Saudi Journal of Biological Sciences 29 (2022) 103420

HOSTED BY

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

# A sequential transformation method for validating soybean genome editing by CRISPR/Cas9 system

Duy Dinh Trinh [a,1], Ngoc Thu Le [a], Thao Phuong Bui [a,d], Thao Nhu Thi Le [a], Cuong Xuan Nguyen [b], Ha Hoang Chu [a,c], Phat Tien Do [a,c,*]

[a] Institute of Biotechnology, Vietnam Academy of Science and Technology, Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam
[b] Division of Plant Sciences, University of Missouri, Columbia, MO, United States
[c] Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam
[d] University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam

ARTICLE INFO

*Article history:*
Received 3 May 2022
Revised 13 July 2022
Accepted 12 August 2022
Available online 17 August 2022

*Keywords:*
*Agrobacterium rhizogenes*
CRISPR/Cas9
Guide RNA
Hairy roots
Sequential transformation
Soybean

ABSTRACT

This study was performed to evaluate the sequential transformation for soybean genome editing using the CRISPR/Cas9 system as well as to show a strategy for examining the activity of CRISPR/Cas9 constructs, especially the designed guide RNAs (gRNAs). The gRNAs for targeted mutations of an exogenous gene and multiple endogenous genes were constructed and transferred into a stably-overexpressed-Cas9 soybean line using *Agrobacterium rhizogenes*-mediated hairy root induction system. The targeted mutations were identified and characterized by the poly-acrylamide gel electrophoresis (PAGE) heteroduplex method and by sequencing. Induced mutations of the exogenous gene (*gus*) were observed in 57% of tested transgenic hairy roots, while 100% of the transgenic root lines showed targeted mutations of the endogenous (*SACPD-C*) gene. Multiple gRNAs targeting two endogenous genes (*SACPD-C* and *SMT*) induced mutation rates of 75% and 67%, respectively. Various indels including small and large deletions as well as insertions were found in target sites of the tested genes. This sequential transformation method could present the targeting efficacy of different gRNAs of each tested gene. Additionally, in this study differences in gRNA ratings were found between bioinformatics predictions and actual experimental results. This is the first successful application of the sequential transformation method for genome editing in soybean using the hairy root system. This method could be potentially useful for validating CRISPR/Cas9 constructs, evaluating gRNA targeting efficiencies, and could be applied for other research directions.


## 1. Introduction

The CRISPR/Cas9 system has become an important tool for gene function studies and plant genetic improvement. A critical component of the system is the guide RNA (gRNA), which recognizes the targeted sequences in the genome and directs the Cas9 endonuclease for editing (Doudna and Charpentier, 2014). Different gRNAs of a single gene can have different editing efficiencies (Naim et al., 2020). Performing stable plant transformation is usually associated with a substantial investment of both time and resources. Therefore, selecting the most efficient gRNA target sequences is a critical step in genome editing by CRISPR/Cas systems.

Predicting the effectiveness of gRNAs has usually been carried out through computational tools. However, non-identical outcomes have been reported (Haeussler et al., 2016; Uniyal et al., 2019). These *in silico* predictions have also differed from experimental results in plant genome editing (Naim et al., 2020). As a result, the editing efficiency of CRISPR/Cas9 constructs with the selected gRNAs still needs to be tested in empirical systems such as protoplasts (Brandt et al., 2020; Shan et al., 2014; Wang et al., 2016) or the *Agrobacterium rhizogenes*-mediated hairy root system (Do et al., 2019; Zheng et al., 2020). Normally, the whole CRISPR

* Corresponding author at: Institute of Biotechnology, Vietnam Academy of Science and Technology, Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam.
*E-mail addresses:* trinhdinhduy@ibt.ac.vn (D.D. Trinh), lethungoc@ibt.ac.vn (N.T. Le), buiphuongthao@ibt.ac.vn (T.P. Bui), lethinhuthao@ibt.ac.vn (T.N.T. Le), xxnwxc@umsystem.edu (C.X. Nguyen), chuhoangha@ibt.ac.vn (H.H. Chu), dtphat@ibt.ac.vn (P.T. Do).
[1] Current address: Division of Plant Sciences, University of Missouri, Columbia, MO, United States.

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2022.103420
1319-562X/

construct – including the Cas9 sequences, the gRNAs, and other components – would be delivered at once into plant cells to induce targeted mutations. This requires large transgenic vectors and limits both the capacity of testing systems as well as the transformation efficiency. In addition, the insertion sites and expression levels of both Cas9 and gRNA may differ across transgenic events derived from different constructs, leading to different editing outcomes. Cas9 expression level has been shown to correlate with editing efficiency (Mikami et al., 2015b). In some cases, targeted mutations were observed only in the subsequent transgenic generations, when the activity of Cas9 protein had become stable (Kanazashi et al., 2018; Le et al., 2020). This makes the comparison and selection of the gRNAs with the most potential for the stable transformation challenging. Recently, sequential transformation method for genome editing by CRISPR/Cas9 system has been successfully utilized in several plant species to study the influence of individual components on overall activity (Mikami et al., 2015b; 2015a), as well as achieve higher editing efficiency (Ellison et al., 2020; Miki et al., 2018; Zhang et al., 2016). However, there has been no attempt to apply this approach in soybean, one of the most recalcitrant plants for transformation.

In this study, the sequential transformation method for genome editing by CRISPR/Cas9 was performed in soybean and validated utilizing hairy root induction. The feasibility of the sequential transformation method was confirmed by induced mutations of both exogenous and endogenous genes. This system was then successfully utilized to compare the editing potential of different gRNAs of single and multiple genes using a single transgenic vector. This result indicates a good utility of the sequential transformation system for frequent and rapid screening of gRNAs for subsequent stable soybean genome editing as well as further analysis of CRISPR/Cas9 activities and other plant biology studies.

## 2. Materials and methods

### 2.1. Target selection and vector construction

The β-glucuronidase reporter gene (*gus*) on the binary vector pCAMBIA1301 (CAMBIA Company, Canberra, Australia), the stearoyl-ACP desaturase (*SACPD-C*) gene (NCBI Gene ID: 100037478) and the gene coding for selenocysteine methyltransferase (*SMT*) (NCBI Gene ID: 100790567) were selected to test CRISPR/Cas9 editing by sequential transformation in soybean hairy root system (Fig. 1A). The gRNA target sequences of the three genes (Supplemental Table 1) were identified and selected via the online program CCTop (Labuhn et al., 2018; Stemmer et al., 2015).

The gRNA cassettes were constructed by overlap extension PCR as previously described (Li et al., 2020), using the listed primers (Supplementary Table 2) and the pBlu/gRNA vector, a gift from the Robert Stupar's laboratory (RRID: Addgene_59188) (Michno et al., 2015) as a template. The gRNA cassettes for exogenous, endogenous and multiple genes were inserted to respective transformation vectors – pCAMBIA1301 and pFGC5941 (Kerschen et al., 2004) using restriction enzymes *Sac*I, *Eco*RI, *Bam*HI, and *Hind*III and T4 ligation. The green fluorescence protein (GFP) cassette from pK7WG2D (Karimi et al. 2002) was inserted into transformation vectors harboring gRNAs targeting *gus* and *SACPD-C* in order to identify transformed hairy roots (Supplementary Fig. 1). All constructs (Fig. 1B) were multiplied and selected via *E. coli* and confirmed by Sanger sequencing.

### 2.2. Hairy root transformation and reporter gene assays

*In vitro* hairy root transformation was performed using mature seeds at T4 generation of a stable transgenic Cas9 soybean line generated from the previous study (Le et al., 2020). The *Cas9* gene was under the control of the 35SPPDK promoter (the maize C4PPDK basal promoter and the cauliflower mosaic virus 35S enhancer) (Yoo et al., 2007) (RRID: Addgene_52254). The presence of *Cas9* gene was confirmed by PCR before use (data not shown).

*Agrobacterium rhizogenes*-mediated hairy root transformation was conducted using K599 strain according to the previous report (Chen et al., 2018). Induced hairy roots were subsequently collected for DNA extraction and detection of reporter genes. GFP expression was observed under reflected light with Axio Vert.A1 (FL-LED Stand) inverted light microscope and captured via Axiocam 105 colors (Carl Zeiss MicroImaging GmbH) and GUS histochemical assay was performed using X-Gluc solution as described by Jefferson et al. (1987).

### 2.3. Mutant identification and characterization

Genomic DNA was extracted from *in vitro* soybean hairy root using the modified CTAB method (Dellaporta et al. 1983) and was subjected to PCR amplification of expanded target regions with site-specific primers (Supplementary Table 3). Cas9-induced mutations were screened by heteroduplex analysis on native poly-acrylamide gel electrophoresis (PAGE) (Zhu et al., 2014). Briefly, tested DNA was mixed with homologous wild-type DNA to undergo denaturation at high temperature and then random annealing at room temperature. Then, the product was separated on 15% poly-acrylamide gel at 70 V for 16 h, or 160 V for 5 h. The formation of heteroduplexes between mutant and wild-type DNA strands should result in differentiated migration through the gel. The induced mutations were further confirmed and characterized by Sanger sequencing, in which PCR products were inserted into pBT vector as described previously (Phan et al., 2005) and sequenced with recommended primers. Sample sequences were aligned to wild-type sequences via the tool MUSCLE (Madeira et al., 2019) for mutation analysis.

## 3. Results

### 3.1. Editing of an exogenous gene by sequential transformation

To investigate genome editing by CRISPR/Cas9 in soybean via sequential transformation, we first tested the system with a reporter gene (*gus*). The transgenic vector carrying expression cassettes of GFP, GUS and gRNAs targeting *gus* gene was transferred into hairy roots (Fig. 2A, B). Transgenic hairy roots were identified via the expression of GFP (Fig. 2C). Under a fluorescence microscope, 31% of tested roots (20/65) showed GFP expression (Supplementary Table 4). Then, each GFP positive root was grown on culture medium as individual lines for PCR analysis using specific primers (Supplementary Fig. 2). For further analysis, seven GFP-expressing hairy roots (gus-1 to gus-7) were randomly selected for GUS detection. Three transgenic lines showed no GUS expression, the others displayed GUS activity as indicated by the blue in the entire root, most pronounced at the root tip (Fig. 3A).

Fig. 3B shows induced mutations in the *gus* gene after targeted regions were amplified and analyzed on poly-acrylamide gel electrophoresis (PAGE). The extra DNA bands compared to WT on the PAGE gel indicated the indels of the *gus* gene in three root lines without GUS expression (gus-1, gus-5, gus-6). Although gus-7 root line was GUS-positive, shifted DNA bands were still observed on the gel, suggesting the incomplete loss of function of *gus* gene in this line. The heteroduplex analysis result was also consistent with sequencing results of four roots lines (gus-1, gus-2, gus-6 and gus-7) (Fig. 3C). Specifically, no indel was found in the target sequence of the gus-2 line, while large deletions (-75 bp) were observed in two lines (gus-1 and gus-6). Other indels were also observed on

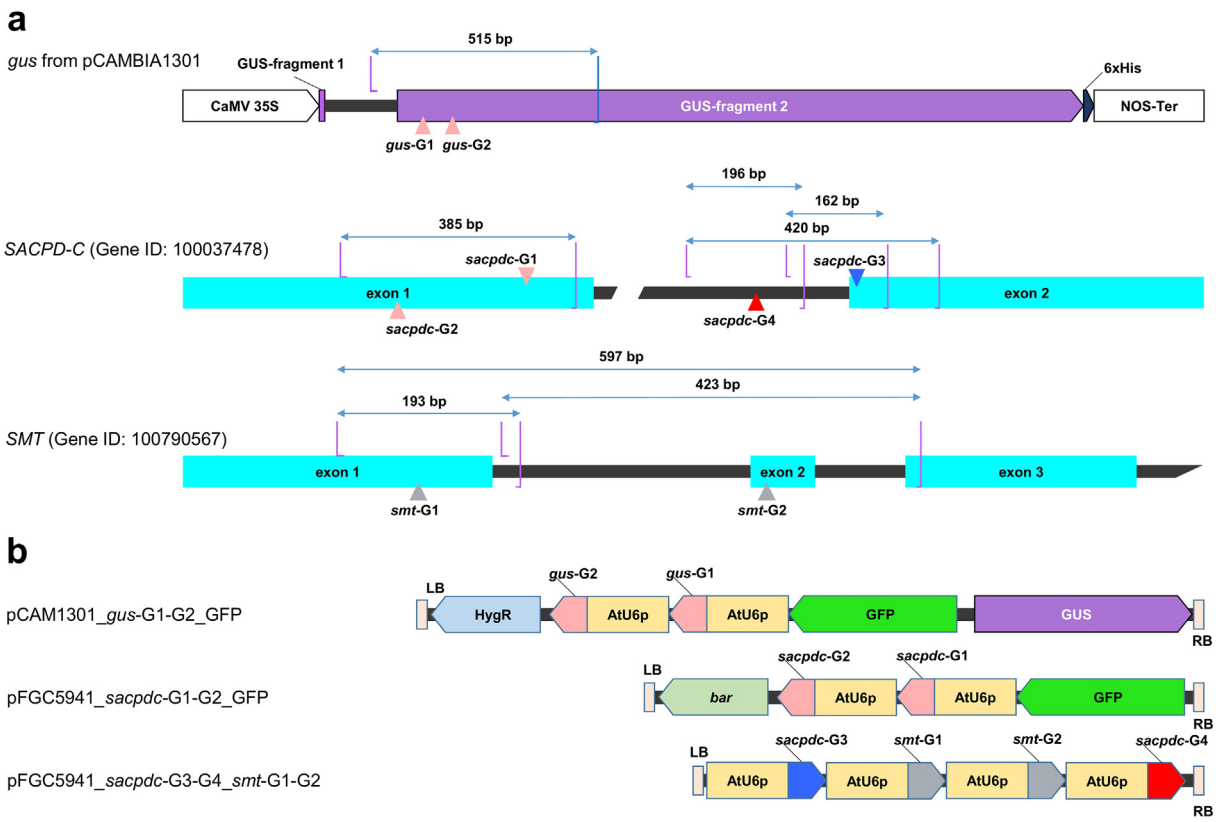


**Fig. 1.** gRNA target locations on tested genes and transgenic vector structures. (a) Locations of gRNA target sequences on each tested gene. Target sequences are presented as triangles, gene-specific primers for genotyping as purple 'L' shapes. Lengths of PCR products are also indicated in base pairs (bp). (b) T-DNA regions for the targeted editing of *gus* (pCAM1301_*gus*-G1-G2_GFP), *SACPD-C* (pFGC5941_*sacpdc*-G1-G2_GFP), and both *SACPD-C* and *SMT* genes (pFGC5941_*sacpdc*-G3-G4_*smt*-G1-G2), respectively. CaMV 35S, Cauliflower mosaic virus 35S promoter. 6xHis, 6xHis affinity tag. NOS-Ter, Nopaline synthase terminator. HygR, Hygromycin resistance gene. AtU6p, *Arabidopsis thaliana* U6 promoter. GFP, green fluorescence protein. *gus*, β-glucuronidase gene. *bar*, bialaphos resistance gene. LB/RB, T-DNA left and right borders, respectively.

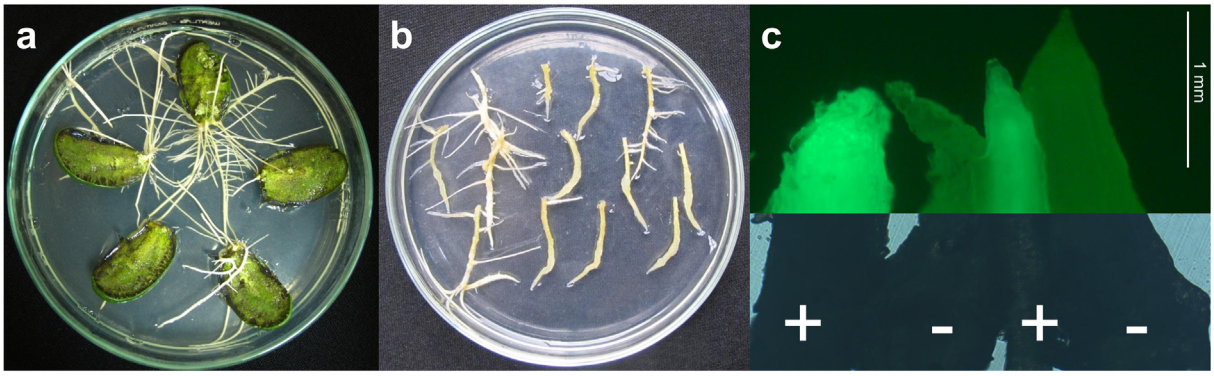


**Fig. 2.** Transgenic hairy root induction and selection. (a) Induced hairy roots from soybean cotyledons. (b) Individual hairy root lines on selection medium. (c) Hairy roots with (+)/without (-) green fluorescence, indicating presence/absence of transgenic T-DNA insertion.

the target sites of the tested gene of these two lines. Mutations occurring on both target sites of *gus* gene in the gus-1 and gus-6 lines illustrate the simultaneous excisions of Cas9 at the two target sites. The gus-7 line also carried heterozygous mutations, which was consistent with the respective GUS activity in these roots. These results demonstrate that sequential transformation of gRNAs into the stable Cas9-expressing soybean is capable of inducing mutations in delivered exogenous genes.

### *3.2. Editing of a single endogenous gene by sequential transformation*

To test the editing of endogenous genes by sequential transformation, soybean hairy roots were transformed with pFGC5941_*sacpdc*-G1-G2_GFP (Fig. 1B), where constructed gRNAs targeted the first exon of the *SACPD-C* gene (Fig. 1A). Transgenic hairy roots sufficiently identified by GFP expression were randomly selected for induced mutant analysis in the *SACPD-C* gene. All analyzed hairy roots showed extra DNA bands compared to the WT root on PAGE (Fig. 4A). Therefore, mutations of *SACPD-C* gene had been induced in the tested transgenic root lines. Sequence analysis of sac-2, sac-4 and sac-6 lines also confirmed the targeted indels of *SACPD-C* gene (Fig. 4B). Similar to *gus*-targeted lines, large indels (−219 bp and −220 bp) were also found between two target sites of *SACPD-C* in lines sac-2 and sac-6. Moreover, small indels (from −2 to −8 bp) were observed at individual target sites. Therefore, the sequential transformation system was

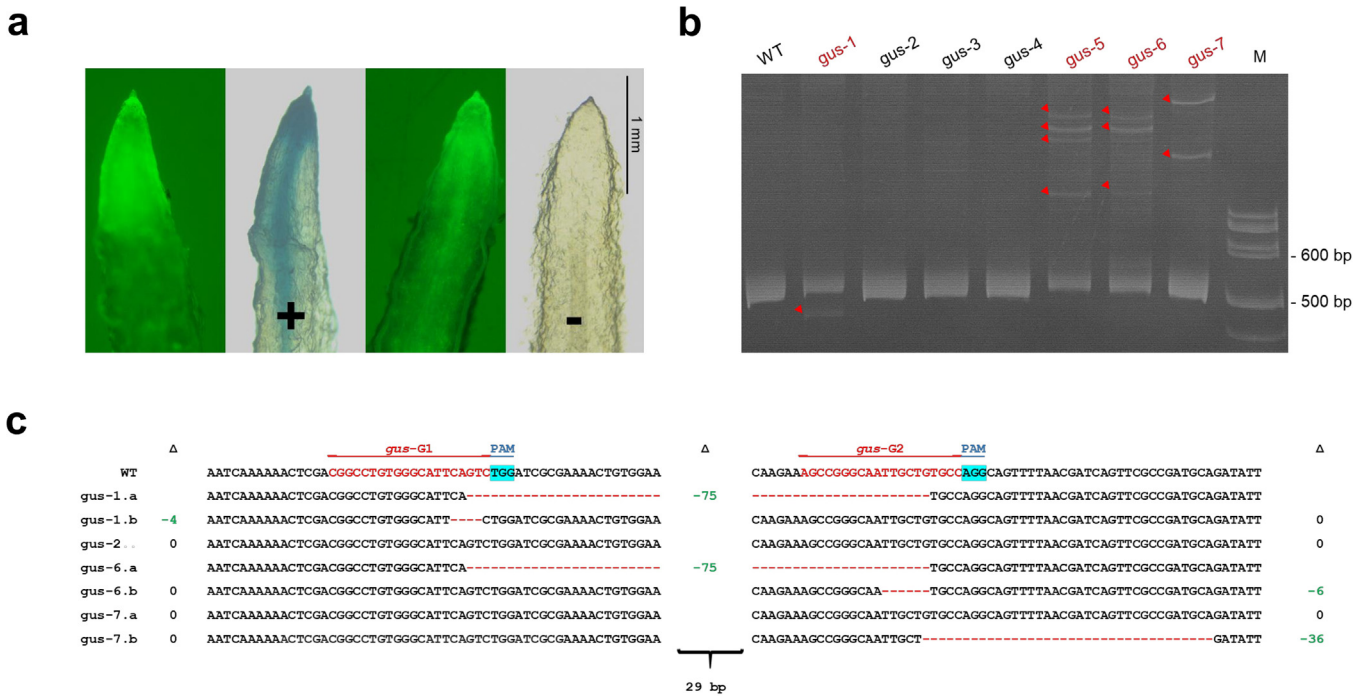


**Fig. 3.** Targeted editing of *gus* gene in soybean transgenic hairy roots. (a) GFP-expressing hairy roots with (+)/without (-) GUS activity. (b) Induced mutations of *gus* detected by heteroduplex analysis. Lines with expected mutations are indicated in red. The red arrowheads indicate DNA band shifts which could be from heteroduplex formation. (c) Indel characterization of representative samples by sequencing. bp, base pair(s). Δ, types of mutation. Different alleles detected in each sample are marked by lower case letters. Red letters are targeted sequences. Blue highlights are PAM sequences. Deletions are presented as red dashes. Mutations at the left target, right target, or large deletions in between two targets are indicated accordingly.

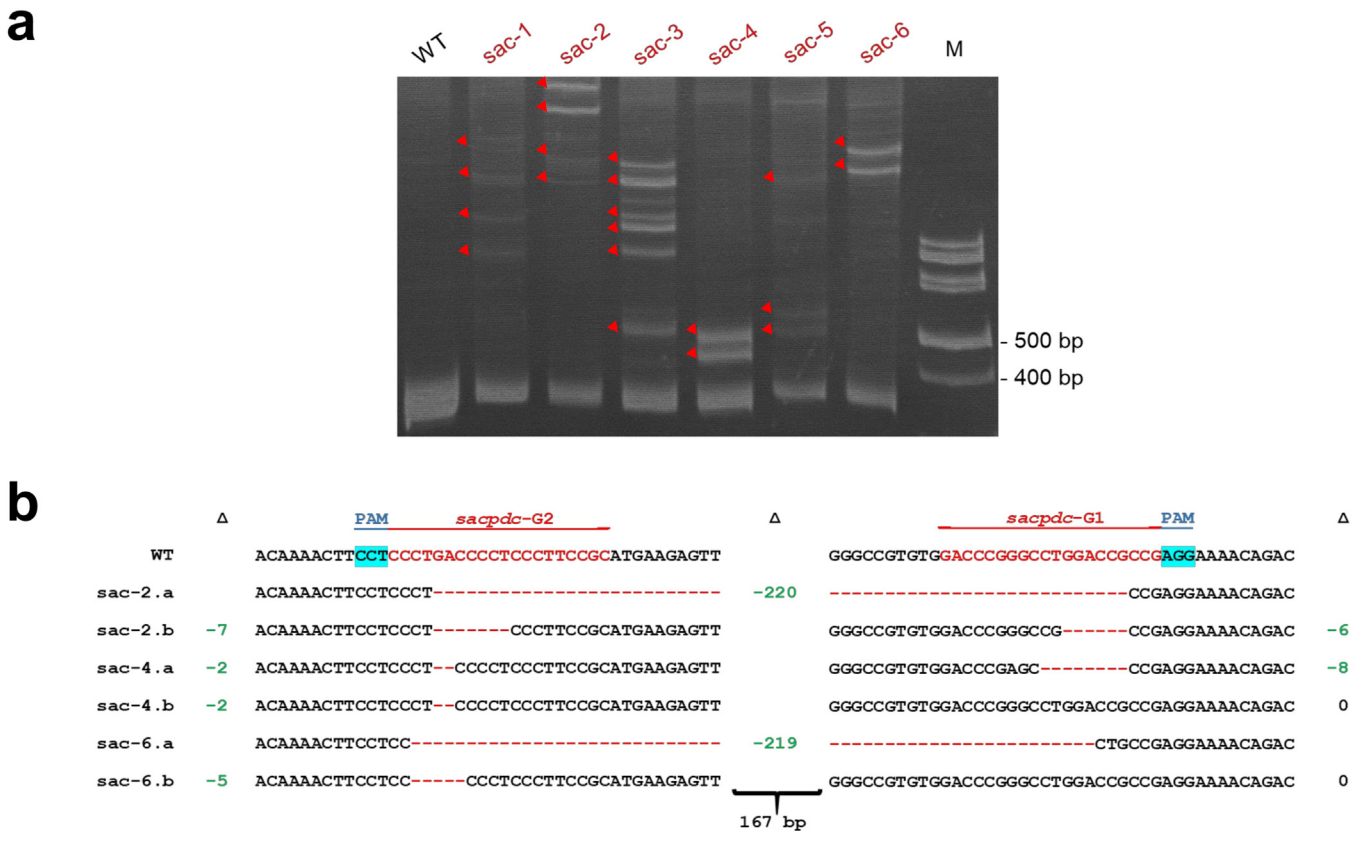


**Fig. 4.** Targeted editing of *SACPD-C* gene in soybean transgenic hairy roots. (a) Induced mutations of *SACPD-C* detected by heteroduplex analysis. Lines with expected mutations are indicated in red. The red arrowheads indicate DNA band shifts which could be from heteroduplex formation. (b) Indel characterization of representative samples by sequencing. bp, base pair(s). Δ, types of mutation. Different alleles detected in each sample are marked by lower case letters. Red letters are targeted sequences. Blue highlights are PAM sequences. Deletions are presented as red dashes. Mutations at the left target, right target, or large deletions in between are indicated accordingly.

also demonstrated to have capacity for inducing targeted mutations of endogenous genes in soybean.

### 3.3. Editing of multiple genes by sequential transformation

This study also tested the ability of the sequential transformation method to simultaneously edit multiple genes and to evaluate the targeting capabilities of different gRNAs. Two endogenous genes, *SACPD-C* and *SMT*, were selected for mutagenesis. The gRNAs with the most and least potential for *SACPD-C* gene were selected using the CRISPRater feature in CCTop (Labuhn et al., 2018). Two gRNAs with similar ratings were selected to target *SMT* gene (Supplemental Table 1). These four gRNAs were integrated into a single transformation vector pFGC5941_sacpdc-G3-G4_smt-G1-G2 (Fig. 1B). Transgenic root lines were identified by PCR with T-DNA-specific primers (data not shown).

The PAGE heteroduplex analysis in Figs. 5 and 6 indicates that mutations were induced at all target sites of both *SACPD-C* and *SMT* genes. Particularly, 9 out of 12 examined transgenic hairy root lines exhibited induced mutations on *SACPD-C* gene, among which, 3 lines (M2, M8, M9) (25%) exhibited induced mutations at both target sites, 5 lines (M1, M4, M5, M7, M12) (42%) had indels at the *sacpdc*-G4 target, and 1 line (M11) (8%) showed mutations only at the *sacpdc*-G3 target (Fig. 5A). For the *SMT* gene, mutations were detected in 8 of 12 tested samples (Fig. 6A), 2 of which (M2, M9) (17%) showed mutations at both targets, while 6 lines (M1, M4, M5, M7, M8, M12) (50%) carried mutations at only the *smt*-G2 target.

To confirm and characterize the targeted mutations induced by the sequential transformation system, the targeted regions were amplified from representative samples and subjected to sequencing (Figs. 5B and 6B). The sequencing results were consistent with the PAGE heteroduplex analysis, in which, M9 line contained mutations at both target sites of each gene, M5 line had mutations at the *sacpdc*-G4 and *smt*-G2 targets but not at *sacpdc*-G3 and *smt*-G1, and M6 line had no mutation at any target site. From sequencing data, we also found different types of mutations in the tested genes, including large and small deletions.

Overall, these results demonstrate that our sequential transformation system in soybean has the potential to induce mutations of multiple genes as well as to evaluate the targeted mutation efficacies of different selected gRNAs.

## 4. Discussion

### 4.1. Demonstration of sequential transformation system for soybean genome editing

The sequential transformation for genome editing by CRISPR/Cas9 system has been reported in several plant species including *Arabidopsis thaliana* (Lei et al., 2021; Miki et al., 2018), tobacco (Ellison et al., 2020; Uranga et al., 2021), rice (Mikami et al., 2015b; 2015a) and petunia (Zhang et al., 2016). In some of these studies, gRNAs were transferred into the Cas9-overexpressing plants via viral vectors (Ellison et al., 2020; Lei et al., 2021; Uranga et al., 2021) and the induced mutations were identified and confirmed in the next generations of the virus-infected plants. Alternatively, targeted mutations were also generated by sequentially transforming gRNA expression constructs into the Cas9-overexpressing plants (Mikami et al., 2015b; 2015a; Miki et al., 2018; Zhang et al., 2016). In soybean, the *Agrobacterium rhizogenes*-mediated hairy root system has been used to evaluate and confirm CRISPR/Cas9-induced mutations of exogenous as well as endogenous genes (Cai et al., 2015; Jacobs et al., 2015; Le et al., 2020; Michno et al., 2015). Here, we report the first success in utilizing sequential transformation system for soybean genome editing by CRISPR/Cas9. The effectiveness of this system was confirmed by the induction of targeted mutations in exogenous and endogenous – both single and multiple – genes. A range of indels in the targeted genes including small deletions, larger deletions and insertions

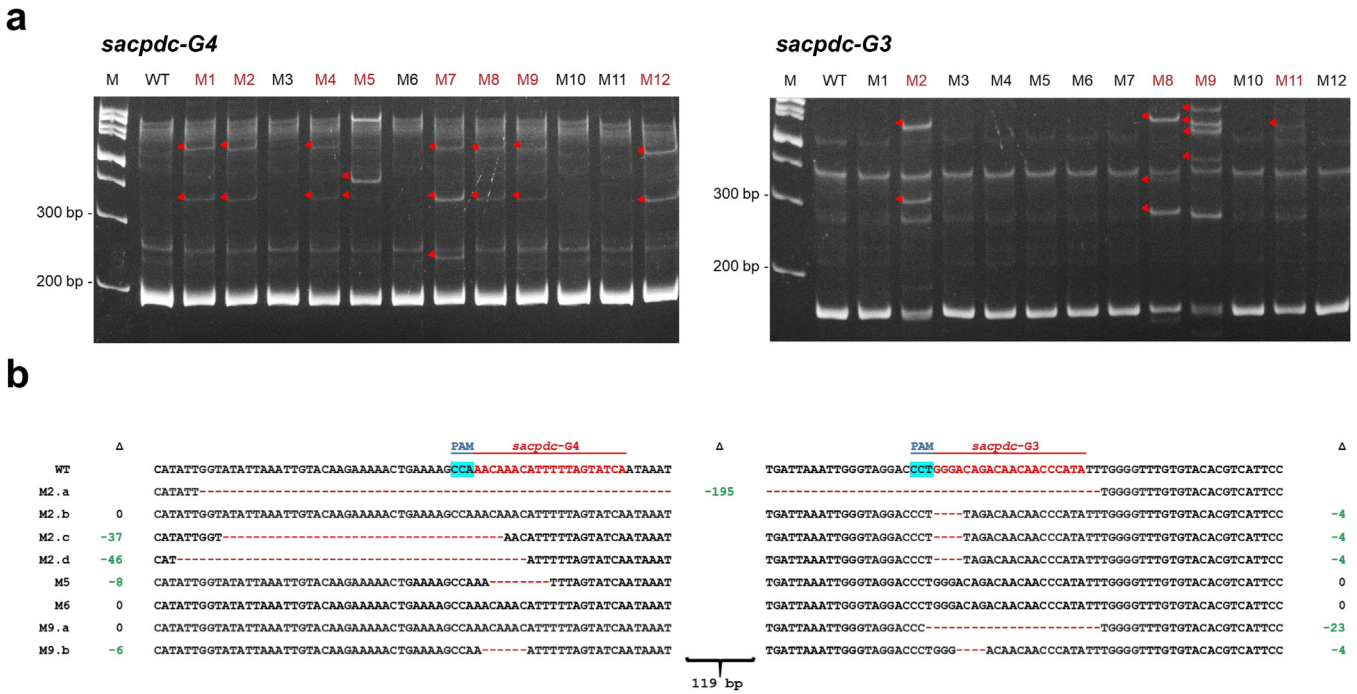


**Fig. 5.** Identification and characterization of *SACPD-C* indels in soybean hairy roots from multiple-gRNA construct. (a) Induced mutations of *SACPD-C* detected by heteroduplex analysis. Lines with expected mutations are indicated in red. The red arrowheads indicate DNA band shifts which could be from heteroduplex formation. (b) Indel characterization of representative samples by sequencing. bp, base pair(s). Δ, types of mutation. Different alleles detected in each sample are marked by lower case letters. Red letters are targeted sequences. Blue highlights are PAM sequences. Deletions are presented as red dashes. Mutations at the left target, right target, or large deletions in between are indicated accordingly.

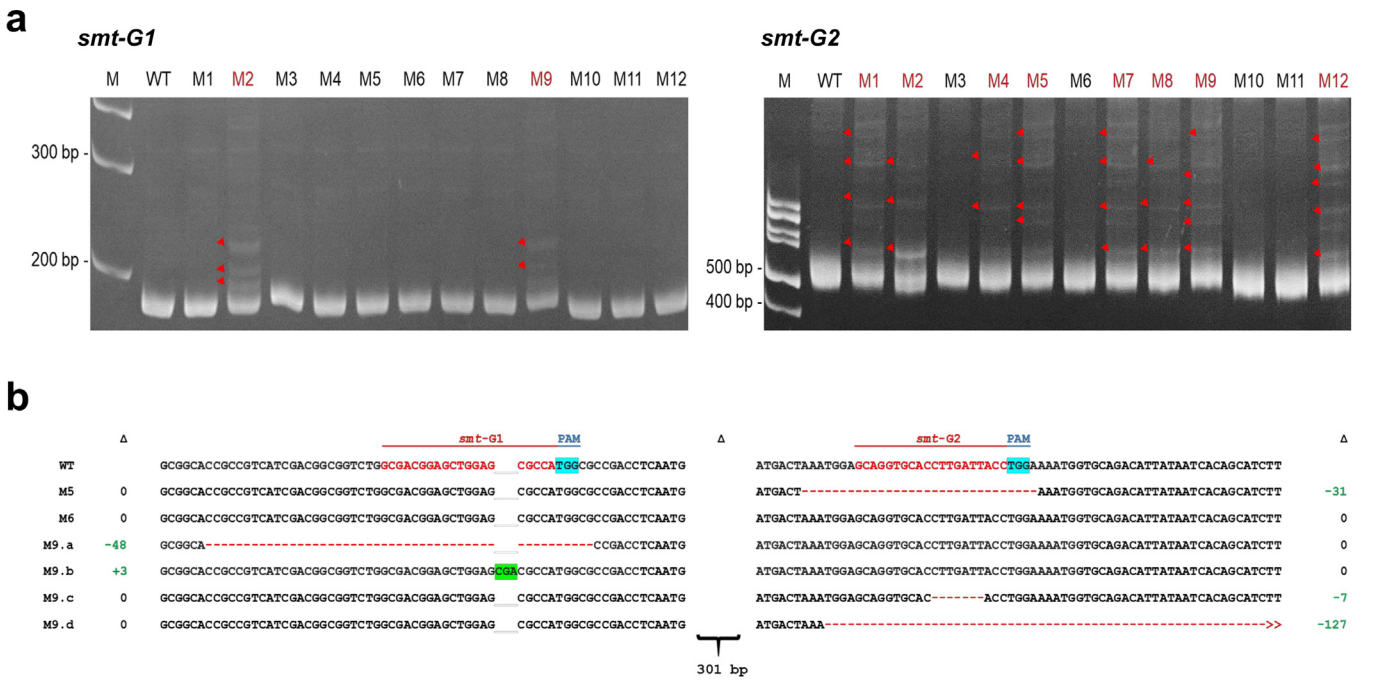


**Fig. 6.** Identification and characterization of *SMT* indels in soybean hairy roots from multiple-gRNA construct. (a) Induced mutations of *SMT* gene detecting by heteroduplex analysis. Lines with expected mutations are indicated in red. The red arrowheads indicate DNA band shifts which could be from heteroduplex formation. (b) Indel characterization of representative samples by sequencing. bp, base pair(s). Δ, types of mutation. Different alleles detected in each sample are marked by lower case letters. Red letters are targeted sequences. Blue highlights are PAM sequences. Deletions are presented as red dashes, insertions as green highlights. Mutations at the left target, right target, or large deletions in between are indicated accordingly.

generated by soybean hairy root sequential transformation indicates the utility of this system for validation of CRISPR/Cas activities and gene function studies.

### *4.2. High editing efficiency achieved by sequential transformation in soybean*

In this report, the sequential transformation was performed using soybean hairy roots to induce targeted mutations of endogenous and co-delivered exogenous genes. The induced mutant rates observed in experiments reached up to 100% indicating the usefulness of this system for genome editing studies in soybean. Previously, the hairy root transformation system had been used to test and validate CRISPR/Cas9 activity at the very beginning of soybean genome editing. For example, the stable exogenous gene GFP and multiple endogenous genes were edited using hairy root with the overall efficiencies of 95% (Jacobs et al., 2015). Moreover, the soybean hairy root system was also utilized to evaluate targeting ability of individual gRNAs, which exhibited induced mutation rates from 10% to 93% (Cai et al., 2015). A limited number of targeted mutations of the exogenous gene *gus* were generated by co-delivery of this gene and CRISPR/Cas components in the same T-DNA construct into soybean hairy roots (Michno et al., 2015). The hairy root system has also been used as an adequate predictive tool to verify the activity of CRISPR/Cas9 constructs before performing stable transformations (Do et al., 2019; Le et al., 2020; Zheng et al., 2020). However, Cas9 and gRNA cassettes constructed and delivered in one or separated vectors for transformation could increase the time required and limit the number of targeted gRNAs.

### *4.3. Comparing and validating efficiencies of multiple designed gRNAs via the soybean sequential transformation*

Recently, the targeting ability of gRNAs has been predicted employing various bioinformatics programs utilizing genome database of each plant species (Xie et al., 2014; Stemmer et al., 2015; Bae et al., 2014). However, disagreements in gRNA ratings among different programs and with experimental results have been reported (Haeussler et al., 2016; Naim et al., 2020; Uniyal et al., 2019). These differences may have resulted from the genome insertion sites and expression levels of Cas9 and gRNAs from individual binary vectors, posing challenges to the evaluation of targeting ability of gRNAs. In our sequential transformation system, all gRNAs resided in one T-DNA of a construct which was delivered into stable Cas9-expressing transgenic soybean hairy roots. In this way, variations generated from different vectors or transgenic performance can be minimized. The sequential transformation system using soybean hairy roots has proven to have potential for showing differences in the targeted ability of gRNAs as well as disagreements between software prediction and real experimental performance. For example, mutations in *sacpdc*-G3 and *sacpdc*-G4 target sites occurred at 33% (4/12) and 67% (8/12) respectively, although *sacpdc*-G3 and *sacpdc*-G4 were rated as "HIGH"/"LOW"-potential gRNAs, respectively (Supplementary Table 1). On the other hand, *smt*-G1 and *smt*-G2 targets, both rated as "MEDIUM"-potential gRNAs exhibited differences in induced mutation efficiency. Although sample sizes for the present editing study were not large and therefore need additional replicates, our results show a usefulness of sequential transformation using hairy roots for validating and comparing the targeting ability of selected gRNAs in soybean as well as other crops.

### *4.4. Further perspectives*

Success in utilizing sequential transformation to edit exogenous DNA provides further research strategies to control and target invading DNA. For instance, sequential transformation approach could be used to target and destroy pathogenic viral DNA instead of performing regular transformation as in previous reports (Baltes et al., 2015; Ji et al., 2015). In addition, sequential

transformation could be combined with viral vectors for *in vivo* genome editing as well as generating stable mutant soybean.

## 5. Conclusion

Here, we show the first success in sequential transformation for genome editing of soybean using hairy root transformation. The genome editing efficiency of sequential transformation was confirmed by inducing mutations of exogenous and endogenous – both single and multiple – genes in soybean. This system should be instrumental for validating the CRISPR/Cas9 construct activities, gRNA targeting ability as well as other research purposes.

## Funding

This research was supported by the National Foundation for Science and Technology of Vietnam (106.03-2019.11).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors thank Drs. Van Tuong Nguyen, Zhanyuan J Zhang and Sterling Evans for proofreading of the manuscript and helpful suggestions, Dr. Anh Van Thi Le and M.S. Nhung Hong Nguyen for initial microscopy training.

## Author contributions

PD and HC conceived and supervised the study; PD, CN and DT designed the experiments; NL and DT performed CRISPR/Cas9 vector constructions; TB and TL conducted soybean hairy root transformation; DT identified and characterized induced mutations; PD and DT analyzed the data and prepared the manuscript; HC and CN revised and proof-read the manuscript.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103420.

## References

Bae, S., Park, J., Kim, J., 2014. Sequence analysis Cas-OFFinder : a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30, 1473–1475. https://doi.org/10.1093/bioinformatics/btu048.

Baltes, N.J., Hummel, A.W., Konecna, E., Cegan, R., Bruns, A.N., Bisaro, D.M., Voytas, D.F., 2015. Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. Nat. Plants 1, 15145. https://doi.org/10.1038/nplants.2015.145.

Brandt, K.M., Gunn, H., Moretti, N., Zemetra, R.S., 2020. A Streamlined Protocol for Wheat (Triticum aestivum) Protoplast Isolation and Transformation With CRISPR-Cas Ribonucleoprotein Complexes. Front. Plant Sci. 11, 1–14. https://doi.org/10.3389/fpls.2020.00769.

Cai, Y., Chen, L.i., Liu, X., Sun, S., Wu, C., Jiang, B., Han, T., Hou, W., Xu, X., 2015. CRISPR/Cas9-mediated genome editing in soybean hairy roots. PLoS ONE 10 (8), e0136064.

Chen, L., Cai, Y., Liu, X., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T., Hou, W., 2018. Soybean hairy roots produced in vitro by Agrobacterium rhizogenes-mediated transformation. Crop J. 6, 162–171. https://doi.org/10.1016/j.cj.2017.08.006.

Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Report. 1, 19–21. https://doi.org/10.1007/BF02712670.

Do, P.T., Nguyen, C.X., Bui, H.T., Tran, L.T.N., Stacey, G., Gillman, J.D., Zhang, Z.J., Stacey, M.G., 2019. Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous GmFAD2-1A and GmFAD2-1B genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean. BMC Plant Biol. 19, 311. https://doi.org/10.1186/s12870-019-1906-8.

Doudna, J.A., Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. Science (80-.) 346. https://doi.org/10.1126/science.1258096.

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

Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J.-B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.-S., Concordet, J.-P., 2016. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 17, 148. https://doi.org/10.1186/s13059-016-1012-2.

Jacobs, T.B., LaFayette, P.R., Schmitz, R.J., Parrott, W.A., 2015. Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol. 15, 1–10. https://doi.org/10.1186/s12896-015-0131-2.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907. https://doi.org/10.1002/j.1460-2075.1987.tb02730.x.

Ji, X., Zhang, H., Zhang, Y., Wang, Y., Gao, C., 2015. Establishing a CRISPR–Cas-like immune system conferring DNA virus resistance in plants. Nat. Plants 1, 15144. https://doi.org/10.1038/nplants.2015.144.

Kanazashi, Y., Hirose, A., Takahashi, I., Mikami, M., Endo, M., Hirose, S., Toki, S., Kaga, A., Naito, K., Ishimoto, M., Abe, J., Yamada, T., 2018. Simultaneous site-directed mutagenesis of duplicated loci in soybean using a single guide RNA. Plant Cell Rep. 37, 553–563. https://doi.org/10.1007/s00299-018-2251-3.

Karimi, M., Inzé, D., Depicker, A., 2002. GATEWAY™ vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. 7, 193–195. https://doi.org/10.1016/S1360-1385(02)02251-3.

Kerschen, A., Napoli, C.A., Jorgensen, R.A., Müller, A.E., 2004. Effectiveness of RNA interference in transgenic plants. FEBS Lett. 566, 223–228. https://doi.org/10.1016/j.febslet.2004.04.043.

Labuhn, M., Adams, F.F., Ng, M., Knoess, S., Schambach, A., Charpentier, E.M., Schwarzer, A., Mateo, J.L., Klusmann, J.-H., Heckl, D., 2018. Refined sgRNA efficacy prediction improves large- and small-scale CRISPR–Cas9 applications. Nucleic Acids Res. 46, 1375–1385. https://doi.org/10.1093/nar/gkx1268.

Le, H., Nguyen, N.H., Ta, D.T., Le, T.N.T., Bui, T.P., Le, N.T., Nguyen, C.X., Rolletschek, H., Stacey, G., Stacey, M.G., Pham, N.B., Do, P.T., Chu, H.H., 2020. CRISPR/Cas9-Mediated Knockout of Galactinol Synthase-Encoding Genes Reduces Raffinose Family Oligosaccharide Levels in Soybean Seeds. Front. Plant Sci. 11, 1–13. https://doi.org/10.3389/fpls.2020.612942.

Lei, J., Dai, P., Li, Y., Zhang, W., Zhou, G., Liu, C., Liu, X., 2021. Heritable gene editing using FT mobile guide RNAs and DNA viruses. Plant Methods 17, 20. https://doi.org/10.1186/s13007-021-00719-4.

Li, Z.H., Meng, H., Ma, B., Tao, X., Liu, M., Wang, F.Q., Wei, D.Z., 2020. Immediate, multiplexed and sequential genome engineering facilitated by CRISPR/Cas9 in Saccharomyces cerevisiae. J. Ind. Microbiol. Biotechnol. 47, 83–96. https://doi.org/10.1007/s10295-019-02251-w.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R.N., Potter, S.C., Finn, R.D., Lopez, R., 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47, W636–W641. https://doi.org/10.1093/nar/gkz268.

Michno, J.M., Wang, X., Liu, J., Curtin, S.J., Kono, T.J., Stupar, R.M., 2015. CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. GM Crops Food 6, 243–252. https://doi.org/10.1080/21645698.2015.1106063.

Mikami, M., Toki, S., Endo, M., 2015a. Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. Plant Cell Rep. 34, 1807–1815. https://doi.org/10.1007/s00299-015-1826-5.

Mikami, M., Toki, S., Endo, M., 2015b. Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. Plant Mol. Biol. 88, 561–572. https://doi.org/10.1007/s11103-015-0342-x.

Miki, D., Zhang, W., Zeng, W., Feng, Z., Zhu, J.K., 2018. CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. Nat. Commun. 9, 1–9. https://doi.org/10.1038/s41467-018-04416-0.

Naim, F., Shand, K., Hayashi, S., O'Brien, M., McGree, J., Johnson, A.A.T., Dugdale, B., Waterhouse, P.M., He, Z.-Y., 2020. Are the current gRNA ranking prediction algorithms useful for genome editing in plants? PLoS ONE 15 (1), e0227994.

Phan, T.H., Nong, V.H., Le, T.B., Chu, H.H., 2005. using XcmI enzyme to construct pBT T-vector for pcr cloning. Vietnam Journnal Biotechnol. 3, 459–463.

Shan, Q., Wang, Y., Li, J., Gao, C., 2014. Genome editing in rice and wheat using the CRISPR/Cas system. Nat. Protoc. 9 (10), 2395–2410.

Stemmer, M., Thumberger, T., Keyer, S., Wittbrodt, J., Mateo, J.L., 2015. CCTop : An Intuitive, Flexible and Reliable CRISPR / Cas9 Target Prediction Tool. PLoS ONE 9, 1–11. https://doi.org/10.1371/journal.pone.0124633.

Uniyal, A.P., Mansotra, K., Yadav, S.K., Kumar, V., 2019. An overview of designing and selection of sgRNAs for precise genome editing by the CRISPR-Cas9 system in plants. 3. Biotech 9, 223. https://doi.org/10.1007/s13205-019-1760-2.

Uranga, M., Aragonés, V., Selma, S., Vázquez-Vilar, M., Orzáez, D., Daròs, J.-A., Marta, V., Orz, D., 2021. Efficient Cas9 multiplex editing using unspaced sgRNA arrays engineering in a Potato virus X vector. Plant J. 106, 555–565. https://doi.org/10.1111/tpj.15164.

Wang, W., Akhunova, A., Chao, S., Akhunov, E., 2016. Optimizing multiplex CRISPR/Cas9-based genome editing for wheat. bioRxiv 051342. https://doi.org/10.1101/051342.

Xie, S., Shen, B., Zhang, C., Huang, X., Zhang, Y., Khodursky, A.B., 2014. SgRNAcas9: A software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. PLoS ONE 9 (6), e100448.

Yoo, S.D., Cho, Y.H., Sheen, J., 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572. https://doi.org/10.1038/nprot.2007.199.

Zhang, B., Yang, X., Yang, C., Li, M., Guo, Y., 2016. Exploiting the CRISPR/Cas9 System for Targeted Genome Mutagenesis in Petunia. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep20315.

Zheng, N., Li, T., Dittman, J.D., Su, J., Li, R., Gassmann, W., Peng, D., Whitham, S.A., Liu, S., Yang, B., 2020. CRISPR/Cas9-Based Gene Editing Using Egg Cell-Specific Promoters in Arabidopsis and Soybean. Front. Plant Sci. 11, 1–15. https://doi.org/10.3389/fpls.2020.00800.

Zhu, X., Xu, Y., Yu, S., Lu, L., Ding, M., Cheng, J., Song, G., Gao, X., Yao, L., Fan, D., Meng, S., Zhang, X., Hu, S., Tian, Y., 2014. An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. Sci. Rep. 4, 1–8. https://doi.org/10.1038/srep06420.