Targeted Gene Editing in Plants using CRISPR-Cas9, Single-Stranded DNA Oligonucleotides, and Protoplast Regeneration

Chen-Tran Hsu[1,#], Yu-Hsuan Yuan[1,#], Yao-Cheng Lin[1,#,*], Steven Lin[2,#], Qiao-Wei Cheng[1], Fu-Hui Wu[1], Jen Sheen[3], Ming-Che Shih[1], and Choun-Sea Lin[1,*]

[1] Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

[2] Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

[3] Department of Molecular Biology and Centre for Computational and Integrative Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114, USA

[#] The authors contributed equally to this work

*Correspondence: cslin99@gate.sinica.edu.tw

ORCID: orcid.org/0000-0001-9566-2952

Abstract

Genome editing requires insertion of DNA sequences into specific locations. Protocols involving clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins rely on homology-directed repair, require laborious vector construction, and have low efficiency. DNA oligonucleotides can be used as donors for targeted insertion via nonhomologous end joining. Our simple protocol eliminates the need for expensive equipment and vector construction by using polyethylene glycol to deliver non-modified single-stranded DNA oligonucleotides and CRISPR-Cas9 ribonucleoprotein into protoplasts. We achieved targeted insertion frequencies of up to 50.0% in *Nicotiana benthamiana* and 13.6% in rapid cycling *Brassica oleracea* without antibiotic selection. Using a 60-nt donor containing 27 nt in each homologous arm, 6 of 22 regenerated plants showed targeted insertions, and 1 contained a precise insertion of a 6-bp *Eco*RI site. Whole-genome sequencing showed that the DNA inserted only in the targeted positions, and genetic analysis showed that the inserted sequences transmitted to the next generation.

Key words

Nicotiana benthamiana, rapid cycling *Brassica oleracea*, synthetic single-stranded oligonucleotide DNA, ribonucleoprotein

Introduction

To insert DNA into a specific location in the plant genome, a DNA double-strand break (DSB) must be induced at the target position and the donor DNA (DD) must be inserted into this position via homology-directed repair (HDR)[1-12] or nonhomologous end joining (NHEJ).[13-14] Many tools for creating DSBs are currently available.[15-17] Due to its convenience and efficiency, the combination of clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR associated (Cas) proteins has become a favorite tool among genetic engineers.[4] In addition to CRISPR-Cas reagents, DD must be delivered into the target cells; increasing the amount of delivered DD enhances targeted insertion (TI) efficiency. For example, in maize (*Zea mays*), targeted mutagenesis using CRISPR has been achieved via different methods, although TI plants were obtained only via biolistic methods rather than *Agrobacterium*-mediated transformation because the number of DD copies delivered by the latter method is low.[2]

Protoplasts offer an alternative substrate for genetic transformation and transfection, since they enable the delivery of high numbers of DNA copies[18,19], and the DNA can be inserted into the genome.[18] Like the transcription activator-like effector nuclease genome editing system[20], CRISPR reagents [ribonucleoprotein (RNP) or plasmid DNA] can be delivered into protoplasts via polyethylene glycol (PEG)-mediated transfection, and targeted mutations can be achieved.[21-28] The mutated protoplasts can then be regenerated into plants and, in contrast to other tissue culture-based transformation protocols in dicot plants where transformants are chimeric and some of the mutated alleles are not heritable, the mutated alleles are passed onto the progeny.[21, 23, 24] Here, we describe a simple, high-TI-efficiency protoplast-based strategy for genome editing in plants using CRISPR that does not require expensive equipment.

Methods

Protoplast isolation, transfection, and regeneration

Nicotiana benthamiana and rapid cycling *Brassica oleracea* (RCBO) plants were propagated in ½-strength Murashige and Skoog (½ MS) medium supplemented with 30 g/L sucrose under a 12-h/12-h light/dark cycle at 25°C. Protoplast isolation was performed according to Lin *et al.*[23] and Hsu *et al.* [28] except for the digestion solution [½ MS medium supplemented with 30 g/L sucrose, 0.4 M mannitol, 1% cellulose, 0.5% Macerozyme, 1 mg/L 1-naphthaleneacetic acid (NAA) and 0.3 mg/L kinetin] and digestion time (3 days) in *N. benthamiana*. The protoplasts were co-transfected with RNP and 50 μg synthetic single-stranded oligonucleotide DNA (ssODN; Genomics, Taipei, Taiwan) according to Woo *et al.*[21] Transfected protoplasts were incubated in a 5-cm-diameter Petri dish containing liquid callus medium (½ MS, 0.4 M mannitol, 1 mg/L NAA, 0.3 mg/L kinetin) for 3 weeks. RCBO calli were additionally incubated in 1 mg/L NAA, 1 mg/L 6-benzyladenine (BA), and 0.25 mg/L 2,4-dichlorophenoxyacetic acid for 3 days in the dark. The calli were transferred to liquid shooting medium (containing 2 mg/L BA for *N. benthamiana*, 0.1 mg/L thidiazuron for RCBO) in a 9-cm-diameter Petri dish and incubated at 25°C for 3–4 weeks in the light (16-h/8-h light/dark, 3000 lux). Green explants larger than 5 mm were incubated in solid shooting medium and subcultured every 4 weeks. Shoot clusters with leaves were then transferred to solidified rooting medium (HB1: 3 g/L Hyponex, 2 g/L tryptone, 20 g/L sucrose, 1 g/L activated charcoal, 2.2 g/L Gelrite, pH

5.2).

Cas9 protein purification, single-guide RNA synthesis, and Cas9 RNP nucleofection

Preparation of Cas9 protein and single-guide RNA (sgRNA) and Cas9 RNP nucleofection were performed according to Huang *et al.*[29] Cas9 recombinant protein was overexpressed in *Escherichia coli* BL21 harboring the plasmid pMJ915 (Addgene # 69090). Cas9 protein was purified and stored at −80°C in Cas9 RNP buffer (20 mM HEPES at pH 7.5, 150 mM KCl, 10% glycerol, and 1 mM β-mercaptoethanol). The sgRNAs were synthesized by *in vitro* transcription (IVT) using T7 RNA polymerase (New England Biolabs; M0251L). The DNA oligonucleotides used for IVT template assembly are listed in Table S10. The final sgRNA products were dissolved in Cas9 RNP buffer, quantified using a NanoDrop Lite (Thermo Fischer Scientific), and stored as aliquots at −80°C. Cas9 RNP complexes were assembled immediately before nucleofection by mixing equal volumes of 40 μM Cas9 protein and 88.3 μM sgRNA at a molar ratio of 1:2.2 and incubating at 37°C for 60 min.

Validation of targeted insertions in protoplasts and regenerants

Genomic DNA was extracted from pooled protoplasts and regenerants using a Mini GenoPlus Genomic DNA Extraction Kit (GG2002, Viogene, New Taipei City, Taiwan). To amplify the genomic region targeted by the sgRNA, the corresponding pairs of primers were designed. Primer sequences are shown in Table S10. The PCR conditions were 94°C for 5 min, 35 cycles of 94°C for 30 s, annealing at 55–63°C for 30 s, polymerization at 72°C for 30 s, followed by 72°C for 3 min. The polymerase chain reaction (PCR) products were digested using the appropriate restriction enzyme or RNP and subjected to electrophoresis. The PCR products were cloned into the T&A vector (FYC002-20P; Yeastern Biotech Co. Ltd., New Taipei City, Taiwan). Putative colonies containing the edited DNA were confirmed by sequencing.

Whole-genome sequencing for off-target DD insertion analysis

Leaves of *N. benthamiana* plants regenerated from protoplasts were collected for genomic DNA purification. Genomic DNA used for genome sequencing was extracted from the samples using a Plant DNA Purification Kit (DP320, Tiangen, http://www.tiangen.com/en/). Paired-end libraries were constructed from the DNA using a NEBNext Ultra DNA Library Prep Kit for Illumina with 2 × 150 bp with an average insert size of ~900 bp and sequenced on the NovaSeq 6000 platform. Three technical replicates were performed for each sample. A total of 120 Gbp reads were obtained per regenerant, with a sequencing depth >30×. High-quality Illumina reads were converted to fasta format files and used as a BLAST database for target sequence searches. The DD sequence (Exp. 1, TTTGCGATGCCTAACAAGCTTCAGGGGGAGTTCAGCCGCTT) was used as the query in a highly sensitive BLASTN search strategy (-dust no -soft_masking false -word_size 4 -gapopen 1 -gapextend 2 -penalty -1 -reward 1 -evalue 10 -perc_identity 90 -num_alignments 10000). The sequences were used as queries to perform BLASTN searches of *N. benthamiana* wild-type (WT) genome sequences. If the query sequence was identical to the published sequence in the genome, it was considered that the same sequences as the DD already existed and were not caused by TI. If there was a difference from the published WT genome sequence, and the difference was the same as the DD, it was regarded as an off-target TI. The raw reads were deposited in the NCBI SRA database (BioProject: PRJNA667297).

Results and Discussion

The protoplast regeneration protocol (Figure 1) used in the present study was modified from previously published protocols.[23, 24, 28] A key to our approach is the observation that the phase of the cell cycle largely governs the choice of pathway used for DNA repair: NHEJ is the major DNA repair pathway during the G1, S, and G2 phases, whereas HDR occurs only during the late S and G2 phases.[30, 31] Cell cycle synchronization is an effective strategy for enhancing TI efficiency in human embryonic kidney 293T cells.[32] Here, ethynyl deoxyuridine (EdU) staining was used for detection of S-phase cell-cycle progression.[33] To increase the number of cells in the late S and G2 phases, *N. benthamiana* leaves were incubated in solid medium containing ½ MS, 0.4 M mannitol, 1 mg/L NAA, and 0.3 mg/L kinetin (1N0.3K) for 3 days before protoplast isolation (Figure S1A). Comparison with 1N0.3K treatments, no EdU signal was identified in protoplasts incubated in ½ MS, 0.4 M mannitol medium (Figure S1B). Based on single cell analysis[23], the TI efficiency increased after incubation in 1N0.3K (Figure S1C). For *N. benthamiana*, to simplify the procedure, we added 1 mg/L NAA and 0.3 mg/L kinetin to the digestion solution and incubated the material for 3 days. However, this method was not suitable for RCBO because most of the cells were broken after digestion.

To avoid the need for vector construction and to reduce the instability of sgRNA and *Cas* gene expression, we used an RNP as the CRISPR-Cas reagent. For the DD, we used short non-modified synthetic ssODN, which is relatively inexpensive and easy to obtain. The RNP and ssODN were delivered into protoplasts using PEG-mediated transfection (Figure 1A). After 3 days of incubation in 1N0.3K liquid callus medium, genomic DNA was isolated from the protoplasts and the target gene was amplified by PCR and cloned into the T/A vector for Sanger sequencing to assess the TI efficiency. These protoplasts were cultured and regenerated (Figure 1B).[23, 24, 28] The rooted plants were incubated in the growth chamber and genotyped (Figure 1C). These regenerated plants grew normally and produced seeds. DNA from two types of edited regenerants (TI) and knockout (KO) lines and one non-edited (WT) regenerant was purified for genome-wide sequencing to assess the presence or absence of off-target DD insertion (Figure 1D). DNA from the TI T_1 progeny was extracted for genotyping to test whether the inserted fragment was heritable (Figure 1E).

To evaluate the effect of the length of the homologous arms and the total length of ssODN on TI efficiency, we conducted experiments using a target site in *N. benthamiana PHYTOENE DESATURASE1* (*NbPDS1*) (Figure S2), which produces a visible phenotype when mutated.[34] At the expected sgRNA target position, we inserted a *Hind*III site, and on the left and right sides, we added 7, 17, or 27 homologous arms to make the DD 20 nt, 40 nt, or 60 nt long (Figure S2A). We genotyped *NbPDS1* PCR products from the regenerants. No TI regenerants with the 20-nt ssODN were identified, whereas the 40-nt and 60-nt ssODNs produced TI regeneration efficiencies of 27.3–31.8% without antibiotic or phenotypic selection (Figure S2B). One of the 60-nt TI regenerants (+27#6, Figure S2C, D) contained a precise insertion of a 6-bp *Hind*III recognition site at the target position. These results indicate that HDR also occurred during protoplast regeneration. We hypothesize that the length of the ssODN donor is an important factor in TI efficiency. In a previous study, a 59-nt ssODN (ssADHE) failed to give rise to successful insertions in 23 T_0 rice (*Oryza sativa*) plants,[13] although there was no homologous arm, and a lower DD

concentration was used compared to the current study. Based on these results, we selected 40 nt as the length of the ssODN in our subsequent experiments, except for Experiment (Exp.) 2 (44 nt).

Next, we examined the effect of the insertion length in a 40-nt DD on TI efficiency (Figure 2). In Exp. 1, the *Hind*III site was generated by introducing a 2-nt insertion in the DD (Figure 2A), and TI efficiency in the regenerated plants was 18.2% (Figure 2B, C). In Exp. 2, the protospacer adjacent motif (PAM) position in DD was replaced, 6 nt were added, and the TI efficiency increased to 50.0%. When we increased the insertion length to 15 nt (Exp. 3), we included sites for *Nhe*I and *Bam*HI endonucleases in the insertion, which enabled us to confirm the integrity of TI genotyping by restriction enzyme digestion. As the insertion length increased and the length of the homologous arms decreased (11 nt and 14 nt, respectively), the TI efficiency decreased slightly (40.9%). Three regenerants contained only the *Nhe*I site, indicating partial DD insertion (Figure 2B). Perhaps the ssODN DD was unstable in protoplasts and had been partially degraded before insertion, which would cause the inserted sequence to be incomplete. The presence of a phosphorothioate-linkage modification in DD stabilizes this double-stranded DNA.[13] There was no phosphorothioate-linkage modification in the DD we used in this study, which may have allowed the DD to degrade during TI.

We sequenced all of the *NbPDS1* genes in TI regenerants produced in Exp. 1, 2, and 3 and found that all regenerants contained insertions generated by NHEJ (Tables S1, S2, and S3). However, in a few TI regenerants, one end of the ODN was joined by HDR whereas the other end was joined by NHEJ (Exp. 1#12 and Exp. 2#18). In these NHEJ TI regenerants, the insertion size was 29–445 bp. These differences were caused by insertion of 1–13 repeats of the ssODN, although some of the repeats were incomplete. In rice, only 20% of TI plants had repeat insertion when modified double-stranded DD was used.[13] Both orientations were observed for the TI in our regenerants. Forward and reverse insertions have also been identified in rice.[13]

The accurate replacement of DNA fragments is vital for gene editing. Co-expressing two sgRNAs can lead to fragment deletion in protoplasts.[23] Therefore, two sgRNAs can be designed at both ends of an exon for exon replacement by NHEJ.[14] To aid in TI and DNA replacement, we designed two sgRNAs (L1 and L2) based on both sides of the complementary strands of the original target site (E). These sgRNAs could form a combination of RNPs, including tail-to-head (L1+L2), tail-to-tail (L1+E), or head-to-head (E+L2) orientation (Figure 3A). The ssODN DDs were all located in the same strand of target site E (Figure 3B). In L1+L2 and E+L2, the sequence of ssODN contained 17 bp complementary to L2 sgRNA, which caused the L2 RNP to show reduced efficiency for generating DSBs (Figure S3). The notion that the strand of DD complementary to the target site can reduce RNP activity was demonstrated by experiments using L1 and L2 with complementary ssODN. These results are different from those reported for human cell lines.[32] In human cells, both strands of DD can be used for gene editing. The L1+E experiment had a higher fragment deletion rate than the others (Figure 3C). Except for a decrease in E+L2, the overall TI efficiency was similar to that using a single RNP (Figure 2C, Exp. 2 and 3). Compared with the use of E RNP only, there was a decline in the TI/Edited ratio when two sgRNA RNPs were co-transfected into protoplasts (Figure 3D). Therefore, we conclude that using dual RNPs does not increase TI efficiency. Based on the insertion sequences, all of the TI regenerants contained the NHEJ insertion (Tables S4, S5, and S6).

To determine whether these insertions were heritable, we analyzed the progeny of five *N. benthamiana* regenerants (Figure S4A). We genotyped TI regenerant T_1 seedlings and determined that all TI alleles were inherited (Figure S4B, C). Thus, these protoplast regenerants were not chimeric at the target gene. By contrast, in rice, most T_0 plants appeared to be chimeric.[13] In the current study, no *Cas9* gene was present in the genomes of the regenerants because we used RNP as the CRISPR reagent and therefore no new edited alleles were generated.

We also examined the TI efficiency in RCBO, targeting *BoSnRK1* and *BoGA4.a*[35] (Figure 4). RCBO contains two *BoSnRK1* genes: *BoSnRK1a* and *BoSnRK1b* (Figure 4A). DD was inserted into the target sites (Figure 4B, C, Tables S7 and S8). Sequencing indicated that DD was inserted at an efficiency of 4.5–13.6% (Figure 4C), which is lower than that demonstrated in *N. benthamiana*.

Although we identified an off-target DD insertion in *BoGA4.b* in RCBO, no off-target DD insertion was detected in *N. benthamiana*. *N. benthamiana* contains two *NbPDS* genes, Niben101Scf01283g02002.1 (*NbPDS1*) and Niben101Scf14708g00023.1 (*NbPDS2*), and the sgRNA matched *PDS1* but not *PDS2*. The *PDS2* genes in the regenerants were analyzed, revealing that no off-target mutagenesis or TI occurred. These results indicate that the off-target DD insertion that occurred in RCBO *BoGA4.b* was caused by off-target DSBs that provided an opportunity for DD insertion.

Since NHEJ is the main mechanism for TI, we reasoned that the presence of a homologous arm on the DD designed for HDR might not be necessary. To investigate whether ssODN DD with a non-homologous arm could be used for TI, we co-transfected RCBO protoplasts with *BoGA4.a* sgRNA RNP and *NbPDS1* DD. TI was observed in *BoGA4.a* in only 4.2% of the 24 regenerants (1/24), and none was observed in *BoGA4.b*, even though the editing efficiency was 95.8% (Figure 4C, Table S9). These results indicate that the presence of a homologous arm is not absolutely necessary for TI, but affects the efficiency of this process.

Finally, to explore the off-target DD insertion that occurred in the presence of DSBs without homologous sequences, we performed whole-genome sequencing of three types of plants, in which the regenerant *NbPDS1* gene was (1) the same as the WT, (2) a heterozygous mutant (KO) but without TI, or (3) a bi-allelic TI (TI; Figure 1). The ssODN insertion did not occur in the WT or KO genome. In the genome of the TI regenerant, the DD insertion occurred only in the DSB position created by the CRISPR-Cas RNP. By contrast, in rice that had been transformed using DNA CRISPR-Cas reagent via the biolistic method, quantitative PCR revealed multiple copies (2–10 per plant) of the donor inserts in T_1 plants, which suggested that frequent off-target DD insertion occurred in addition to the intended target site insertions.[13]

Conclusion

In this study, we used protoplast regeneration, RNP, and ssODN to establish a simple and inexpensive DNA TI system that can be used in *N. benthamiana* and RCBO. A synthesized 40-nt ssODN can be used as DD directly without the need for plasmid construction, and the PEG-mediated protoplast transfection method can provide a large amount of DD for TI. In addition, the plant material can be treated during protoplast isolation to increase the proportion of cells in S phase. In stable

transformation systems, the expression of Cas protein is important for knock in,[3] but in this study, we used the RNP as the CRISPR reagent to avoid low expression, and TI occurred after CRISPR-Cas-created DSBs had formed. Thus, this insertion method should be applicable to any gene target site in any plant species that can be regenerated from protoplasts. Because the TI efficiency is high, TI regenerants can be obtained without the need for antibiotic selection or phenotypic screening. Similar to other protocols,[13, 14, 36] the major mechanism of TI in this study was NHEJ. Although the HDR ratio was low, we still obtained a precise insertion regenerant.

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Author Disclosure Statement

No competing financial interests exist.

Author contributions

CSL, YCL, SL, MCS, and JS conceived and designed the experiments. CTH, QWC, and YHY performed the CRISPR-Cas9 experiments. SL performed SpCas9 protein purification. CTH, QWC, YHY, and CSL conducted the protoplast regeneration. CTH, QWC, YHY, and FHW performed the molecular biology experiments and targeted mutagenesis analysis. YCL performed whole genome sequencing and bioinformatics analysis. YCL, MCS, JS, and CSL wrote the manuscript with input from all co-authors. All authors read and approved the final manuscript.

References

1. Čermák T, Baltes NJ, Čegan R, Zhang Y, Voytas DF. High-frequency, precise modification of the tomato genome. *Genome Biol* 2015; **16**: 232.
2. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, *et al.* Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiol* 2015;**169**: 931–945.
3. Endo M, Mikami M, Toki S. Biallelic gene targeting in rice. *Plant Physiol*. 2016; **170**, 667–677.
4. Sauer NJ, Narváez-Vásquez J, Mozoruk J, Miller RB, Warburg ZJ, *et al.* Oligonucleotide-mediated genome editing provides precision and function to engineered nucleases and antibiotics in plants. *Plant Physiol* 2016; **170**, 1917–1928.
5. Dahan-Meir T, Filler-Hayut S, Melamed-Bessudo C, Bocobza S, Czosnek H, Aharoni A, Levy AA. Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system *Plant J* 2018; **95,** 5-16.
6. Miki D, Zhang W, Zeng W, Feng Z, Zhu JK. CRISPR/Cas9-mediated gene targeting in *Arabidopsis* using sequential transformation. *Nat. Commun.* 2018; **9**, 1967.
7. Wolter F, Klemm J, Puchta H. Efficient in planta gene targeting in *Arabidopsis* using egg cell-specific expression of the Cas9 nuclease of *Staphylococcus aureus*. *Plant J* 2018; **94**, 735-746.
8. Wolter F, Puchta H. In planta gene targeting can be enhanced by the use of CRISPR/Cas12a. *Plant J* 2019; **100,** 1083-1094.
9. Li S, Li J, He Y, Xu M, Zhang J, Du W, Zhao Y, Xia L. Precise gene replacement in rice by RNA transcript-templated homologous recombination. *Nat. Biotechnol.* 2019; **37**, 445-450.
10. Vu TV, Sivankalyani V, Kim EJ, Doan DTH, Tran MT, *et al.* Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. *Plant Biotechnol J* 2020; **18**, 2166-2143.
11. Ali Z, Shami A, Sedeek K, Kamel R, Alhabsi A, *et al.* Fusion of the Cas9 endonuclease and the VirD2 relaxase facilitates homology□directed repair for precise genome engineering in rice. *Commun Biol* 2020; **3**, 44.
12. Schindele A, Dorn A, Puchta H. CRISPR/Cas brings plant biology and breeding into the fast lane. *Curr Opin Biotechnol* 2020; **61**, 7-14.
13. Lu Y, Tian Y, Shen R, Yao Q, Wang M, *et al.* Targeted, efficient sequence insertion and replacement in rice. *Nat Biotechnol* 2020; 38, 1402–1407.
14. Li JY, Meng X, Zong Y, Chen K, Zhang H, *et al.* Gene replacements and insertions in rice by intron targeting using CRISPR–Cas9. *Nat. Plants* 2016; **2,** 16139.
15. Weinthal D, Tovkach A, Zeevi V, Tzfira T. Genome editing in plant cells by zinc finger nucleases. *Trends Plant Sci* 2010; **15**, 308–321.

16. Fauser F, Roth N, Pacher M, Ilg G, Sánchez-Fernández R, Biesgen C, Puchta H. In planta gene targeting *Proc Natl Acad Sci USA*, 2012; **109,** 7535-7540.

17. Zhang Y, Zhang F, Li X, Baller JA, Qi Y, *et al.* Transcription activator-like effector nucleases enable efficient plant genome engineering. *Plant Physiol* 2013; **161**, 20–27.

18. Krens FA, Molendijk L, Wullems GJ, Schilperoort RA. *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. *Nature* 1982; **296**: 72–74.

19. Yoo SD, Cho YH, Sheen J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* 2007; **2,** 1565-1572.

20. Li J, Stoddard TJ, Demorest ZL, Lavoie PO, Luo S, *et al.* Multiplexed, targeted gene editing in *Nicotiana benthamiana* for glyco-engineering and monoclonal antibody production. *Plant Biotechnol J* 2016; **14**, 533–542.

21. Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, *et al.* DNA-free genome editing in plants with preassembled CRISPR–Cas9 ribonucleoproteins. *Nat Biotechnol* 2015; **33**, 1162–1164.

22. Andersson M, Turesson H, Nicolia A, Fält AS, Samuelsson M, Hofvander P. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep* 2017; **36**, 117–128.

23. Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, *et al.* Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. *Plant Biotechnol. J.* 2018; **16**, 1295–1310.

24. Hsu CT, Cheng YJ, Yuan YH, Hung WF, Cheng QW, *et al.* Application of Cas12a and nCas9-activation-induced cytidine deaminase for genome editing and as a non-sexual strategy to generate homozygous/multiplex edited plants in the allotetraploid genome of tobacco. *Plant Mol Biol* 2019; **101**, 355–371.

25. Park SC, Park S, Jeong YJ et al. DNA-free mutagenesis of *GIGANTEA* in *Brassica oleracea* var. capitata using CRISPR/Cas9 ribonucleoprotein complexes. *Plant Biotechnol Rep* 2019; **13**, 483–489.

26. Yu J, Tu L, Subburaj S, Bae S, Lee GJ. Simultaneous targeting of duplicated genes in *Petunia* protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Rep* 2020; doi: 10.1007/s00299-020-02593-1.

27. De Bruyn C, Ruttink T, Eeckhaut T, Jacobs T, De Keyser E, Goossens A, et al. Establishment of CRISPR/Cas9 Genome Editing in Witloof (*Cichorium intybus* var. foliosum). *Front Genome Ed*, 2021; 2, 24

28. Hsu CT, Lee WC, Cheng YJ, Yuan YH, Wu FH, Lin CS. Genome editing and protoplast regeneration to study plant–pathogen interactions in the model plant *Nicotiana benthamiana*. *Front. Genome Ed.*, 2021; **2**, 39.

29. Huang RS, Shih HA, Lai MC, Chang YJ, Lin S. Enhanced NK-92 Cytotoxicity by CRISPR genome engineering using Cas9 ribonucleoproteins. *Front Immunol* 2020; **11**, 1008.

30. Hiom K. Coping with DNA double strand breaks. *DNA Repair* 2010; **9**,1256–1263

31. Puchta H, Fauser F. Synthetic nucleases for genome engineering in plants: prospects for a bright future. *Plant J* 2014; **78**, 727–741.

32. Lin S, Staahl BT, Alla RK Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* 2014; **3**, e04766.
33. Kotogány E, Dudits D, Horváth GV, Ayaydin F. A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. *Plant Methods* 2010; **6**:5.
34. Kaya H, Mikami M, Endo A, Endo M, Toki S. Highly specific targeted mutagenesis in plants using *Staphylococcus aureus* Cas9. *Sci Rep* 2016; **6**, 26871.
35. Lawrenson T, Shorinola O, Stacey N, *et al.* Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol* 2015; **16**, 258.
36. Dong OX, Yu S, Jain R, Zhang N, Duong PQ, *et al.* Marker-free carotenoid-enriched rice generated through targeted gene insertion using CRISPR-Cas9. *Nat Commun* 2020; **11,** 1178.

Figure legends

Figure 1. Strategy for targeted DNA insertion using ribonucleoprotein and single-stranded oligonucleotide DNA in *Nicotiana benthamiana*. (A) Ribonucleoprotein (RNP) and single-stranded oligonucleotide DNA (ssODN) are delivered to S-phase protoplasts by polyethylene glycol–mediated transfection (Figure S1). (B) Transfected protoplasts are regenerated. (C) DNA from the regenerants is amplified by the polymerase chain reaction and genotyped using restriction enzymes or RNP. (D) DNA is purified from targeted insertion (red), knockout (green), and non-edited (yellow) regenerants and sequenced to determine whether ssODN was inserted into other positions. (E) Offspring are genotyped and sequenced to test whether the inserted sequence is heritable.

Figure 2. Effect of 40-nt single-stranded oligonucleotide DNA donor DNA homology arm length, the inserted sequence, and the modified protospacer adjacent motif sequence on targeted insertion into *NbPDS1* in *Nicotiana benthamiana* regenerants. (A) Donor sequences used in Exp. 1–3. Lowercase: insertion or replacement nucleotides. The protospacer adjacent motif (PAM) is underlined. (B) Polymerase chain reaction (PCR) of the target gene in the regenerated plants was performed in each experiment, and different restriction enzymes were used for genotyping. (black: *Bst*NI, Edited; red: *Hind*III, targeted insertion). M: marker. -: wild-type control. +: restriction enzyme control. Targeted insertion PCR product sequences are shown in Tables S1–S3. (C) Edited and targeted insertion efficiencies of different ssODN donors. T: total length of DD. I: insertion. H: homologous arm. L: left arm. R. right arm. R. E.: restriction enzyme.

Figure 3. Targeted insertion using two ribonucleoproteins in *Nicotiana benthamiana*. (A) Relative positions and directions of the three ribonucleoproteins (RNPs) used. The target site is shown below. The protospacer adjacent motif is underlined. (B) Donor DNA (DD) used in each combination. Lowercase: inserted

*Eco*RI site. (C) Restriction enzyme analysis of the target efficiency of regenerants derived from different RNP combinations and DD transfected protoplasts. TI: targeted insertion. (D) Summary of edited and TI efficiencies of RNP and single-stranded oligonucleotide DNA. R.E.: restriction enzyme.

Figure 4. Targeted insertion using single-stranded oligonucleotide DNA donor DNA in rapid cycling *Brassica oleracea*. (A) Target site of *BoSnRK1* and the donor sequence. The protospacer adjacent motif is underlined. Lowercase: insertion sequence. (B) Genotyping of the regenerants derived from ribonucleoprotein (RNP) and single-stranded oligonucleotide DNA (ssODN) transfected protoplasts. The edited efficiency by RNP was assessed. Targeted insertion was assessed using the *Eco*RI site inserted by the ssODN. (C) Summary of edited and targeted insertion efficiencies. R.E.: restriction enzyme site inserted by ssODN. HA: homologous arm. TI: targeted insertion.

Supplementary Material

Figure S1. 5-ethynyl-2'-deoxyuridine (EdU) staining of different materials. (A) *N. benthamiana* leaves were cut into strips, placed in 1N0.3K (1/2 MS, 0.4 M mannitol, 1 mg/L NAA, 0.3 mg/L kinetin), and incubated for 0, 24, 48, or 72 hours. The incubated leaves and the protoplasts derived from these leaves were stained with EdU. *N. benthamiana* protoplasts derived from BY2 cells during cell division were used as a positive control. Bar = 50 μm. (B) Protoplasts obtained using different incubation media (1/2 MS, 0.4 M mannitol 1N0.3K) were stained with EdU. Bar = 50 μm. (C) Protoplasts obtained using different incubation media were subjected to targeted insertion (TI) using RNP (target sequence: TTGCGATGCCTAACAAGCCAG) and donor DNA (TGCGATGCCTAACAAGCaagcttCAGGGGAGTTCAGCCGC). *NbPDS1* was subjected to PCR and analyzed by digestion with *Bst*NI (for Edited) and *Hind*III (for TI). M: DNA marker. %: the efficiency of Edited (black) and TI (red).

Figure S2. Effect of homologous arm length and total length of the single-stranded oligonucleotide DNA donor DNA on targeted insertion in *NbPDS1* in the *N. benthamiana* regenerant. (A) Sequences of donor DNA (DD). The protospacer adjacent motif (PAM) is underlined. Lower case: restriction enzyme site. (B) Protoplasts transfected with different lengths of single-stranded oligonucleotide DNA (ssODN). Regenerant genotypes were determined. PCR of the target gene *NbPDS1* was performed, and the PCR product was analyzed by digestion with *Bst*NI (for Edited) and *Hind*III (for TI). (C) Editing and TI efficiency for different lengths of ssODN. T: total length. I: insertion. H: homologous arm. L: left arm. R: right arm. R. E.: restriction enzyme. (D) Results of Sanger sequencing of +27#6, the precise *Hind*III insertion regenerant (blue background). (E) BLASTN results of +27#6 *NbPDS1* (Query) and wild-type *NbPDS1* (Sbjct).

Figure S3. Editing efficiencies of regenerants from Exp. 1–3 assessed using restriction enzyme digestion and ribonucleoprotein.

Figure S4. Progeny of targeted insertion regenerants in *N. benthamiana*. (A) DNA was isolated from 21 progeny seedlings from Exp. 3#7 and the target gene *NbPDS1* was amplified by PCR. The products were digested using two restriction enzymes (*Nhe*I and *Bam*HI), for which sites were added in the donor single-sranded oligonucleotide DNA (ssODN). (B) Summary of the five targeted insertion (TI) regenerants in the T_1 progeny. The genotype was determined as described in (A). (C) Summary of sequences of the TI alleles. Detailed sequence information for each regenerant is shown in Table S3 (Exp.3#7) and Table S5 (L1+E#2, 6, 9, and 10).

Table S1. Exp. 1 sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S2. Exp. 2 sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. –number: deletion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S3. Exp. 3 sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. –number: deletion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S4. L1+L2 sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. –number: deletion length. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S5. L1+E sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

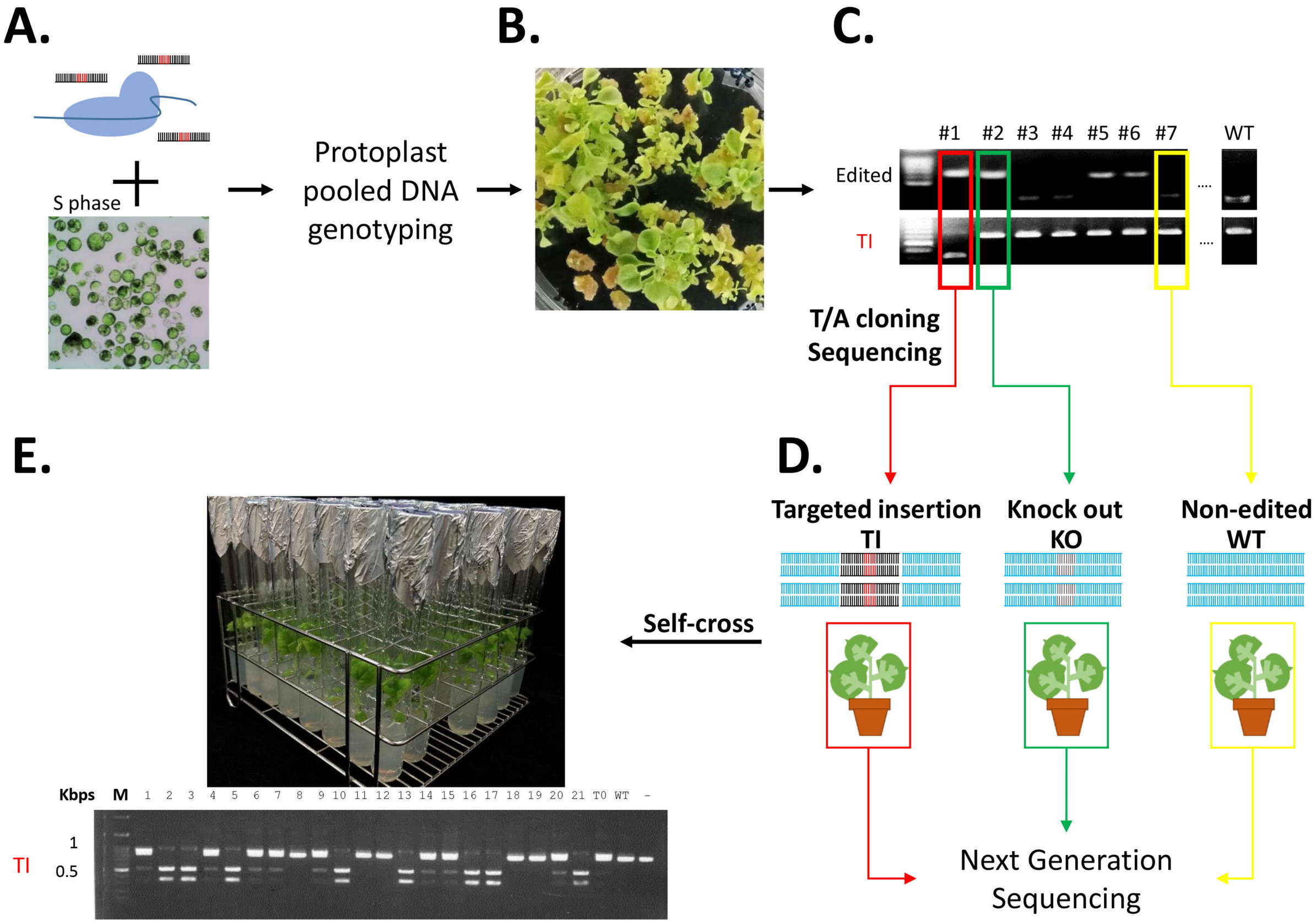
Table S6. E+L2 sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S7. *BoSnRK1* sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S8. *BoGA4.a* sequences. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

Table S9. *BoGA4.a* sequences of the RCBO TI regenerants by using *N. benthamiana NbPDS1* donor DNA. (A) Summary of the TI regenerants. Underlined: PAM. +number: insertion length. –number: deletion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orientation column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underlined: PAM.

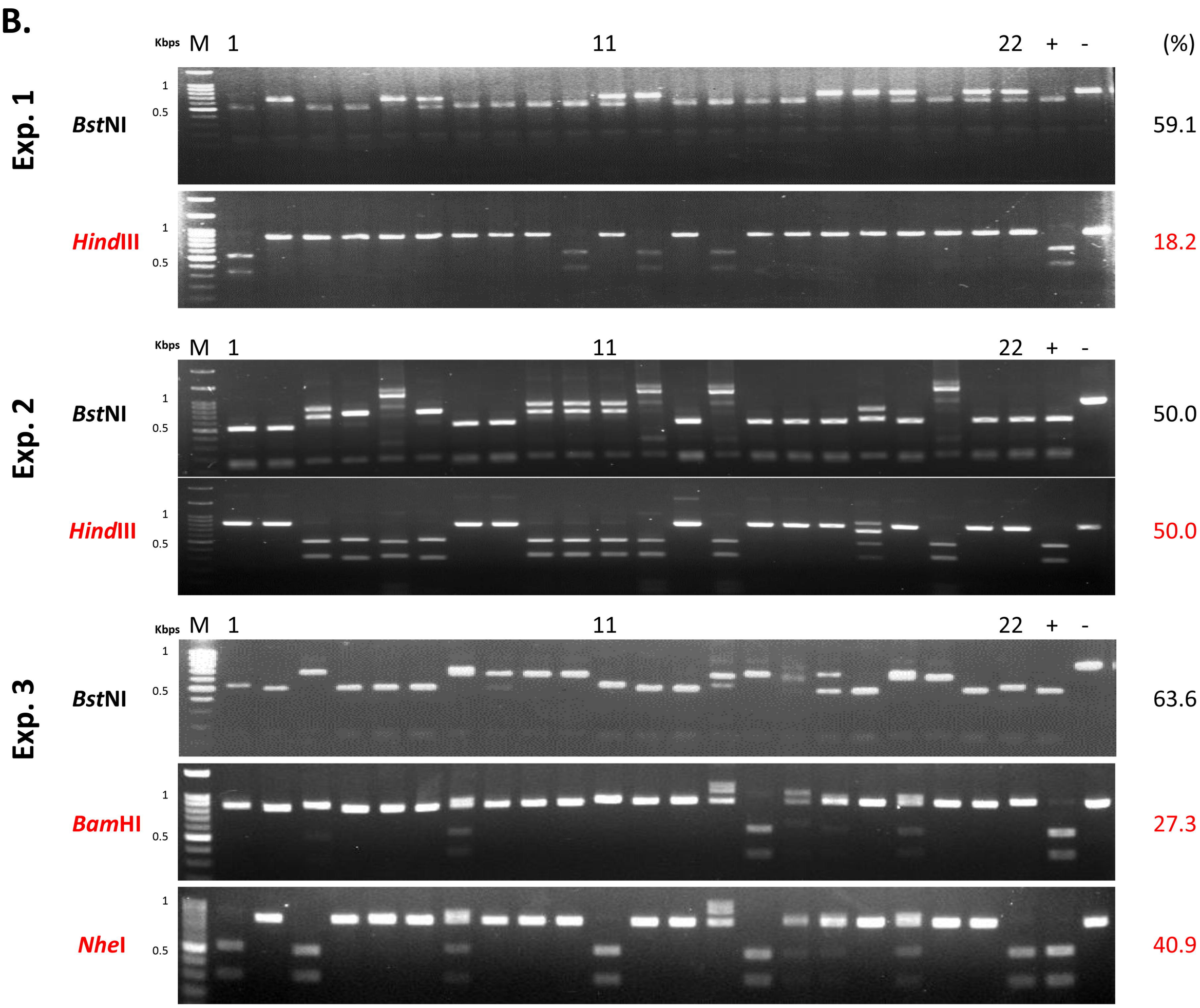
Table S10. Primer information.


A.
S phase
Protoplast
pooled DNA
genotyping
B.
C.
#1 #2 #3 #4 #5 #6 #7 WT
Edited
TI
T/A cloning
Sequencing
D.
Targeted insertion
TI
Knock out
KO
Non-edited
WT
Self-cross
Next Generation
Sequencing
E.
Kbps M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 T0 WT -
1
0.5
TI

A.

WT	TTTGCGATGCCTAACAAGC------CAGGGGAGTTCAGCCGCTT
Donor DNA sequences	
Exp. 1	TTTGCGATGCCTAACAAGCtt----CAGGGGAGTTCAGCCGCTT
Exp. 2	TTTGCGATGCCTAACAAGCtttttCAtttGAGTTCAGCCGCTT
Exp. 3	ATTTGCGATGCgctagcggatcc------tttAGTTCAGCCGCTTT

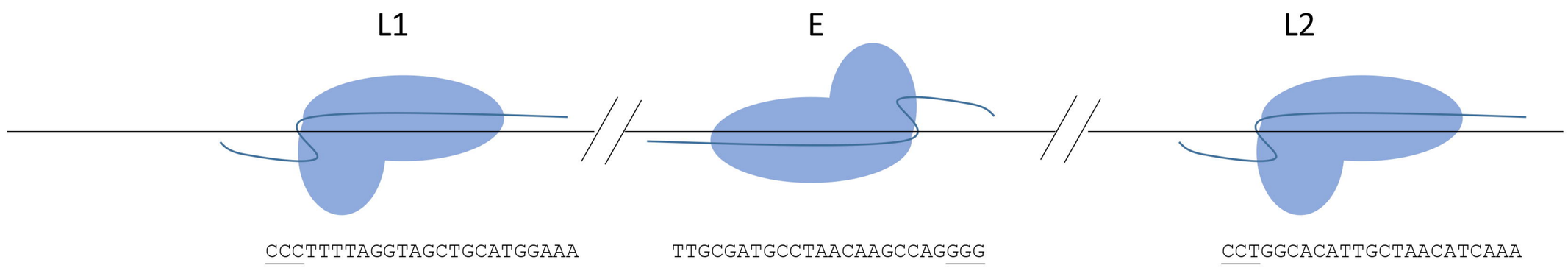
B.



C.

Exp	Donor					Efficiency (%)	
	Length (nt)				R.E.	Regenerates	
	T	I	H			Edited (%)	TI (%)
			L	R			
1	40	2	19	19	*Hind*III	59.1 (13/22)	18.2 (4/22)
2	44	11	19	14	*Hind*III	50.0 (11/22)	50.0 (11/22)
3	40	15	11	14	*Bam*HI, *Nhe*I	63.6 (14/22)	40.9 (9/22)

A.



B.

WT TACTTGTACCTCCCTTTTAGGTAGCTGCATGGAAA-(345)-TTGCGATGCCTAACAAGCCAGGGGAGTTCAGCCGC-(126)-CCTGGCACATTGCTAACATCAAA

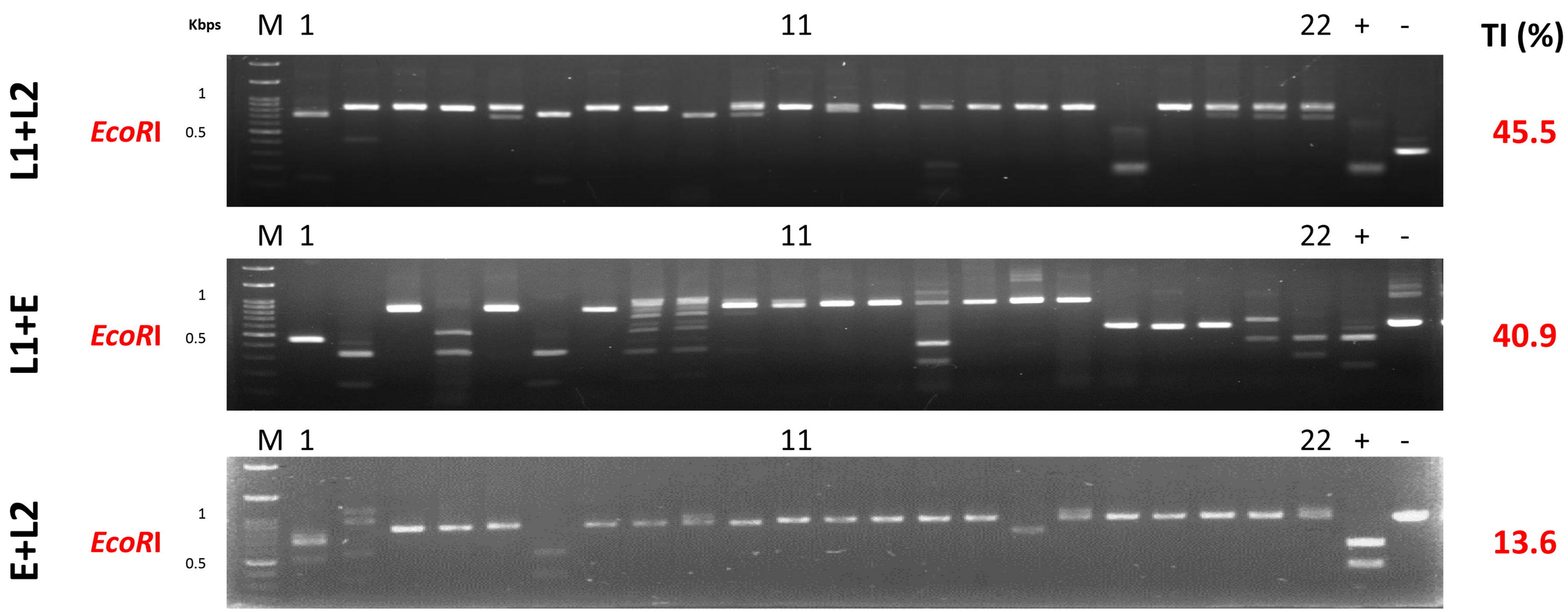
Donor DNA sequences

L1+L2 TACTTGTACCTCCCTTTgaattc----------ACATTGCTAACATCAAA

L1+E TACTTGTACCTCCCTTTgaattc----------CAGGGGAGTTCAGCCGC

E+L2 TGCGATGCCTAACAAGC-gaattc----------ACATTGCTAACATCAAA

C.



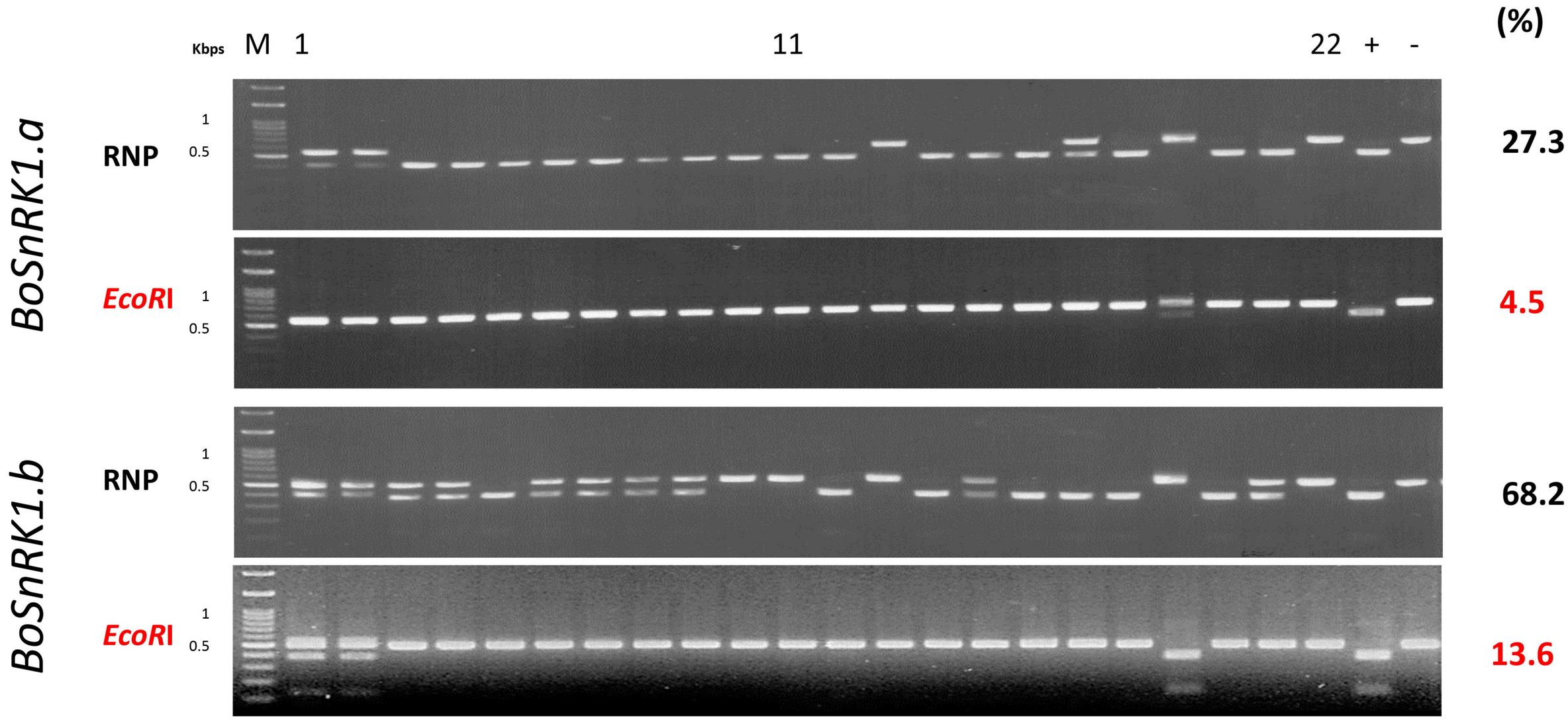
D.

Target site	Donor		Efficiency (%)				
	Length (nt)	R.E.	Regenerates				
			Edited				TI
L1+L2	40	*Eco*RI	L1	72.7 (16/22)	L2	13.6 (3/22)	45.5 (10/22)
L1+E	40	*Eco*RI	L1	81.8 (18/22)	E	72.7 (16/22)	40.9 (9/22)
E+L2	40	*Eco*RI	E	63.6 (14/22)	L2	4.5 (1/22)	13.6 (3/22)

A.

WT	*BoSnRK1.a*	TCATAGAGACGGATGAT------GTGAGGATGCATAAAGAGTCTCAAGATT
	BoSnRK1.b	TCATAGAGACGGATGAT------GTGAGGATGCATAAAGAGACTCATGATT
Donor DNA sequences		
Donor DNA		TCATAGAGACGGATGATgaattcGTGAGGATGCATAAAGA

B.



C.

sgRNA	Donor		Efficiency (%)		
	HA gene	R.E.		Edited	TI
BoSnRK1	*BoSnRK1.ab*	*Eco*RI	*BoSnRK1.a*	27.3 (6/22)	4.5 (1/22)
			BoSnRK1.b	68.2 (15/22)	13.6 (3/22)
BoGA4.a	*BoGA4.a*	*Eco*RI	*BoGA4.a*	13.6 (3/22)	4.5 (1/22)
			BoGA4.b	18.2 (4/22)	9.1 (2/22)
	NbPDS1	*Eco*RI	*BoGA4.a*	95.8 (23/24)	4.2 (1/24)
			BoGA4.b	95.8 (23/24)	0.0 (0/24)