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A New Protocol for Targeted insertion using CRISPR-Cas9, Oligo Single-Stranded DNA 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

Summary

Precise insertion of DNA sequences into specific genome locations is essential for genome editing. Current Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)–CRISPR associated protein (Cas) protocols rely on homology-directed repair (HDR). These protocols require laborious vector construction and suffer from low efficiency. Oligo DNA can be used as donor DNA (DD) for precise DNA insertion, or targeted insertion (TI) via nonhomologous end joining (NHEJ) in many species. Here, we report a simple protocol that eliminates the need for expensive equipment and vector construction by using polyethylene glycol (PEG) to deliver non-modified synthetic single-stranded oligo DNA (ssODN) and CRISPR-Cas9 ribonucleoprotein (RNP) into protoplasts. Up to 50.0% targeted insertion was achieved in *Nicotiana*

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benthamiana and 13.6% in Rapid Cycling *Brassica oleracea* (RCBO) without antibiotic selection. Using 60 nt DD that contained 27 nt homologous arms, 6 out of 22 regenerated plants showed TI, and one of them had a precise insertion of 6 bp *Eco*RI (4.5%) in *N. benthamiana*. Based on whole-genome sequencing, DD inserted only in the double-strain break (DSB) positions that were induced by the CRISPR-Cas RNP. Importantly, the analysis of T1 progenies indicated that the TI sequences were successfully transmitted into the next generation.

Key words

Nicotiana benthamiana, Rapid Cycling *Brassica oleracea*, synthetic single-stranded oligo DNA, Ribonucleoprotein

Introduction

For DNA insertion at specific location, it is necessary to induce a DNA double-strand break (DSB) at the target position and then insert the donor DNA (DD) by DNA repair pathways: homology-directed repair (HDR) (Čermák *et al.*, 2015; Svitashev *et al.*, 2015; Endo *et al.*, 2016; Sauer *et al.*, 2016; Dahan-Meir *et al.*, 2018; Miki *et al.*, 2018; Wolter *et al.*, 2018; Wolter and Puchta 2019; Li *et al.*, 2019; Vu *et al.* 2020; Ali *et al.*, 2020; Schindele *et al.*, 2020) or nonhomologous end joining (NHEJ) (Lu *et al.*, 2020; Li *et al.*, 2016). There are many tools to create such DSBs (Weinthal *et al.*, 2010; Fauser *et al.*, 2012; Zhang *et al.* 2013). Because of its convenience and efficiency, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)–CRISPR associated protein (Cas) is now a favourite tool among genetic engineers (Sauer *et al.*, 2016). In addition to the CRISPR-Cas reagents, DDs need to be delivered into the target cells, and increasing the amount of delivered DDs enhances targeted insertion (TI) efficiency. For example, in maize, targeted mutagenesis using CRISPR has been achieved via different transformation 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 (Svitashev *et al.*, 2015). Protoplasts offer an alternative substrate for genetic transformation and transfection, since they allow delivery of high numbers of DNA copies (Krens *et al.* 1982; Yoo *et al.*, 2007) and DNA can insert into genome (Krens *et al.* 1982). As for the transcription activator-like effector nuclease genome editing system (Li *et al.*, 2016), CRISPR reagents [ribonucleoprotein (RNP) or plasmid DNA] can be delivered into protoplasts by polyethylene glycol (PEG), and targeted mutations can be achieved (Schindele *et al.*, 2020; Woo *et al.*, 2015; Andersson et al, 2017; Lin et al, 2018; Hsu *et al.*, 2019; Yu

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et al., 2020). 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 on to the progeny (Woo *et al.*, 2015; Lin et al, 2018; Hsu *et al.*, 2019). In this study, we describe a simple, high TI efficiency protoplast-based strategy for genome editing in plants using CRISPR, with no need for expensive equipment.

Results and Discussion

The protoplast regeneration protocol (Figure 1) used was modified from those previously published (Lin *et al.*, 2018; Hsu *et al.*, 2019). Key to our approach is the fact that the phase of the cell cycle largely governs the choice of pathway. NHEJ is the major DNA repair pathway in G1, S and G2 phases, whereas HDR occurs only in late S and G2 phases (Hiom, 2010; Puchta and Fauser, 2014). Cell cycle synchronization has been effective to enhance TI efficiency in the human embryonic kidney 293T cells (Li et al., 2014). To increase late S and G2 phases cells, tobacco leaves were incubated in solid medium containing ½-strength MS, 0.4 M mannitol, 1 mg/L naphthaleneacetic acid (NAA) and 0.3 mg/L kinetin (1N0.3K) for three days before protoplast isolation (Figure S1). In *N. benthamiana*, to simplify the procedure, we add 1 mg/L NAA and 0.3 mg/L kinetin to the digest solution for three days. That method was not suitable for Rapid Cycling *Brassica oleracea* (RCBO), however, because most of the cells were broken after digest.

To avoid constructing vectors and reduce the instability of sgRNA and Cas protein gene expression, we used a RNP as the CRISPR-Cas reagent. For the DD, we used short non-modified synthetic ssODN, which is relatively economical and easy to obtain. The RNP and ssODN were delivered into protoplasts using PEG transfection (Figure 1A). After three days of incubation in the 1N0.3K liquid medium, genomic DNA was isolated from the protoplasts and the target gene was amplified by PCR and cloned into T/A vector for Sanger sequencing to assess the TI efficiency. These protoplasts were cultured using a slightly modified version of the previously published protocol (Lin *et al.*, 2018; Hsu *et al.*, 2019; Figure 1B). 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 knock out (KO) and one non-edited (WT) regenerant were purified for genome-wide sequencing to assess the presence or absence of off-targeted insertions (Figure 1D). DNA from the TI T_1 progeny was extracted for

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genotyping to test whether the inserted fragment was heritable (Figure 1E).

To evaluate the effect of the length of homologous arms and total length of ssODN on TI efficiency, we conducted experiments using the *N. benthamiana PHYTOENE DESATURASE1* (*PDS1*) target site (Kaya *et al.*, 2016; Figure S2). At the expected sgRNA target position, we inserted an *Hind*III site, and on the left and right sides we added 7, 17, or 27 homologous arms to make the DD 20, 40, or 60 nt (Figure S2A). Regenerant DNA *PDS1* PCR products were genotyped, and the results indicated no TI regenerants with the 20 nt ssODN, whereas the 40 and 60 nt ssODN produced TI regeneration efficiencies of 27.3-31.8 % without antibiotic and phenotype selection (Figure S2B). One of the 60-nt TI regenerants (+27#6, Figure S2C, D) had a precise insertion of a 6-bp *Hind*III recognition site at the target position. This result indicates that HDR also occurred during protoplast regeneration. We hypothesize that length of ssODN donor is an important factor of TI efficiency. In rice, a 59-nt ssODN (ssADHE) gave rise to no successful insertion in 23 T0 plants (Lu *et al.*, 2020), although there was no homologous arm and lower DD concentration comparison with this study. Based on these results, except Exp. 2 (44 nt), we selected 40 nt as the length of the ssODN in our subsequent experiments.

Next, we determined the influence of insertion length on DD in 40 nt (Figure 2). In Experiment 1 (Exp. 1), the *Hind*III site was generated by a 2-nt insertion in the DD (Figure 2A), and TI efficiency was 18.2% in the regenerated plants (Figure 2B, C). In Exp. 2, the PAM position in DD was replaced, 6 nt was added, and the TI efficiency was increased to 50.0 %. When we tested increasing the insertion length to 15 nt (Exp. 3), we included sites for *Nhe*I and *Bam*HI endonucleases at the insertion, which enabled us to confirm the integrity of TI genotyping by restriction enzyme digest. As the insertion length was increased and homozygous arms reduced (11 and 14 nt), the TI efficiency decreased slightly (40.9 %). There were three regenerants that had only the *Nhe*I site, indicating partial DD insertion (Figure 2B). It is possible the that ssODN DD was unstable in protoplasts and had been partially degraded before insertion, which would cause the inserted sequence to be incomplete. The phosphorothioate-linkage modification DD was designed to stabilize the double-stranded DNA (Lu *et al.*, 2020). There was no phosphorothioate-linkage modification in the DD we used in this study, which may have allowed DD degradation during TI.

All of the *PDS1* genes of TI regenerants in Exp. 1, 2 and 3 were sequenced, revealing that all regenerants contained insertions generated by NHEJ (Table S1, 2, and 3). 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 to 445 bp. These differences were caused by

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ssODN 1-13 repeat insertions, although some of the repeats were incomplete. In rice, only 20% of TI plants had repeat insertion when using modified double-strain DD (Lu *et al.*, 2020). Both orientations were observed for the TI in our regenerants. There were also forward and reverse insertions identified in rice (Lu *et al.*, 2020).

For gene editing, accurate replacement of DNA fragments is vital. Co-expression two sgRNAs can cause fragment deletion in protoplasts (Lin *et al.*, 2018). Therefore, two sgRNAs can be designed at both ends of an exon for exon replacement by NHEJ (Li et al., 2016). To aid in TI and DNA replacement, we designed two sgRNAs (L1 and L2), on both sides of the complementary strands of the original target site (E). These could form a combination of RNPs, including tail to head (L1+L2), tail to tail (L1+E), head to head (E+L2) (Figure 3A). The ssODN DD were all in the same strand of target site E (Figure 3B). In L1+L2 and E+L2, the sequence of ssODN had 17 bp complementary to L2 sgRNA, which caused the L2 RNP to reduce DSB efficiency (Figure S3). The target site complementary strain DD can reduce the RNP function is demonstrated by experiments using L1 and L2 with complementary ssODN. These results are different from those in human cell lines (Lin *et al.*, 2014). Both strain DD can be used for gene editing. The L1+E experiment had a higher fragment deletion rate (Figure 3C). Except for a decrease in E+L2, the overall TI efficiency was similar to that with a single RNP (Figure 2C, Exp. 2 and 3). Compared with the E RNP only, there was a decline in the TI/Edited ratio when two sgRNA RNPs were co-transfected (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 (Table S4, 5, and 6).

To determine if these insertions were heritable, the progeny of five *N. benthamiana* regenerants were analyzed (Figure S4A). TI regenerant T_1 seedlings were genotyped and the results indicated that all TI alleles were inherited (Figure S4B, C). Thus, these protoplast regenerants were not chimeric at the target gene. In rice, most T_0 plants appeared to be chimeric (Lu *et al.*, 2020). In our study, there was no *Cas9* gene in the genomes because the RNP was used as the CRISPR reagent, so no new edited alleles were generated.

We also examined TI efficiency in RCBO, targeting *SnRK1* and *GA4.a* (Figure 4). In RCBO, there are two *SnRK1* genes: *SnRK1a* and *SnRK1b* (Figure 4A). DD was inserted into the target sites (Figure 4B, C, Table S7 and 8). Sequencing indicated that DD was inserted with an efficiency of 4.5–13.6 % (Figure 4C, D), which is lower than that demonstrated in tobacco.

Although we identified an off-targeted insertion in *GA4.b* in RCBO, no off-

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targeted insertion was observed in *N. benthamiana*. There are two *N. benthamiana PDS* genes, Niben101Scf01283g02002.1 (*PDS1*) and Niben101Scf14708g00023.1 (*PDS2*), and the sgRNA matches *PDS1*, but not *PDS2*. The *PDS2* genes in the regenerants were genotyped, revealing that no off-targeted mutagenesis or TI occurred. This result indicates that off-target TI which occurred in RCBO *GA4.b* is caused by off-target DBSs that provide an opportunity for DD insertion.

Since NHEJ is the main mechanism for TI, the homologous arm on the DD designed for HDR use may not be necessary. To understand whether non-homozygous arm ssODN DD could be used for TI, we used *BoGA4.a* sgRNA RNP and *NbPDS* DD to co-transfect RCBO protoplasts. Among 24 regenerants, only 4.2% (1/24) TI was observed in *BoGA4.a* but none in *GA4.b* even the edited efficiency is 95.8% (Figure 4C, Table S9). This result indicates that the homologous arm is not absolutely necessary in TI, but it affects the efficiency of TI.

To understand the off-targeted insertion that occurred without homozygous sequence DSBs, we performed whole-genome sequencing of three types of plants, in which the regenerant *PDS1* gene was (1) the same as wild-type (WT), (2) heterozygous mutant (KO) but without TI or (3) bi-allelic TI (TI; Figure 1). The results indicate that ssODN insertion did not occur in the WT and 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 which used DNA CRISPR-Cas reagent via biolistic method, qPCR revealed multiple copies (2–10 copies per plant) of the donor inserts in T1 plants, which suggested frequent off-targeted insertions besides the intended target site insertions (Lu *et al.*, 2020).

In this study, we used protoplast regeneration, RNP, and ssODN to establish a simple and cheap DNA TI system that can be used in *N. benthamiana* and RCBO. Synthesized 40 nt ssODN can be used as DD directly without 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, the expression of Cas protein is important for knock in (Endo *et al.*, 2016; Miki *et al.*, 2018), but in this study we used the RNP as the CRISPR reagent and the TI occurred after CRISPR-Cas-created DSBs were formed. Thus, this insertion method should be applicable to any gene target site in any plant species which can regenerate from protoplast. Because the TI efficiency is high, TI regenerants can be obtained without antibiotic selection and phenotype screening. Similar to other protocols (Lu *et al.*, 2020; Dong *et al.*, 2020), the major mechanism of TI in this study is NHEJ. Although the HDR ratio is low, we did still obtain regenerant via HDR. In the future, we plan to modify this

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protocol based on understanding of the plant HDR mechanism or using TR-HDR (Lu *et al.*, 2020) to perform precise genome editing.

Methods

Protoplast isolation, transfection, and regeneration

N. benthamiana and RCBO were grown and propagated in ½ MS medium supplemented with 30 g/L sucrose under a 12/12 h light/dark cycle at 25 °C. Protoplast isolation was performed according to Lin *et al.* (2018), except that the digestion solution and digestion time were as described above, and RNP and 50 µg ssODN co-transfection according to Woo *et al.* (2015). 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). Before incubation in liquid shooting medium, RCBO calli were incubated in 1 mg/NAA, 1 mg/L 6-benzyladenine (BA) and 0.25 mg/L 2,4-dichlorophenoxyacetic acid for three days in the dark. After 3-week incubation in the dark, calli were incubated in liquid shooting medium (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 light (16/8 h light/dark, 3000 lux). Green explants larger than 5 mm were incubated in solid shoot medium and subcultured every 4 weeks. Shoot clusters with leaves were then transferred to solidified root medium (HB1: 3 g/L HYPONeX, 2 g/L tryptone, 20g/L sucrose, 1 g/L activated charcoal, 2.2 g/L gelrite, pH5.2).

Cas9 protein purification, sgRNA synthesis, and Cas9 RNP Nucleofection

Preparation of Cas9 Protein and sgRNA and Cas9 RNP nucleofection were performed according to Huang *et al.* (2020). Cas9 recombinant protein was over-expressed in *E. coli* BL21 from 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) by using T7 RNA polymerase (New England Biolabs; M0251L). The DNA oligonucleotides for IVT template assembly were listed in Table S10. The final sgRNA products were dissolved in Cas9 RNP buffer, quantitated by 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 of Cas9 protein and 88.3 µM of sgRNA at molar ratio of 1: 2.2 and incubating at 37°C for 60 min.

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Validation of targeted insertion in protoplasts and regenerants

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

Whole genome sequencing for off-targeted insertion analysis

Leaves of *N. benthamiana* protoplast regenerants were collected for Genomic DNA purification. Genomic DNA for genome sequencing was extracted using a Plant DNA Purification Kit (DP320, Tiangen, http://www.tiangen.com/en/). Paired-end libraries of DNA were constructed by the NEBNext Ultra DNA Library Prep Kit for Illumina with 2 × 150 bp with average insert size ~900 bp and sequenced on a NovaSeq 6000 platform. Three technical replicates were performed for each sample. Total reads were 120 Gbp per regenerant and the sequencing depth was more than 30×. High-quality Illumina reads were converted to fasta format files and used as a BLAST database for target sequence searches. Genomic sequence of *PDS* genes (Niben101Scf01283, *PDS1* and Niben101Scf14708, *PDS2*) were used as the query in a high 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). Only reads within ± 150 bp of the candidate sequence region (Exp. 1 DD sequence, TTTGCGATGCCTAACAAGCTTCAGGGGGAGTTCAGCCGCTT) were retained for the local multiple sequence alignment. If the query sequence was identical to the published sequence on the WT genome, it was concluded that these same sequences as 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-targeted insertion. The raw reads were deposited in the NCBI SRA database (BioProject: PRJNA667297).

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Competing interests

The authors declare that they have no competing interests.

Author contributions

CSL, 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,

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and FHW performed the molecular biology experiments and targeted mutagenesis analysis. YCL performed bioinformatics analysis. YCL, MCS, JS, and CSL wrote the manuscript with input from all co-authors. All authors read and approved the final manuscript.

Figure legends

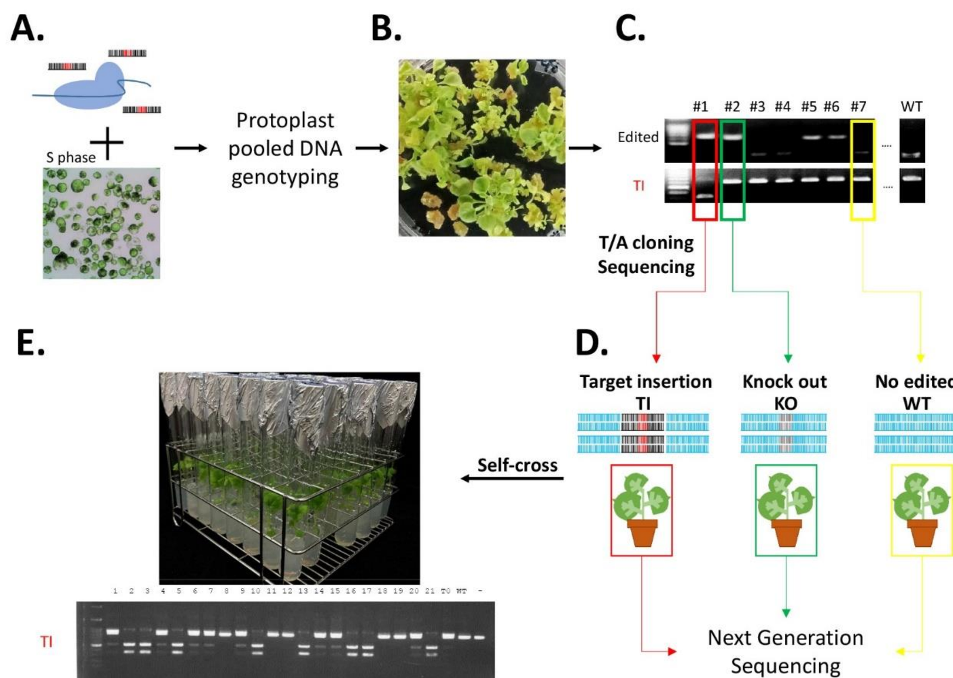

Figure 1. Strategy for *N. benthamiana* targeted DNA insertion using RNP and ssODN. (A). RNP and ssODN were delivered to S phase protoplasts by PEG method (Figure S1). (B) Transfected protoplasts were regenerated. (C) DNA from the regenerants was PCR amplified and genotyped using restriction enzymes or RNP. (D) Targeted insertion (red), knock out (green), and non-edited regenerant (yellow) DNA was purified and sequenced to determine whether ssODN was inserted in other positions. (E) Offspring were genotyped and sequenced to test whether the inserted sequence was heritable.

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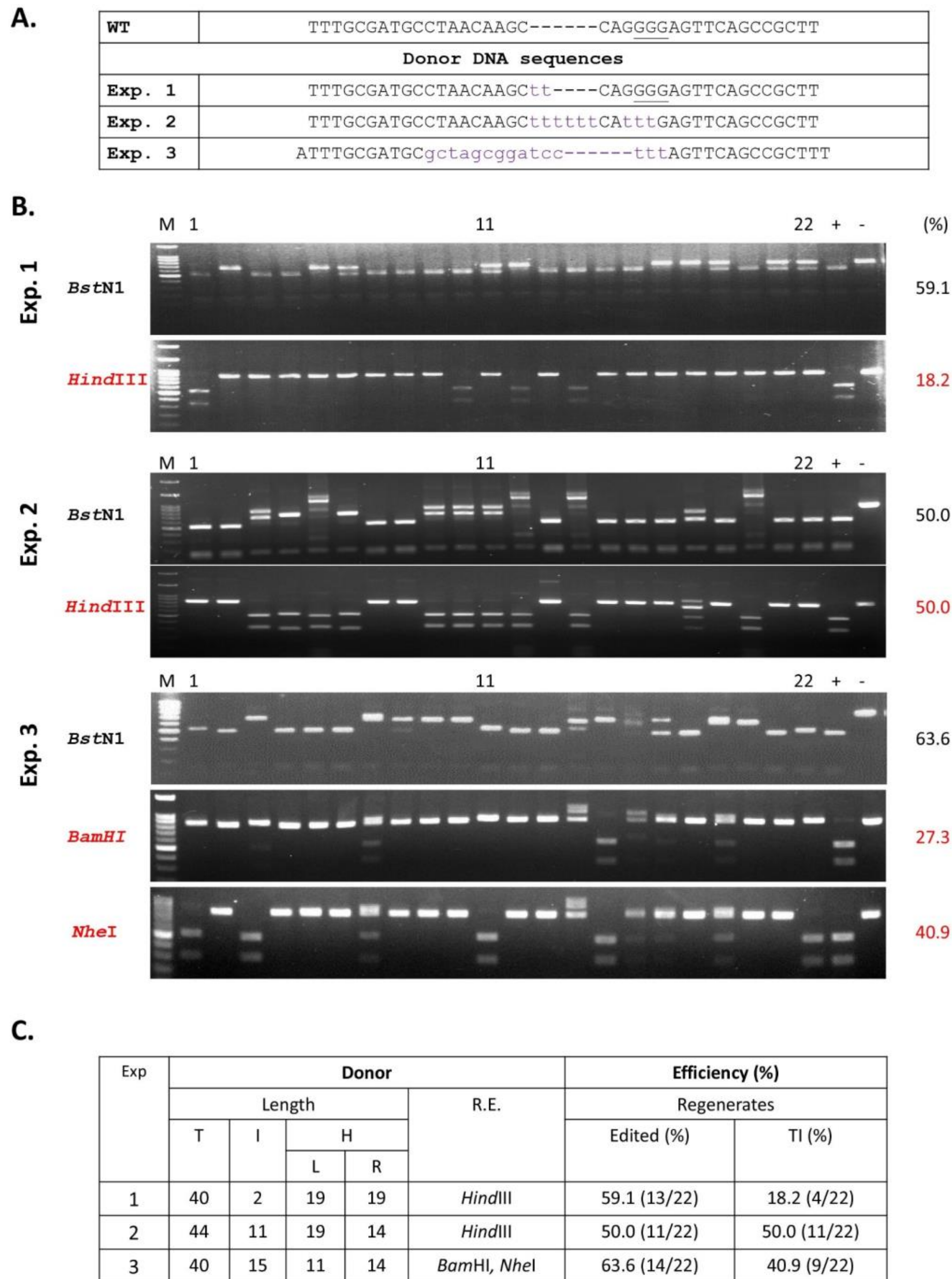
A.

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

Exp	Donor					Efficiency (%)	
	Length				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)

Figure 2. Effect of 40 nt ssODN DD homology arm length, inserted sequence and modified PAM sequence on the targeted insertion in the *N. benthamiana* regenerant *PDS1*. (A) Donor sequences of Exp. 1–3. Lower case: insertion or replacement nucleotides. Underline: PAM. (B) Target gene PCR in the regenerated plants was performed in each experiment and different restriction enzymes 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 Table S1-3. (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.

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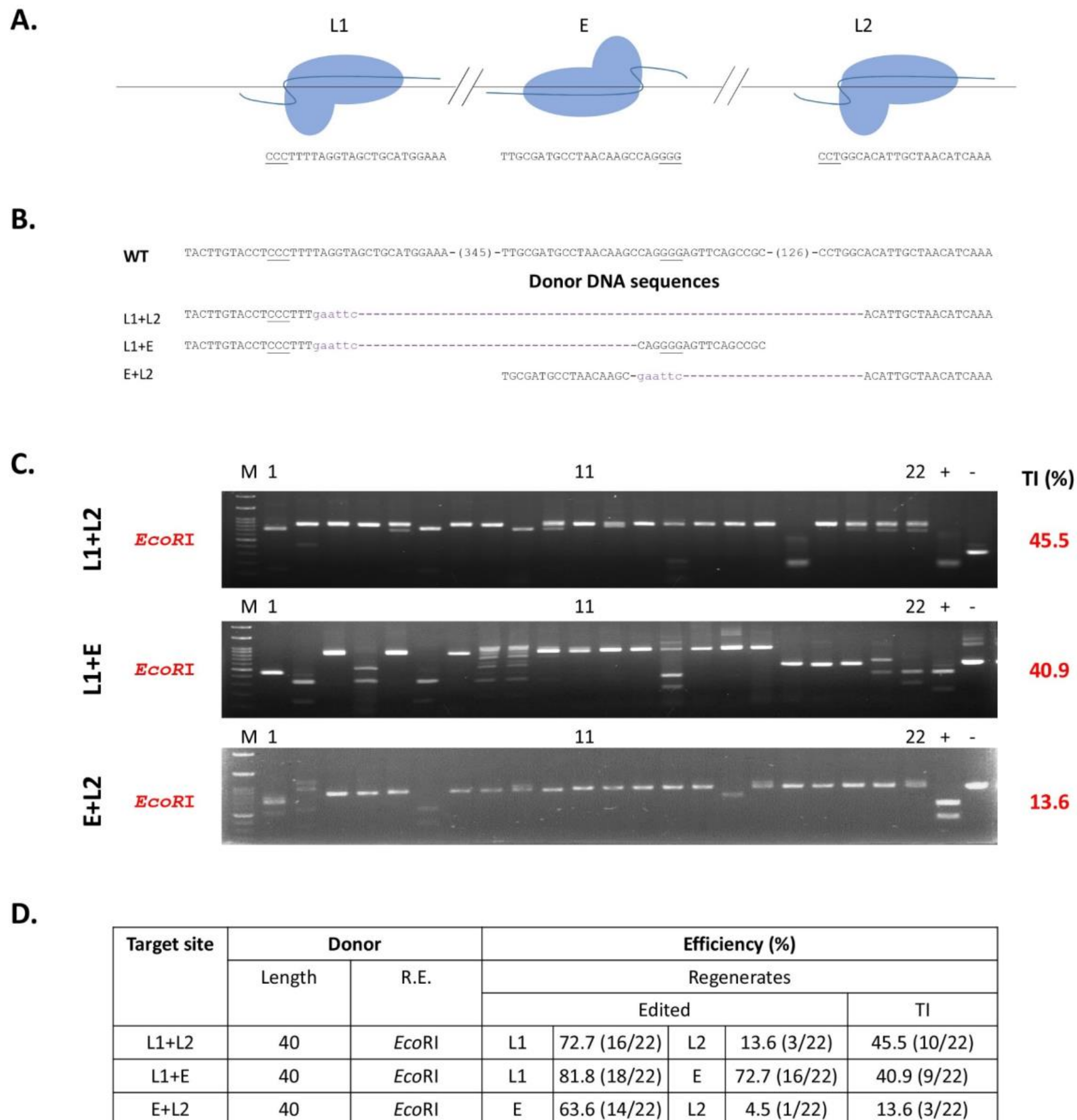

Target site	Donor		Efficiency (%)				
	Length	R.E.	Regenerates				
			Edited				TI
L1+L2	40	*Eco*RI	L1	72.7 (16/22)	L2	13.6 (3/22)	45.5 (10/22)
L1+E	40	*Eco*RI	L1	81.8 (18/22)	E	72.7 (16/22)	40.9 (9/22)
E+L2	40	*Eco*RI	E	63.6 (14/22)	L2	4.5 (1/22)	13.6 (3/22)

Figure 3. Targeted insertion using two RNPs in *N. benthamiana*. (A). Relative position and direction of the three RNPs used. Below is the target site. Underline: PAM. (B). DD used in each combination. Lower case: inserted *Eco*RI site. (C). Restriction enzyme analysis of 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 ssODN. R. E.: restriction enzyme.

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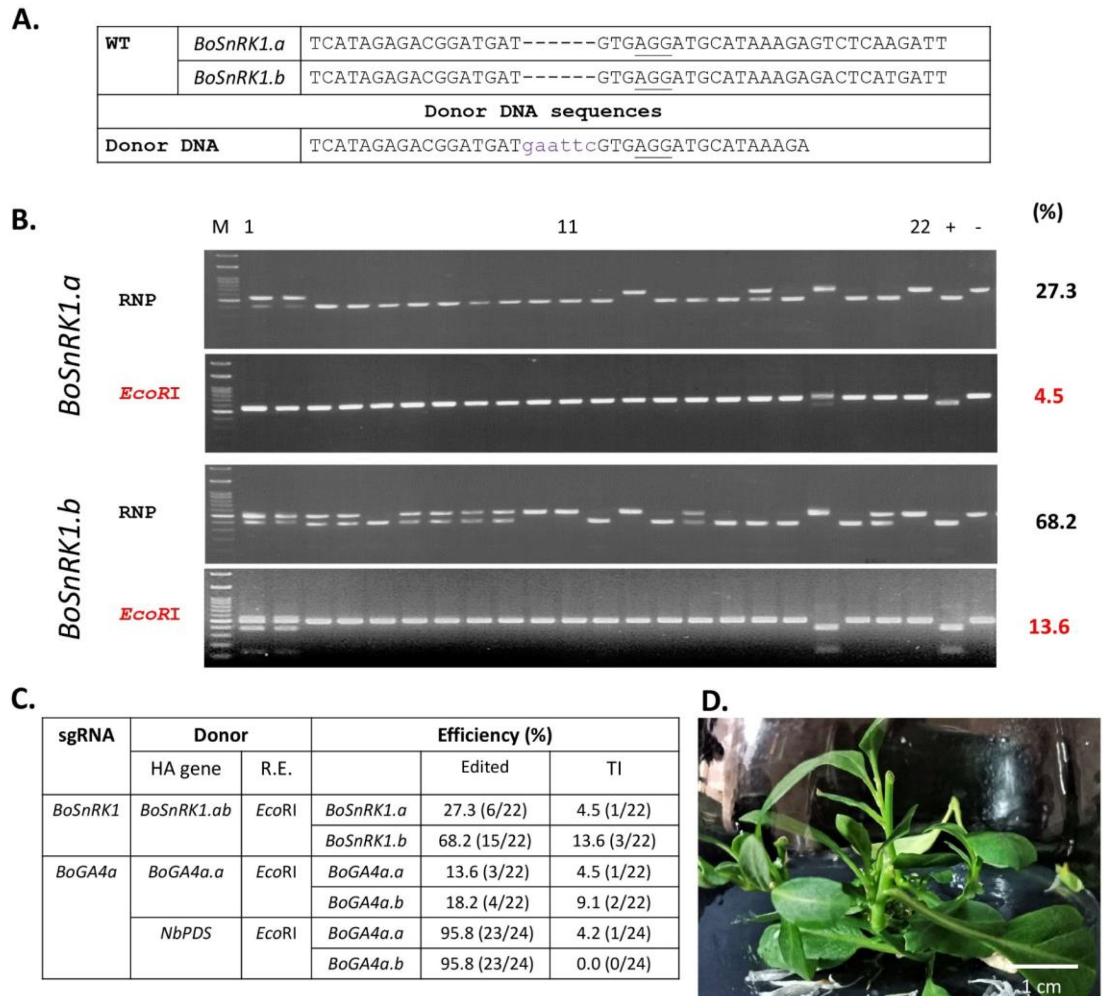

Figure 4. Targeted insertion using ssODN DD in RCBO. (A) Target site of *BoSnRK1* and donor sequence. Underline: PAM; Low case: insertion sequence. (B) Genotyping of the regenerants derived from RNP and ssODN transfected protoplasts. Edited efficiency was assessed by RNP. Targeted insertion assessed using the *Eco*RI site inserted by the ssODN. (C) Summary of edited and targeted insertion efficiency. R.E.: restriction enzyme site inserted by ssODN. HA: homologous arm. TI: targeted insertion. (D) Regenerant BoSnRK1_BoSnRK1#19, with targeted insertion. Bar = 1 cm.

Supporting Information

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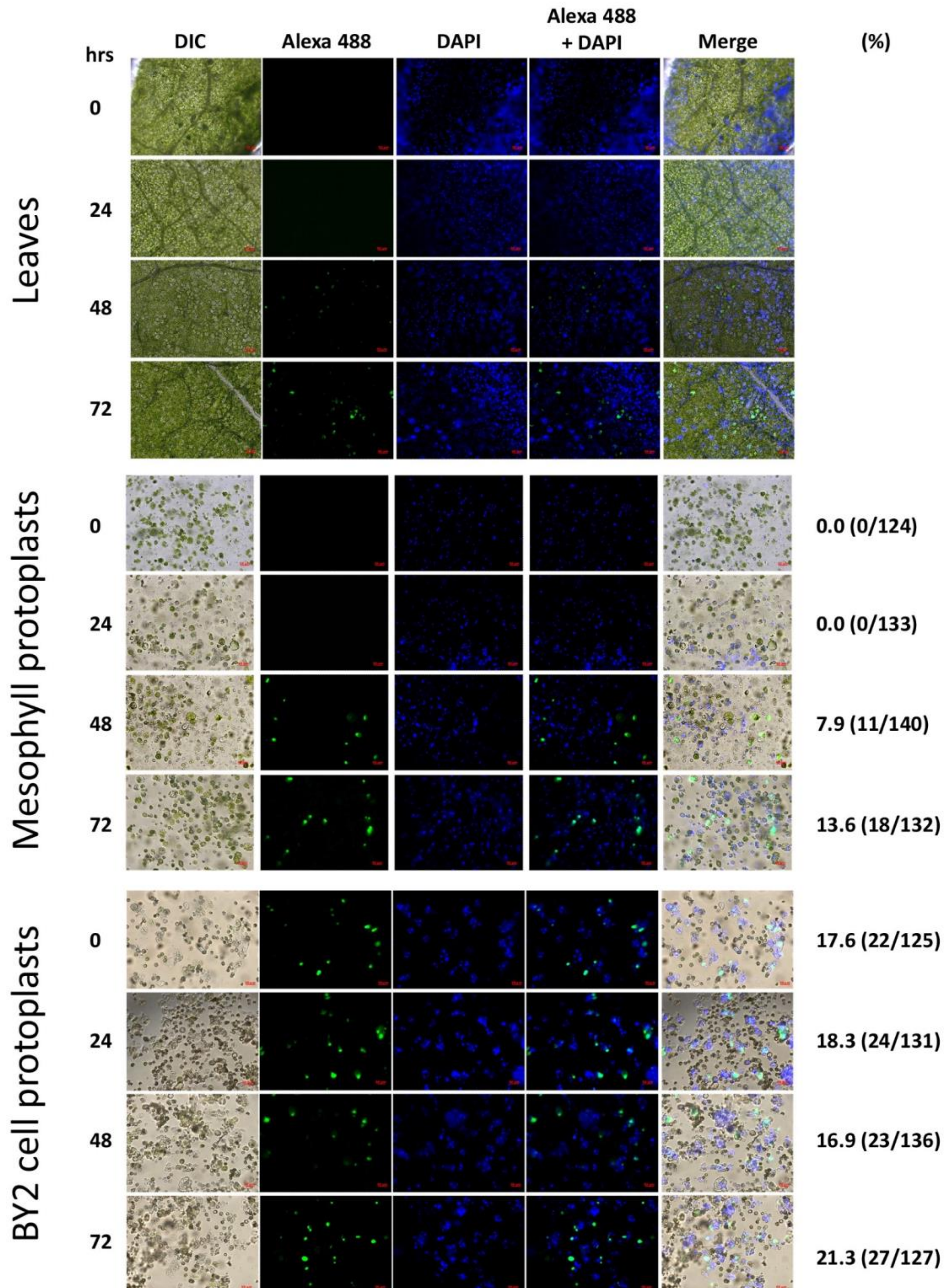

Figure S1. 5-ethynyl-2'-deoxyuridine (EdU) staining for different materials. *N. benthamiana* leaves were cut into strips and placed in 1N0.3K (1/2 MS, 0.4 M mannitol, 1 mg/L NAA, 0.3 mg/L kinetin), and incubated for 0, 24, 48, or 72 hours. The incubated leaves and the protoplasts derived from incubated leaves were stained with EdU. Tobacco protoplasts which derived from BY2 cells in cell division were used as positive control. Bar = 50 µm.

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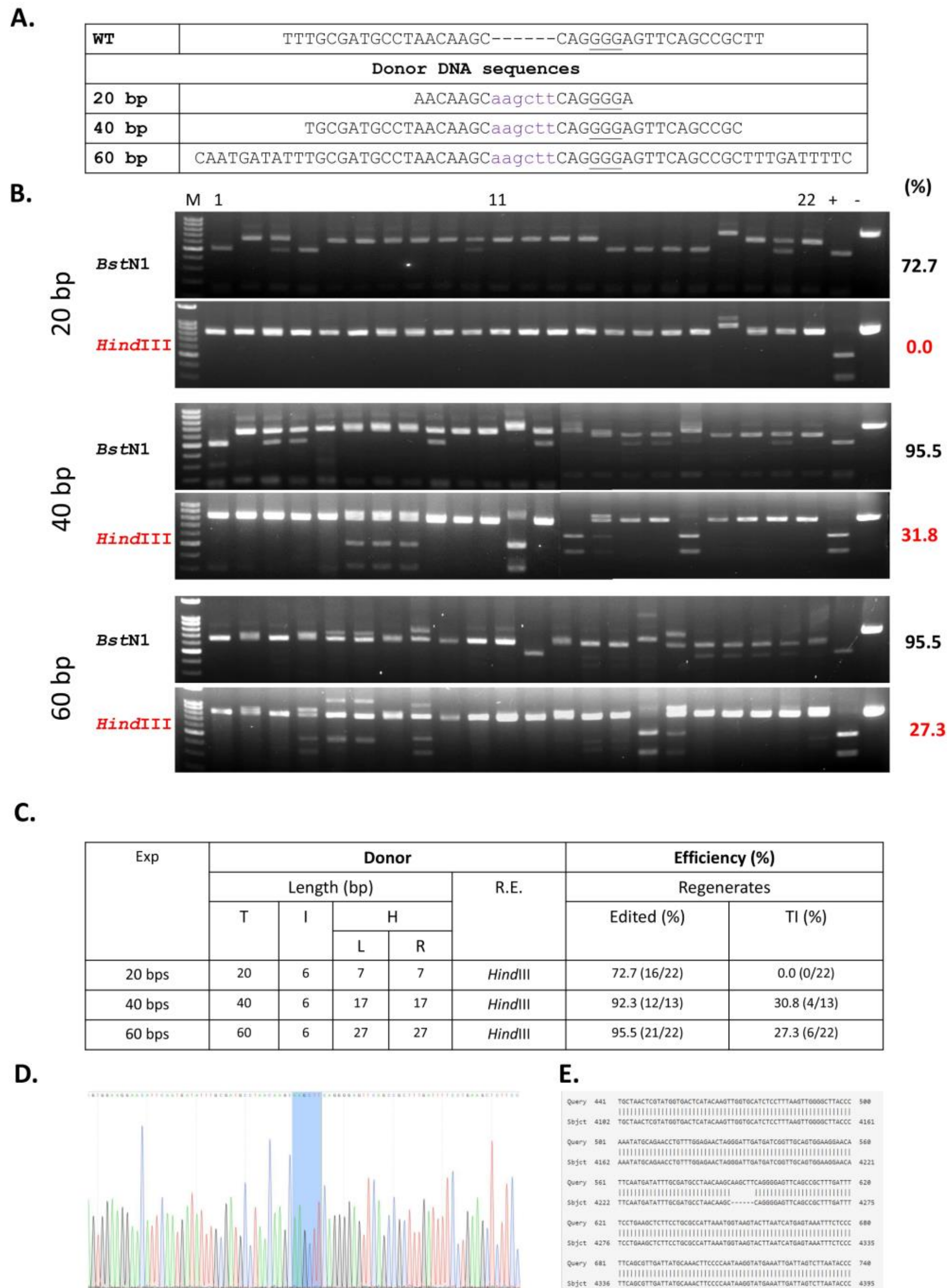
A.

WT	TTTGCGATGCCTAACAAGC------CAGGGGAGTTCAGCCGCTT
	Donor DNA sequences
20 bp	AACAAGCaagcttCAGGGGA
40 bp	TGCGATGCCTAACAAGCaagcttCAGGGGAGTTCAGCCGC
60 bp	CAATGATATTTGCGATGCCTAACAAGCaagcttCAGGGGAGTTCAGCCGCTTTGATTTTC

C.

Exp	Donor					Efficiency (%)	
	Length (bp)				R.E.	Regenerates	
	T	I	H			Edited (%)	TI (%)
			L	R			
20 bps	20	6	7	7	*Hind*III	72.7 (16/22)	0.0 (0/22)
40 bps	40	6	17	17	*Hind*III	92.3 (12/13)	30.8 (4/13)
60 bps	60	6	27	27	*Hind*III	95.5 (21/22)	27.3 (6/22)

Figure S2. Influence of homologous arm length and total length of the ssODN DD on targeted insertion in *N. benthamiana* regenerant *PDS1*. (A). Sequences of DD. Letter underline: PAM. Low case: restriction enzyme site. (B). Protoplasts transfected with different lengths of ssODN. Regenerants genotypes were determined. Target gene *PDS1* PCR was performed and analyzed by *Bst*NI (for Edited) and *Hind*III (for TI). (C). Edited 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) The Sanger sequencing results of +27#6, the precise *Hind*III insertion regenerant (blue background). (E) The BlastN results of +27#6 *PDS* (Query) and wild type Niben101Scf01283Ctg022 (Sbjct).

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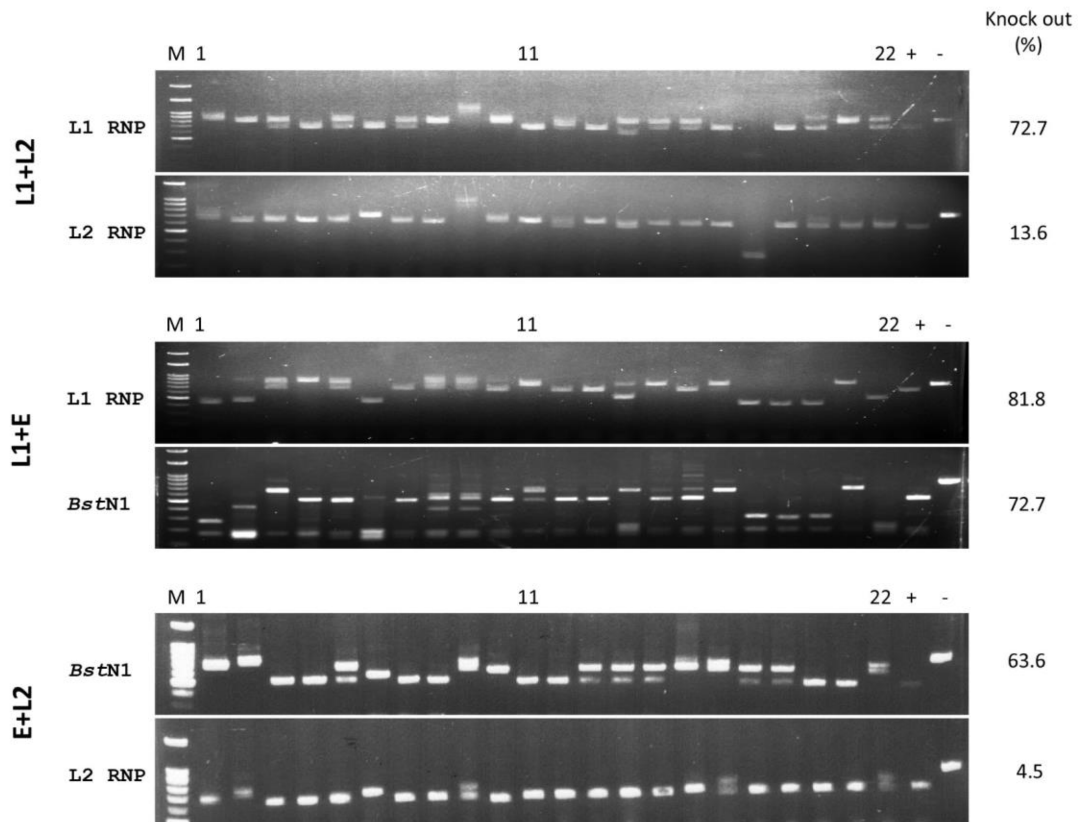

Figure S3. Editing efficiency in Exp. 1–3 regenerants assessed using restriction enzyme and RNP.

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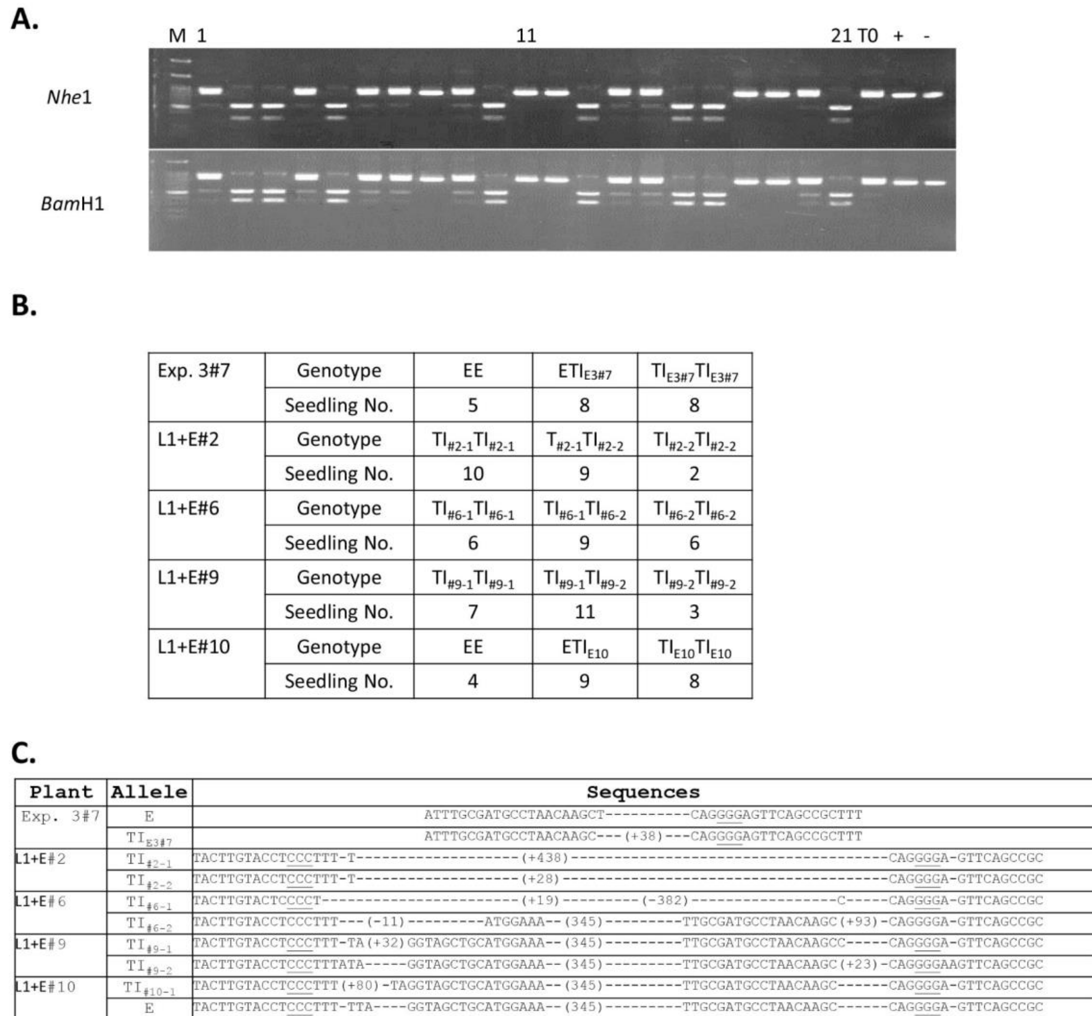

Exp. 3#7	Genotype	EE	$ETI_{E3\#7}$	$TI_{E3\#7}TI_{E3\#7}$
	Seedling No.	5	8	8
L1+E#2	Genotype	$TI_{\#2-1}TI_{\#2-1}$	$T_{\#2-1}TI_{\#2-2}$	$TI_{\#2-2}TI_{\#2-2}$
	Seedling No.	10	9	2
L1+E#6	Genotype	$TI_{\#6-1}TI_{\#6-1}$	$TI_{\#6-1}TI_{\#6-2}$	$TI_{\#6-2}TI_{\#6-2}$
	Seedling No.	6	9	6
L1+E#9	Genotype	$TI_{\#9-1}TI_{\#9-1}$	$TI_{\#9-1}TI_{\#9-2}$	$TI_{\#9-2}TI_{\#9-2}$
	Seedling No.	7	11	3
L1+E#10	Genotype	EE	ETI_{E10}	$TI_{E10}TI_{E10}$
	Seedling No.	4	9	8

Plant	Allele	Sequences
Exp. 3#7	E	ATTTGCGATGCCTAACAAGCT----------CAGGGGAGTTCAGCCGCTTT
	$TI_{E3\#7}$	ATTTGCGATGCCTAACAAGC---(+38)---CAGGGGAGTTCAGCCGCTTT
L1+E#2	$TI_{\#2-1}$	TACTTGTACCTCCCTTT-T-------------------(+438)-------------------------------------CAGGGGA-GTTCAGCCGC
	$TI_{\#2-2}$	TACTTGTACCTCCCTTT-T-------------------(+28)--------------------------------------CAGGGGA-GTTCAGCCGC
L1+E#6	$TI_{\#6-1}$	TACTTGTACTCCCCT-----------------------(+19)---------(-382)-----------------C-----CAGGGGA-GTTCAGCCGC
	$TI_{\#6-2}$	TACTTGTACCTCCCTTT---(-11)---------ATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+93)-CAGGGGA-GTTCAGCCGC
L1+E#9	$TI_{\#9-1}$	TACTTGTACCTCCCTTT-TA(+32)GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGCC-----CAGGGGA-GTTCAGCCGC
	$TI_{\#9-2}$	TACTTGTACCTCCCTTTATA-----GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+23)-CAGGGGAAGTTCAGCCGC
L1+E#10	$TI_{\#10-1}$	TACTTGTACCTCCCTTT(+80)-TAGGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC------CAGGGGA-GTTCAGCCGC
	E	TACTTGTACCTCCCTTT-TTA----GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC------CAGGGGA-GTTCAGCCGC

Figure S4. Progeny of targeted insertion regenerants in *N. benthamiana*. (A) DNA from twenty-one progeny seedlings of Exp. 3#7 was isolated and the target gene *PDS1* was amplified by PCR. The products were digested by two restriction enzymes (*Nhe*I and *Bam*HI), for which sites were added in donor ssODN. (B) Summary of the five targeted insertion (TI) regenerants T_1 progeny. WT: wild type. The genotype is determined by the method described in (A). (C) Summary of sequences of TI alleles. The detail sequence information of each regenerants are shown in Table S3 (Exp.3#7) and Table S5 (L1+E#2, 6, 9, and 10).

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A.

Wild type	TTTGCGATGCCTAACAAGC		CAGGGGAGTTCAGCCGCTT		
	L	I	R		
Donor sequence	TTTGCGATGCCTAACAAGC	tt	CAGGGGAGTTCAGCCGCTT		
Plant no.				**Repeat**	**Orient**
#1	TTTGCGATGCCTAACAAGC	+32	CAGGGGAGTTCAGCCGCTT	1	+
	TTTGCGATGCCTAACAAGC	+30	CAGGGGAGTTCAGCCGCTT	1	+
#10	TTTGCGATGCCTAACAAGC	+60	CAGGGGAGTTCAGCCGCTT	2	+, -
	TTTGCGATGCCTAACAAGC	+60	CAGGGGAGTTCAGCCGCTT	1	+, -
#12	TTTGCGATGCCTAACAAGC	+42	CAGGGGAGTTCAGCCGCTT	2	+
#14	TTTGCGATGCCTAACAAGC	+30	CAGGGGAGTTCAGCCGCTT	1	+
	TTTGCGATGCC--------	+40	CAGGGGAGTTCAGCCGCTT	2	+

B.

Plant no.	Insertion sequences
#1	TTTGCGATGCCTAACAAGCTTCAGGGGAGTTC
	TGCGATGCCTAACAAGCTTCAGCGGAGTTC
#10	TGCGATGCCTAACAAGCTTCAGGGAGTTTCAGCGGCTGAACTCCACTGAAGCTTGTTAGC
	TGCGATGCCTAACAGGCTTCAGGGGGGTTCAGCGGCTGAACTCCCCTGAAGCTTGTTAGC
#12	TTTTGCGATGCCTAACAAGCTTCAGGGGAGTTTTACAAGCTT
#14	TGCGATGCCTAACAAGCTTCAGCGGAGTTC
	AAACAAGCTTTGCGATGCCTAACAAGCTTCAGGGGAGTTC

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

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A.

Wild type	TGCGATGCCTAACAAGC		CAGGGGAGTTCAGCCGC		
	L	I	R		
Donor sequence	TGCGATGCCTAACAAGC	ttttttCAttt	GAGTTCAGCCGC		
Plant no.				Repeat	Orient
#3	TGCGATGCCTAA-----	+29	GAGTTCAGCCGC	1	+
	TGCGATGCCTAA-----	+99	---TTCAGCCGC	3	+
#4	TGCGATGCCTA------	+88	-------GCCGC	2	+,-
#5	TGCGATGCCTAACAAGC	+321	CAGGGGAGTTCAGCCGC	9	+,-
	TGCGATGCCTAACAAGC	+384	CAGGGGAGTTCAGCCGC	10	+,-
#6	TGCGATGCCTA------	+88	-------(-18)----	2	+,-
#9	TGCGATGCCTAA-----	+29	GAGTTCAGCCGC	1	+
	TGCGATGCCTAA-----	+99	---TTCAGCCGC	2	+,-
#10	TGCGATGCCTAA-----	+29	GAGTTCAGCCGC	1	+
	TGCGATGCCTAA-----	+99	---TTCAGCCGC	3	+,-
	TGCGATGCCTAA-----	+166	---TTCAGCCGC	4	+,-
#11	TGCGATGCCTAA-----	+29	GAGTTCAGCCGC	1	+
	TGCGATGCCTAA-----	+92	---TTCAGCCGC	3	+,-
#12	TGCGATGCCTAACAAGC	+321	CAGGGGAGTTCAGCCGC	9	+,-
	TGCGAT-----------	+332	CAGGGGAGTTCAGCCGC	10	+,-
	TGCGATGCCTAACAAGC	+189	CAGGGGAGTTCAGCCGC	5	+,-
	TGCGATGCCTAACAAGC	+127	CAGGGGAGTTCAGCCGC	4	+,-
#14	TGCGATGCCTAACAAGC	+445	------(-123)------	13	+,-
	TGCGATGCCTAACAAGC	+321	CAGGGGAGTTCAGCCGC	9	+,-
#18	------(-35)------	+68	GAGTTCAGCCGC	2	+,-
#20	TGCGATGCCTAACAAGC	+321	CAGGGGAGTTCAGCCGC	9	+,-
	TGCGATGCCTAACAAGC	+377	CAGGGGAGTTCAGCCGC	10	+,-

B.

Plant no.	Insertion sequences
#3	GCCTAACAAGCTTTTTTCATTTGAGTTCA
	ATGCGATGCCTAACAAGCTTTTTTGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTTGCGATGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTT
#4	TGAAAAAAGTTGTTAGGCATCTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTCAGCCGCTGAACTCAAATGAAAAAAGCTTGTTAG
#5	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
	TTTGCGATGCCTAACAAGCTTTTTTCATTTTCCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTGCGATGCCTAAGCGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAAAAGCGGCTGAA CTAAGCGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
#6	TGAAAAAAGTTGTTAGGCATCTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTCAGCCGCTGAACTCAAATGAAAAAAGCTTGTTAG
#9	GCCTAACAAGCTTTTTTCATTTGAGTTCA
	ATGCGATGCCTAACAAGCTTTTTTGCCTAACAAGCTGAACTCAAATGAAAAAAGTTTTTGCGATGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTT
#10	GCCTAACAAGCTTTTTTCATTTGAGTTCA
	ATGCGATGCCTAACAAGCTTTTTTGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTTGCGATGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTAT
	ATGCGATGCCTAACAAGCTTTTTTGCCTAACAAGCTGAACTCAAATGAAAAAAGCCTGTTAGGCATCGCAAAATTTGCGATGCTTAACAAGCTTTTTTCATTTGAACTCAAATGAAAAAAGCTTTTGCGATGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTT
#11	GCCTAACAAGCTTTTTTCATTTGAGTTCA
	ATGCGATGCCTAACAAGCTTTTTTCATTTGAACTCAAATGAAAAAAGCTTTTGCGATGCCTAACAAGCTGAACTCAAATGAAAAAAGCTTTT
#12	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
	GTCTAACAAGCTTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTG TTAGGCATCGCATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTGCGATGCCTAAGCGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAAAAGCGGCTGAACTAAGCGGCTGAACTCAAATGAAA AAAGCTTGTTAGGCATCG
	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
#14	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTCTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAATGCGATGCCTAACAAGCT TTTTTCATTTGAGTTCAGCCGTCGCAAAAAGCTTGTTAGGCATCGCAAAAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAAAAAGCTTGTTAGGCATCGCA
	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
#18	CGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAAATGCGATGCCTAACAAGCTTTTTTCATTT
#20	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCTGAACTCAAATGAAAAAAGCTTGTTAGGCATCG
	TTTGCGATGCCTAACAAGCTTTTTTCATTTTTCCTAACATTTTGCGATGCCTAACAAGCTTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTGAGATGCCTAACAAGCTTTTTTCATTTGAGTTTTGCGATGCCTAACTCAAATGAAAAAACTTGTTAGGCATCGC ATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGATGCCTAACAAGCTTTTTTCATTTGAGTTTTTGCGATGCCTAACAAGCTTTTTGCGACGGCCGAACTCAAATGAAAAAAGCTTGTTAGGCATCGCAATGCGATGCCTAACAAGCT TTTTTCATTTGAGTTCAGCCGCTTGTTAGGCATCG

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

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A.

Wild type	ATTTGCGATGCCTAACAAGC		CAGGGGAGTTCAGCCGCTTT			
	L	I	R			
Donor sequence	ATTTGCGATGC	gctagcggatccttt	AGTTCAGCCGCTTT			
Plant no.				**Repeat**		**Orient**
				BamHI	***NheI***	
#1	ATTTGCGATGCCTAACAAGC	+30	CAGGGGAGTTCAGCCGCTTT	0	1	+
#3	ATTTGCGATGCCTA------	+49	-----GAGTTCAGCCGCTTT	2	2	+
#7	ATTTGCGATGCCTAACAAGC	+38	CAGGGGAGTTCAGCCGCTTT	1	1	+
#11	ATTTGCGATGCCTAACAAGC	+30	CAGGGGAGTTCAGCCGCTTT	0	1	+
#15	ATTTGCGATGCCTA------	+49	-----GAGTTCAGCCGCTTT	2	2	+
#16	ATTTGCGATGCCTA------	+49	-----GAGTTCAGCCGCTTT	2	2	+
#17	ATTTGCGATGCCTAACAAGC	+29	CAGGGGAGTTCAGCCGCTTT	1	1	+
#19	ATTTGCGATGCCTAACAAGC	+38	CAGGGGAGTTCAGCCGCTTT	1	1	+
#22	ATTTGCGATGCCTAACAAGC	+30	CAGGGGAGTTCAGCCGCTTT	0	1	+

B.

Plant no.	**Insertion sequences**
#1	GATCCTTTAGTTCAGCCGCTAGCGCATCGC
#3	GCGCTAGCGGATCCTTTAGTTTTGCGATGCGCTAGCGGATCCTTTTTAT
#7	ATATCGGATCCTTTAGTTCTGCGTGGCTAGCGGCTGAA
#11	GATCCTTTAGTTCAGCCGCTAGCGCATCGC
#15	GCGCTAGCGGATCCTTTAGTTTTGCGATGCGCTAGCGGATCCTTTTTAT
#16	GCGCTAGCGGATCCTTTAGTTTTGCGATGCGCTAGCGGATCCTTTTTAT
#17	TGAACTAAAGGATCCGCTAGCGCATCGCA
#19	ATATCGGATCCTTTAGTTCTGCGTGGCTAGCGGCTGAA
#22	GATCCTTTAGTTCAGCCGCTAGCGCATCGC

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

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A.

Wild type	TACTTGTACCTCCCTTT-------TAGGTAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA		
Donor sequence	TACTTGTACCTCCCTTT-----------------------------------gaattc-----------------ACATTGCTAACATCAAA		
Plant No.		Repeat	Orient
#1	TACTTGTACCTCCCTTTTA--(+81)--TAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA	3	-
	TACTTGTACCTCCCTTT-(+29)-TAGGTAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA	1	+
#5	TACTTGTACCTCCCTTT-(+17)--AGGTAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA	1	-
#6	TACTTGTACCTCCCTTTT(+83)--AGGTAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA	3	+,-
#9	(-72)-------------(+413)-------------------------------ATT---(490)----CCTGGCACATTGCTAACATCAAA	12	+,-
	(-72)-------------(+244)-------------------------------ATT---(490)----CCTGGCACATTGCTAACATCAAA	7	+,-
#10	TACTTGTACCTCCCTTT-(+30)-TAGGTAGCTGCATGGAAAGATGATGATGGAGATT---(490)----CCTGGCACATTGCTAACATCAAA	1	-
#14	TACTTGTACCTCCCTTTT(+306)---ACATTGCTAACATCAAA	9	+,-
#18	TACTTGTACCTCCCT---(+20)---ATTGCTAACATCAAA	1	+
#20	TACTTGTACCTCCCTTT(+78)----AGGTAGCTGCATGGAAAGATGATGATGGAGAT---(490)----CCTGGCACATTGCTAACATCAAA	3	+,-
#21	TACTTGTACCTCCCTTTT(+28)-------AGCTGCATGGAAAGATGATGATGGAGAT---(490)----CCTGGCACATTGCTAACATCAAA	1	+
#22	TACTTGTACCTC-----(+35)--------------ATGGAAAGATGATGATGGAGAT---(490)----CCTGGCACATTGCTAACATCAAA	1	+,-

B.

Plant no.	Insertion sequences
#1	TTTTGAATTCAAAGGGAGGTAGCAATGTGAATTCAAATTCAAAGGGAGGAATGTAAGCAATGTGAATTCAAAGGGAAACAA
	CTTGTACCTCCCTTTGAATTCACATTGCT
#5	AATGTGAATTCAAAGGG
#6	TTGAATTCAAAGGGAATTCAAAGGAAATTCAAAGGGAGGAATGTGAATTCTTCACATTGCTAACATTACTTGTACCTTCCTTT
#9	GTACCTCCCTTTGAATTCAAAGCTTCCTTTGAATTCACATTGCTTTGTACCTCCCTTTGAATTCAAATTGAATTCACATTGCTAACATTACTTGTATCTCCCTTTGAATTCAAAGGGTACTTGTACCTCCCTTTGATGTTAGCAATGTGAATTCAAAACCTTCCTTTGAATTCAA TTCACAGGGAGATACTATGTGAATTCAATCCTCCCTTTGATGTTAGCAATGTGAATTCAAACTTGTACCTCCCTTTGATGTTAGCAATGTCCCTTTGGATTCAAAGGGAGGTACAAAGGGAGGTACAAGTAAATTAGCAATGTGAATTCAAAGGGAGGTACAAGTACTTGTACCT CCCTTTGAATTCCCTTTGAATTCTCTCTTTGAATTACTTGTACCTCTTTTTTACTTGTACCTC
	GTACCTCCCTTTGAATTCAAAGCTTCCTTTGAATTCACATTGCTTTGTACCTCCCTTTGAATTCAAACTTGTACCTCCCTTTGATGTTAGCAATGTCCCTTTGAATTCAAAGGGAGGTACAAAGGGAGGTACAAGTAAATTAGCAATGTGAATTCAAAGGGAGGTACAAGTACTT GTACCTCCCTTTGAATTCCCTTTGAATTCTCTCTTTGAATTACTTGTACCTCTTTTTTACTTGTACCTC
#10	ATCAATGTGAATTCAAAGGGAGGTACAAGT
#14	CCCTTTGAATTCAAAGGGAGGTACAAGTAATATTCCTCTCTTTGAATTCACATTGCTACCTCCCTTTGAATTCATCCTCCCTTTGAATTACTTGTACCTCCCTTTGAATTCACATTGCTTTACTTGTACCTCCCTTTGAATTTACTTGTACCTCCCTTTGAATTCACATTCCTTT TAATTTCTCCCTTTGAATTACTTGTACCTCCCTTTGAATTCACATTGCTATTACTTGTACCTCCCTTTGAATTCACATTTTTGAATTCACATTGCTTTTGAATTCACATTGCTATCTTGTACCTCCCTTTG
#18	GTACCTCCCTTTGAATTCAA
#20	GTGAATTCAAAGGGAGGTACAATGTGAATTCAAAGGGAGGTACGAGTAATACTTGTACCTCCCTTTGAATTCAAAGGG
#21	TGTACCTCCCTTTGAATTCACATTGCTA
#22	AAGGAGGTACCTCCCTTTGAATTCAAAGGGAGGTA

Table S4. L1+L2 sequences. (A) Summary of the TI regenerants. Underline: PAM. +number: insertion length. –number: deletion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orient column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underline: PAM.

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A.

Wild type	TACTTGTACCTCCCTTT-TA----GGTAGCTGCATGGAAA---(345)---------TTGCGATGCCTAACAAGC------CAGGGGA-GTTCAGCCGC		
Donor sequence	TACTTGTACCTCCCTTT---------------------gaattc-------------------------------------CAGGGGA-GTTCAGCCGC		
Plant No.		R.	O.
#2	TACTTGTACCTCCCTTT-T-------------------(+438)-------------------------------------CAGGGGA-GTTCAGCCGC	2	+
	TACTTGTACCTCCCTTT-T-------------------(+28)--------------------------------------CAGGGGA-GTTCAGCCGC	1	+,-
#4	TACTTGTACCTCCCTTT---(-8)-------TGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+42)-CAGGGGA-GTTCAGCCGC	1	+
#6	TACTTGTACTCCCCT-----------------------(+19)---------(-382)-----------------C-----CAGGGGA-GTTCAGCCGC	1	-
	TACTTGTACCTCCCTTT---(-11)---------ATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+93)-CAGGGGA-GTTCAGCCGC	2	-
#8	TACTTGTACCTCCCTTTATA-----GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+23)-CAGGGGAAGTTCAGCCGC	1	+
	TACTTGTACCTCCCTTT-TA(+31)GGTAGCTGCATGGAAA--(345)---------TTGCGAAGCCTAACAAGC-----CCAGGGGA-GTTCAGCCGC	1	+
	TACTTGTACCTCCCTTT-TA(+32)GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+23)-CAGGGGAAGTTCAGCCGC	2	+
#9	TACTTGTACCTCCCTTT-TA(+32)GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGCC-----CAGGGGA-GTTCAGCCGC	1	+
	TACTTGTACCTCCCTTTATA-----GGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC(+23)-CAGGGGAAGTTCAGCCGC	1	+
#10	TACTTGTACCTCCCTTT(+80)-TAGGTAGCTGCATGGAAA--(345)---------TTGCGATGCCTAACAAGC------CAGGGGA-GTTCAGCCGC	2	+,-
#14	TACTTGTACCTCCCTTT---------------------(+105)-------------------------------C-----CAGGGGA-GTTCAGCCGC	2	+,-
#21	TACTTGTACCTCCCTT----------------------(+411)-------------------------------------CAGGGGA-GTTCAGCCGC	1	+,-
#22	TACTTGTACCTCCCTTT---------------------(+105)-------------------------------C-----CAGGGGA-GTTCAGCCGC	2	+,-
	TACTTGTACCTCCCTTT---------------------(+105)-------------------------------C-----CAGGGGA-GTTCAGCCGC	2	+,-

B.

Plant no.	Insertion sequences	
Target site	L1	E
#2	GAATTCCAGGGGAGTTTACTTGTACCTCCCTTACCTCCCTTTGAATATCATTGAATGTTCCTTCCACTGCAACCGATCATCAATCCCTAGTTCTCCAAACAGGTTCTGCATATTTGGGTA AGCCCCAACTTAAAGGAGATGCACCAACTTGTATGAGTCACCATACGAGTTAGCAGATAAATAAAGAGAGGCAAGGCAGAATACAGATCGACTGACAAAAATTGGCTATTTGCAAAAGAC AAAAGACAAACAGGGATAGCTATCTGATTAATAAAAGAGATCTTGAAAGAAATTGCTATGACTGTATTAGAAGATTAAAGTAGCATTCTTGAGGAGTCAAACTTACAGAATATGTGCAAC CCAGTCTCGTACCAATCTCCATCATCATCTTTCCATGCAGCTACCTATTGTACCTCCCTTTGAATTCCAGGGGAGGTA	
	TACCTCCCTTTGAATTCCAGGGGAGGTA	
#4		TACTTGTACCTCCCTTTGAATTCCAGGGGAGTTCAAAGGGAA
#6	GGAATTCAAAGGGAGGTAC	
		TCTGGAGTTCAAAGGGAGAATTCAAAGGGAGGTACAACTCCCCTGGAAAAAA GGGAGGTACAAGAAACTCCCCTGGAACTCCCCTGGAATTCA
#8		TACTTGTACCTCCCTTTGAATTC
	CTTGTACCTCCCTTGAATTCCCAGGGAATTA	
	CTTGTACCTCCCTTTGAATTCCCAGGGAATTA	TACTTGTACCTCCCTTTGAATTC
#9	CTTGTACCTCCCTTTGAATTCCCAGGGAATTA	
		TACTTGTACCTCCCTTTGAATTC
#10	ATTTTCCAGGGGAGTTCCCTTTCCAGGGGAGTTTCCCTTTGAATTCCAGGGGAGTTTTTTGAACTCCCCCTGGAATTCAA	
#14	CCTCCCTTTGAATTTACTTGTACCTCCCTTCAAAGGGAGGTACAAGTTCAAAGGGAGGTACAAGTAAACTCCCCTGGAATTCAAAGGGAGAATTCAAAGGGAGGT	
#21	GCTTGTTAGGCATCGCAAATATCATTGAATGTTCCTTCCACTGCAACCGATCATCAATCCCTAGTTCTCCAAACAGGTTCTGCATATTTGGGTAAGCCCCAACTTAAAGGAGATGCACCA ACTTGTATGAGTCACCATACGAGTTAGCAGATAAATAAAGAGAGGCAAGGCAGAATACAGATCGACTGACAAAAATTGGCTATTTGCAAAAGACAAAAGACAAACAGGGATAGCTATCTG ATTAATAAAAGAGATCTTGAAAGAAATTGCTATGACTGTATTAGAAGATTAAAGTAGCATTCTTGAGGAGTCAAACTTACAGAATATGTGCAACCCAGTCTCGTACCAATCTCCATCATC ATCTTTCCATGCAGCTACCTTTGTACCTCCCTTTGAATTCAAAGGGAGGTA	
#22	CCTCCCTTTGAATTTACTTGTACCTCCCTTCAAAGGGAGGTACAAGTTCAAAGGGAGGTACGAGTAAACTCCCCTGGAATTCAAAGGGAGAATTCAAAGGGAGGT	
	CCTCCCTTTGAATTTACTTGTACCTTCCTTCAAAGGGAGGTACAAGTTCAAAGGGAGGTACAAGTAAACTCCCCTGGAATTCAAAGGGAGAATTCAAAGGGAGGT	

Table S5. L1+E sequences. (A) Summary of the TI regenerants. Underline: PAM. +number: insertion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orient column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underline: PAM.

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A.

Wild type	TGCGATGCCTAACAAG------CCAGGGGAGTTCAGCCGC---(126)-CCTGGCACATTGCTAACATCAAA(30)GAATTTCTGCATGTAGCTAAA		
Donor sequence	TGCGATGCCTAACAAGC---------------------------gaattc------------------------------ACATTGCTAACATCAAA		
Plant No.		R.	O.
#1	TGCGATGCCTAACAAGC-------------------------(+93)-----------------------------------CTGCATGTAGCTAAA	3	+
#2	-----(-36/+104)--------CAGGGGAGTTCAGCCGC---(126)-CCTGGCACATTGCTAACATCAAA(30)GAATTTCTGCATGTAGCTAAA	2	+,-
	TGCGATGCCTAACAAG-(+130)-----GAGTTCAGCCGC---(126)-CCTGGCACATTGCTAACATCAAA(30)GAATTTCTGCATGTAGCTAAA	0	+,-
#6	TGCGATGCCTAACAAG-(+57)CCAGGGGAGTTCAGCCGC---(126)-CCTGGCACATTGCTAACATCAAA(30)GAATTTCTGCATGTAGCTAAA	2	+,-

B.

Plant No.	Insertion sequences
#1	GAATTCACATTGCGAATTCACATTGCTAACATTTCACATTATTTGCGATGCCTAACAAGCGAATTCACATTGCTAACATTGATGCCTAACAAG
#2	TGGTTTGACATGATTCGGACATCCGATTGAGAAAATAGAAATTTTAAAGTGTTCTTGAGTTGCCTAACAAGCGAATTCACATCGAATTCGCTTGTTAGGCATCG
	TTCCGTTCTCGAACCTGATAAGAATGAGGTAAATTTTAATCATTTAACATGGGATTGGAGAAATGAAATTATTGCTTTTTTATAGCACGGAATCGTGCCTAATGACAAAGGAAAGGCTCACGCACTTCGC
#6	CTAACAAGCGAATTCACATTGCTAACATCAATGTGAATTCGCTTGTTAGGCATCGCA

Table S6. E+L2 sequences. (A) Summary of the TI regenerants. Underline: PAM. +number: insertion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orient column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underline: PAM.

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A.

Wild type		TCATAGAGACGGATGAT		GTGAGGATGCATAAAGA		
		L	I	R		
Donor sequence		TCATAGAGACGGATGAT	gaattc	GTGAGGATGCATAAAGA		
Plant no.	**Gene**				Repeat	Orient
#1	*BoSnRK1.b*	TCATAGAGACGGATGAT	+42	-----------TAAAGA	1	+
#2	*BoSnRK1.b*	TCATAGAGACGGATGAT	+37	------ATGCATAAAGA	1	+
#19	*BoSnRK1.a*	TCATAGAGACGGATGA-	+33	GTGAGGATGCATAAAGA	1	+
	BoSnRK1.b	TCATAGAGACGGATGA-	+19	GTGAGGATGCATAAAGA	1	+
		TCATAGAGACGGATGAT	+50	GTGAGGATGCATAAAGA	1	+ ; -

B.

Plant no.	Gene	Insertion sequences
#1	*BoSnRK1.b*	AGACGGATGATGAATTCGTGAGGATGCATCCTCGCGAATGCG
#2	*BoSnRK1.b*	AGACGGATGATGAATTCGTGAGGATGCATCCTCACGA
#19	*BoSnRK1.a*	GAGACGGATGATGAATTCGTGAGGATGCATCCT
	BoSnRK1.b	GAGACGGATGATGAATTC
	BoSnRK1.b	CATAGAGACGGATGATGCATCCTCACGAATTCATCATCCGTCTCTATGAT

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

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A.

		L	I	R		
Wild type	*BoGA4a.a*	GTTGAGAGGGGAGCCGG		TGATGGTAAAACCTTCG		
	BoGA4a.b	ATTGAGAGGGGAACCGG		TGATGGTGAAGCCTTCG		
Donor sequence		GTTGAGAGGGGAGCCGG	gaattc	TGATGGTAAAACCTTCG		
Plant no.	**Gene**				Repeat	Orient
#5	*BoGA4a.a*	GTTGAGAGGGGAGCCG-	+76	-GATGGTAAAACCTTCG	2	+,-
	BoGA4a.b	ATTGAGAGGGGAACCGG	+61	TGATGGTGAACCCTTCG	2	+
#17	*BoGA4a.b*	ATTGAGAGGGGAACC--	+31	-GATGGTGAAGCCTTCG	1	+

B.

Plant no.	Insertion sequences
#5	AGAGGGGAGCCGGGAACCATCAGAATTCCCGGCTCCCCTCTCAACTACCATCAGAATTCCCGGCTCCCCTCTCAAC
	GAGAGGGGAGCCGGGAATTCTGAAGGTAAATACCATCAGAATTCCCGGCTCCCCTCTCCCG
#17	TGAGAGGGGAGCCGGGAATTCTGATGGTAAA

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

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A.

		5′	I	3′		
Wild type	*BoGa4a.a*	AGCCGG		TGATGG		
	BoGa4a.b	AACCGG		TGATGG		
Donor sequence			TGCGATGCCTAACAAGCaagcttCAGGGGAGTTCAGCCGC		Repeat	Orient
Plant no.	**Gene**					
#4	*BoGa4a.a*	AGCCGG	+29	--ATGG	1	+,-
		AGCCGG	+29	--ATGG	1	+,-

B.

Plant no.	Insertion sequences
#4	GGAGTTCAGCCCCTGAAGCTTGCTTGTTA
	GGAGTTCAGCCGCTGAAGCTTGCTTGTTA

Table S9. *BoGA4.a* sequences by *N. benthamiana PDS* DD. (A) Summary of the TI regenerants. Underline: PAM. +number: insertion length. –number: deletion length. L: left homologous arm; I: insertion; R: right homologous arm. + in Orient column: forward insertion; -: reverse. (B) Insertion sequences of TI regenerants. Underline: PAM.

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Gene PCR primer		
N.b_PDS	F	TATAGAGCACTATGTTAGTCAGTTT
	R	TAGAGAAATGAGAATAAGAGATACAGG
B.o_GA4.a	F	GCCCAGTCTGAATCTGAATT
	R	AAAACATCCCTCTCATTGAC
B.o_GA4.b	F	ATTGAGTTTCTCACCGGTAG
	R	GAAGAAATATGACGGCACTATAC
B.o_SnRK1.a	F	CTTTGCAGTTTGTTTCAAATTCTTG
	R	TGGCCTCGTCCTCTTGTAGT
B.o_SnRK1.b	F	TGTCTGTGTATTTGCATGTTTTT
	R	CTCGTCCTCCTGTAGTCTACCC
sgRNA in vitro transcription primer		
T7 oligo_T25G	F	TAATACGACTCACTATAG
Bottom_SL297	R	GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATGCTGTTTCCAGCAT
Reverse_SLKS2	R	GCACCGACTCGGTGCCACTTTTTCAAG
Top_E	F	TAATACGACTCACTATAGTTGCGATGCCTAACAAGCCAGGTTTTAGAGCTATGCTGGAAACAGCATAGCAAGTTAA
Top_L1	F	TAATACGACTCACTATAGTTTCCATGCAGCTACCTAAAAGTTTTAGAGCTATGCTGGAAACAGCATAGCAAGTTAA
Top_L2	F	TAATACGACTCACTATAGTTTGATGTTAGCAATGTGCCGTTTTAGAGCTATGCTGGAAACAGCATAGCAAGTTAA
B.o_GA4a	F	TAATACGACTCACTATAGGTTGAGAGGGGAGCCGGTGAGTTTTAGAGCTATGCTGGAAACAGCATAGCAAGTTAA
B.o_SnRK1	F	TAATACGACTCACTATAGTCATAGAGACGGATGATGTGGTTTTAGAGCTATGCTGGAAACAGCATAGCAAGTTAA

Table S10. Primer information.