). Briefly, cotyledon explants were placed in the *Agrobacterium* (AGL1) suspension (OD600 of 0.4) on an incubating shaker at room temperature for 2 h. The tissues were then transferred to co-cultivation medium and maintained at 21°C for 4 days in the dark. The tissues were transferred to the first round of selection medium containing kanamycin. The tissues were maintained at 26°C under a 16 h light/8 h dark cycle for 2 weeks before transferring them onto the second round of selection medium containing kanamycin. When shoots were approximately 3 cm tall, they were excised from the cotyledon explants and transferred to selective rooting medium containing kanamycin. Plantlets were then transferred to soil once roots were established.

Vector construction and guide RNA design

The *S. lycopersicum* ribosomal protein S5 A (*Sl*RPS5A) and *S. lycopersicum* YAO promoter were amplified from the genomic DNA of *S. lycopersicum* by PCR using Phusion High-Fidelity DNA polymerase (NEB) with the primers reported in Table S1. The coding sequence of the intronized SpCas9 was amplified from the pCGS710. These amplified PCR products were then recombined into pCGS710 to generate p*Sl*RPS5A and p*Sl*YAO binary vectors using Gibson Assembly Master Mix (NEB), following the manufacturer's instructions.

All viral vectors used in this study are listed in Table S2. They were constructed using the Golden Gate Assembly cloning protocol, as described previously (Ellison et al., 2020). The target sequences of gRNAs were designed using the CRISPR-P 2.0 web tool (Liu et al., 2017). Primers used in this study were purchased from Integrated DNA Technologies.

Agroinfiltration

Tomato infiltration was performed as previously described with minor modifications (Liu et al., 2002; Tai et al., 1999). Briefly, the TRV1 and TRV2 derivatives were introduced into *A. tumefaciens* GV3101 by the thaw–freeze method. The GV3101 carrying TRV2 derivatives was mixed with GV3101 containing TRV1 in a 1:1 ratio in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 150 μM acetosyringone) to a final OD_{600} of 0.5. After induction in acetosyringone for 3 hours, the mixed solution was co-infiltrated into the cotyledons of 7- to 10-day-old SpCas9-expressing tomato plants. After agroinfiltration, the plants were grown in the growth chamber (14/10 h light/dark photoperiod at 23 ± 2°C) for 6–8 weeks. They were then transplanted into larger pots and moved to the greenhouse. A 40% white shade cloth (Greenhouse megastore) was installed to cover shaded areas in the greenhouse to reduce the light intensity.

Genotyping

Genomic DNA was extracted from leaves and pericarp using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) or the Chelex-100 method (Bio-Rad). The target sites were amplified by PCR using Phusion High-Fidelity DNA polymerase (NEB) and gene-specific primers (Table S1). PCR products were purified using the DNA Clean and Concentrator-5 kit (Zymo Research) for sanger sequencing at the UC Berkeley DNA sequencing facility. The ICE online tool (Synthego) was used to analyze the editing efficiency.

AUTHOR CONTRIBUTIONS

BJS, YO, UN, and SPD-K conceived and designed the experiments. YO, UN, DD, NK, and MJC performed

the experiments. BJS and YO wrote the manuscript. UN and SPD-K edited the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Drs. Daniel Voytas and Colby Starker for kindly providing the pCGS710 vector.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available within the article and its supplementary materials. Raw data are available from the corresponding author on request.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figures S1. Somatic editing of *SlPDS* in M82 tomato plants expressing SpCas9 driven by different promoters using TRV with mutant *Arabidopsis Flowering Locus T*.

Figure S2. Environmental conditions for heritable genome editing in tomato.

Figure S3. Representative image of green and albino phenotypes of M_2 seedlings.

Figure S4. Somatic editing of *SlPDS* in M82 tomato plants expressing SpCas9 driven by different promoters using TRV with $tRNA^{Ileu}$.

Figure S5. Somatic editing of *SlPDS* in fruits from M82 tomato plants expressing SpCas9 driven by different promoters.

Figure S6. No detectable TRV in the photobleached M_1 seedlings.

Figure S7. Heritable virus-induced genome editing in p*Sl*YAO::SpCas9 tomato infected with TRV-g*SlDMR6*-mFT under normal light condition.

Figure S8. Indel frequencies in *SlDMR6* in pericarp in different fruits.

Figure S9. Somatic editing in p35S::SpCas9 and p*Sl*RPS5A::SpCas9 tomato infected with TRV-g*SlDMR6*-mFT under normal and lower light condition.

Table S1. A list of primer sequences used in this study.

Table S2. A list of vector sequences used in this study.

REFERENCES

Altpeter, F., **Springer, N.M.**, **Bartley, L.E.**, **Blechl, A.E.**, **Brutnell, T.P.**, **Citovsky, V.** ***et al.*** (2016) Advancing crop transformation in the era of genome editing. *Plant Cell*, **28**, 1510–1520.

Bhattarai, K.K., **Li, Q.**, **Liu, Y.**, **Dinesh-Kumar, S.P.** & **Kaloshian, I.** (2007) The Mi-1-mediated pest resistance requires Hsp90 and Sgt1. *Plant Physiology*, **144**, 312–323.

Caplan, J. & **Dinesh-Kumar, S.P.** (2006) Using viral vectors to silence endogenous genes. *Current Protocols in Microbiology*, **16**, 16I.6.1–16I.6.13.

Doyle, J.J. & **Doyle, J.L.** (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

Ellison, E.E., **Nagalakshmi, U.**, **Gamo, M.E.**, **Huang, P.**, **Dinesh-Kumar, S.** & **Voytas, D.F.** (2020) Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature Plants*, **6**, 620–624.

Gupta, S. & **Van Eck, J.** (2016) Modification of plant regeneration medium decreases the time for recovery of *Solanum lycopersicum* cultivar M82 stable transgenic lines. *Plant Cell, Tissue and Organ Culture*, **127**, 417–423.

Jinek, M., **Chylinski, K.**, **Fonfara, I.**, **Hauer, M.**, **Doudna, J.A.** & **Charpentier, E.** (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.

Kang, G.H., **Ko, Y.** & **Lee, J.M.** (2025) Enhancing virus-mediated genome editing for cultivated tomato through low temperature. *Plant Cell Reports*, **44**, 22.

LeBlanc, C., **Zhang, F.**, **Mendez, J.**, **Lozano, Y.**, **Chatpar, K.**, **Irish, V.F.** ***et al.*** (2018) Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *The Plant Journal*, **93**, 377–386.

Lee, H., **Baik, J.E.** & **Kim, K.-N.** (2024) Development of an efficient and heritable virus-induced genome editing system in *Solanum lycopersicum*. *Horticulture Research*, **uhae364**. Available from: https://doi.org/10.1093/hr/uhae364

Lee, S.Y., **Kang, B.**, **Venkatesh, J.**, **Lee, J.H.**, **Lee, S.**, **Kim, J.M.** ***et al.*** (2024a) Development of virus-induced genome editing methods in Solanaceous crops. *Horticulture Research*, **11**, uhad233.

Lei, J., **Dai, P.**, **Li, Y.**, **Zhang, W.**, **Zhou, G.**, **Liu, C.** ***et al.*** (2021) Heritable gene editing using FT mobile guide RNAs and DNA viruses. *Plant Methods*, **17**, 20.

Liu, D., **Ellison, E.E.**, **Myers, E.A.**, **Donahue, L.I.**, **Xuan, S.**, **Swanson, R.** ***et al.*** (2024) Heritable gene editing in tomato through viral delivery of isopentenyl transferase and single-guide RNAs to latent axillary meristematic cells. *Proceedings of the National Academy of Sciences of the United States of America*, **121**, e2406486121.

Liu, D., **Xuan, S.**, **Prichard, L.E.**, **Donahue, L.I.**, **Pan, C.**, **Nagalakshmi, U.** ***et al.*** (2022) Heritable base-editing in Arabidopsis using RNA viral vectors. *Plant Physiology*, **189**, 1920–1924.

Liu, H., **Ding, Y.**, **Zhou, Y.**, **Jin, W.**, **Xie, K.** & **Chen, L.L.** (2017) CRISPR-P 2.0: an improved CRISPR-Cas9 tool for genome editing in plants. *Molecular Plant*, **10**, 530–532.

Liu, Y., **Schiff, M.** & **Dinesh-Kumar, S.P.** (2002) Virus-induced gene silencing in tomato. *The Plant Journal*, **31**, 777–786.

Nagalakshmi, U., **Meier, N.**, **Liu, J.Y.**, **Voytas, D.F.** & **Dinesh-Kumar, S.P.** (2022) High-efficiency multiplex biallelic heritable editing in Arabidopsis using an RNA virus. *Plant Physiology*, **189**, 1241–1245.

Nethra, P., **Nataraja, K.N.**, **Rama, N.** & **Udayakumar, M.** (2006) Standardization of environmental conditions for induction and retention of post-transcriptional gene silencing using tobacco rattle virus vector. *Current Science*, **90**, 431–435.

Oh, Y., **Kim, H.** & **Kim, S.G.** (2021) Virus-induced plant genome editing. *Current Opinion in Plant Biology*, **60**, 101992.

Oh, Y. & **Kim, S.-G.** (2021) RPS5A promoter-driven Cas9 produces heritable virus-induced genome editing in *Nicotiana attenuata*. *Molecules and Cells*, **44**, 911–919.

Patil, B.L. & **Fauquet, C.M.** (2015) Light intensity and temperature affect systemic spread of silencing signal in transient agroinfiltration studies. *Molecular Plant Pathology*, **16**, 484–494.

Schindele, P., **Merker, L.**, **Schreiber, T.**, **Prange, A.**, **Tissier, A.** & **Puchta, H.** (2023) Enhancing gene editing and gene targeting efficiencies in Arabidopsis thaliana by using an intron-containing version of ttLbCas12a. *Plant Biotechnology Journal*, **21**, 457–459.

Schindele, P. & **Puchta, H.** (2020) Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing. *Plant Biotechnology Journal*, **18**, 1118–1120.

Senthil-Kumar, M. & **Mysore, K.S.** (2014) Tobacco rattle virus-based virus-induced gene silencing in Nicotiana benthamiana. *Nature Protocols*, **9**, 1549–1562.

Tai, T.H., **Dahlbeck, D.**, **Clark, E.T.**, **Gajiwala, P.**, **Pasion, R.**, **Whalen, M.C.** ***et al.*** (1999) Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. *Plant Biology*, **96**, 14153–14158.

Thomazella, D.P.D.T., **Seong, K.**, **Mackelprang, R.**, **Dahlbeck, D.**, **Geng, Y.**, **Gill, U.S.** ***et al.*** (2021) Loss of function of a DMR6 ortholog in tomato confers broad-spectrum disease resistance. *Proceedings of the National Academy of Sciences*, **118**, e2026152118.

Tsutsui, H. & **Higashiyama, T.** (2017) PKAMA-ITACHI vectors for highly efficient CRISPR/Cas9-mediated gene knockout in *Arabidopsis thaliana*. *Plant & Cell Physiology*, **58**, 46–56.

Uranga, M., **Aragonés, V.**, **García, A.**, **Mirabel, S.**, **Gianoglio, S.**, **Presa, S.** ***et al.*** (2024) RNA virus-mediated gene editing for tomato trait breeding. *Horticulture Research*, **11**, uhad279.

Wang, Q., **Zhang, D.**, **Dai, Y.R.** & **Liu, C.C.** (2024) Efficient tobacco rattle virus-induced gene editing in tomato mediated by the CRISPR/Cas9 system. *Biotechnology Journal*, **19**, 2400204.

Wu, H., **Qu, X.**, **Dong, Z.**, **Luo, L.**, **Shao, C.**, **Forner, J.** ***et al.*** (2020) WUSCHEL triggers innate antiviral immunity in plant stem cells. *Science*, **370**, 227–231.

Yan, L., **Wei, S.**, **Wu, Y.**, **Hu, R.**, **Li, H.**, **Yang, W.** ***et al.*** (2015) High-efficiency genome editing in Arabidopsis using YAO promoter-driven CRISPR/Cas9 system. *Molecular Plant*, **8**, 1820–1823.