The obstacles and potential clues of prime editing applications in tomato, a dicot plant

Tien Van Vu[1,2,*], Jihae Kim[1], Swati Das[1], Jinsu Lee[1], Jae-Yean Kim[1,3,*]

[1]Division of Applied Life Science (BK21 FOUR Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea.

[2]National Key Laboratory for Plant Cell Biotechnology, Agricultural Genetics Institute, Km 02, Pham Van Dong Road, Co Nhue 1, Bac Tu Liem, Hanoi 11917, Vietnam.

[3]Division of Life Science, Gyeongsang National University, 501 Jinju-daero, Jinju 52828, Republic of Korea.

***Correspondence: Tien Van Vu (tienvu.agi@gmail.com; ORCID: 0000-0002-6369-7664). Jae-Yean Kim (kimjy@gnu.ac.kr; ORCID ID: 0000-0002-1180-6232)**.

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ABSTRACT

Precision genome editing is highly desired for crop improvement. The recently emerged CRISPR/Cas technology offers great potential applications in precision plant genome engineering. A prime editing (PE) approach combining a reverse transcriptase (RT) with a Cas9 nickase and a "priming" extended guide RNA has shown a high frequency for precise genome modification in mammalian cells and several plant species. Nevertheless, the applications of the PE approach in dicot plants are still limited and inefficient. We designed and tested prime editors for precision editing of a synthetic sequence in a transient assay and for desirable alleles of 10 loci in tomato by stable transformation. Our data obtained by targeted deep sequencing also revealed only low PE efficiencies in both the tobacco and tomato systems. Further assessment of the activities of the PE components uncovered potential reasons for the inefficiency of the PE complexes. Modifying the pegRNA sequences by shortening or introducing mismatches to the primer binding sequences (PBS) in order to reduce their melting temperatures (Tm) did not enhance the PE efficiency at the SlBMP21, SlALC and SlALS1 loci. Our data show challenges of PE approach in tomato, indicating a further improvement of the PE system for the successful applications such as use of improved expression systems. Our work provides an important clue for the successful application of the PE approach in crop improvement.

Keywords: Prime editor; CRISPR/SpCas9; Precision gene editing; Prime editing; DSB-independent gene editing, Geminiviral replicon.

INTRODUCTION

RNAs have been used for gene correction in human and yeast cells (Meers et al., 2016). CRISPR/Cas-mediated precision editing using a chimeric single guide RNA (sgRNA) and a donor template was shown in rice protoplasts (Butt et al., 2017; Li et al., 2019). However, the gene targeting (GT) frequency recorded using the RNA donor was much lower than that of a single-stranded DNA (ssDNA) donor (Li et al., 2019). Further work needs to be done on the use of RNA as a template for precision plant gene editing.

47 Recently, prime editing (PE) using guide RNA extensions for priming reverse transcription-
48 mediated precise editing appeared to be an excellent precision genome editing technique in
49 mammalian cell lines (Anzalone et al., 2019). The best version of the prime editor used a
50 CRISPR/Cas complex developed by fusing a reverse transcriptase (RT) to the C-terminus of a Cas9
51 nickase (H840A) and a PE gRNA (pegRNA) with a 3' extension that could bind to the 3' nicked
52 strands produced by nCas9. When bound, the nicked strand's free 3'-OH is used as the substrate
53 for the RT to copy genetic information from the pegRNA's 3' extension. If pegRNAs were designed
54 to produce modified nucleotides, these nucleotides would be inserted into the genome during
55 downstream repair processes (Anzalone et al., 2019). A second nick site present downstream of
56 the first nick site would support the retention of the introduced nucleotides. The PE approach
57 may also be an excellent alternative to GT with shorter editing sequence coverage (Van Vu et al.,
58 2019). Prime editors have been shown to work well in monocot plants by Lin and coworkers (Lin
59 et al., 2020). In the same year, other reports also showed the activities of PE complexes in
60 monocots such as rice (Butt et al., 2020; Hua et al., 2020; Li et al., 2020; Tang et al., 2020; Xu et
61 al., 2020) and maize (Jiang et al., 2020). However, the majority of the data showed successful PE
62 at the acetolactate synthase (ALS) locus. Very limited data regarding the applications of PE in
63 dicots were released, with relatively low efficacy in potato (Veillet et al., 2020) and tomato (Lu et
64 al., 2020), and this process would need to be improved for further applications.

65 Therefore, we sought to investigate the activity of PE complexes in plants using a transient assay
66 with a synthetic substrate in tobacco and stable transformation for editing ten loci in tomato.
67 Our data showed low PE efficiency at both the somatic cell and plant levels. Additional analyses
68 revealed the possible negative effects of the PE components on PE performance. Our work shows
69 both obstacles and important clues for the further improvement of the PE approach in plants.

70 **RESULTS AND DISCUSSION**

71 **The PE complexes did not perform better than the BE tool in a transient assay**

72 The PE tool includes an nCas9 fused with an RT enzyme and a pegRNA for binding to the
73 targeted sequence, and its 3' extension primes an RNA-dependent DNA polymerization reaction

74 by annealing to the sequence located upstream of the nicked site on the nontargeted strand
75 (Figure 1a). If a second nick is simultaneously added, we have a PE3 or PE3b approach,
76 depending on the position of the second nick outside or inside the area covered by the RT,
77 respectively (Anzalone et al., 2019). To quickly validate the PE activity in plants, we started our
78 PE experiments with a transient *Agrobacterium*-mediated infiltration system in tobacco leaves
79 that was designed to modify a single nucleotide of a synthetic substrate ((CA)n substrate)
80 (Figure 1b and 1c). A cytidine base editor (CBE) system using PmCDA1 cytosine deaminase was
81 used for comparison of the editing efficiency. The CBE design included a "-sgRNA" control
82 (pCEC1) and two test vectors, one with the T-DNA (pCE01) and one with a geminiviral replicon
83 system (pCE02) (Vu et al., 2020), for amplifying the editing tools (Figure 1c). The PE3b tools
84 were designed with both T-DNA and replicon cargos but also included dual vector systems to
85 avoid possible activity in bacterial cells. One vector carried the nCas9-RT expression cassette,
86 the synthetic substrate, and a sgRNA expression cassette for generating a second nick
87 (pPEsubc1 and pPEsubc2), and the other vector contained the pegRNA expression cassette
88 (pPEsub1 and pPEsub2) (Figure 1b and 1c). RT DNA was chemically synthesized as a tobacco
89 codon-optimized Moloney murine leukemia virus (M-MLV) RT based on the PE2 approach used
90 in the work of Anzalone and coworkers (Anzalone et al., 2019). pPEsubc1 and pPEsubc2 were
91 also used alone as controls for the PE experiments. Three temperature conditions were applied
92 to the infiltrated plants during the incubation stage to study the impacts of temperature on the
93 activities of the BE and PE complexes. Surprisingly, we observed a very strong C to T transition
94 at the 19th nucleotide counted from the PAM by BE in *Agrobacterium* cells (Figure 1d and 1e,
95 *Agrobacteria* panel) that led to indistinguishable BE activity at that position in tobacco leaves
96 (Figure 1d and 1e). By contrast, we observed BE activities in converting C to T at the 15th and
97 17th nucleotides in the tobacco leaves (Figure 1d and 1e). Interestingly, the BE activities were
98 reduced when the temperature increased from 25°C to 37°C (Figure 1e and 1f and
99 Supplemental Table 2). Our data also indicate that the replicon-based BE tool worked much
100 more efficiently than the T-DNA tool under transient expression conditions (Figure 1e and 1f
101 and Supplemental Table 2). Unexpectedly, we failed to show any PE activity with the transient
102 system using both the T-DNA and replicon systems. Furthermore, no improvement was

103 obtained using the temperature treatments (Figure 1g and 1h). One of the reasons for the
104 failure of the PE3b constructs might be due to the inefficiency of the dual *Agrobacterium*-
105 mediated infiltration that required simultaneous transfer of the two T-DNAs carrying PE
106 components into one cell.

107 **The PE tools showed low precise editing efficiencies at 10 tomato loci**

108 We decided to evaluate the ability of the PE system to edit genomic sites in tomato by
109 Agrobacterium-mediated stable transformations. The tomato transformation system was
110 efficiently used for GT experiments in our lab (Supplemental Figure 1) (Vu et al., 2020). We first
111 attempted to edit three tomato loci, SlHKT1;2, SlEPSPS1, and SlOr using PE2 and PE3
112 approaches with the T-DNA and replicon systems (Supplemental Figure 2 and 3 and Data S1).
113 Transformed explants collected at 10 days post-transformation (dpt) were subjected to
114 targeted deep sequencing of the targeted sites. However, in two replicates, we could not reveal
115 any evidence of PE activity that was higher than the background level of the targeted
116 sequencing method (Table 1). PE activity was shown to be locus-specific, and several
117 parameters, such as PBS and RT template lengths, may also affect PE efficiency in plants (Lin et
118 al., 2020). During our study, several PE data were reported that showed comparable PE
119 efficiencies among the PE2, PE3, and PE3b constructs (Butt et al., 2020; Hua et al., 2020; Lin et
120 al., 2020; Tang et al., 2020), and in some cases, the PE2 approach performed better than the
121 PE3 approach (Jiang et al., 2020; Veillet et al., 2020). We then extended the PE experiment to 7
122 more loci using the PE2 approach and single replicon-based vectors (Figure 2a and
123 Supplemental Table 3) to check whether the PE system would work in tomato. Most of the loci
124 were selected to require only single nucleotide changes, and the RT template lengths were
125 designed within the optimal ranges shown in reports on monocots. The PBS lengths were also
126 appropriately selected (Table 1). Using targeted deep sequencing analysis, we found a higher PE
127 performance at SlWH9, SlKD1, SlALC, and SlALS1 than at the mock control, albeit at low
128 absolute values (Table 1). The low PE efficiency might be partially explained by the sample type
129 (cotyledon explants) we collected for targeted deep sequencing. Since the cotyledon explants
130 (0.1 x 0.3 cm in size) contained many untransformed cells due to the nature of the
131 Agrobacterium-mediated transformation, they confer much lower editing efficiency than the

132 protoplast system. Therefore, we also analyzed plants regenerated from the transformed
133 cotyledon explants. We detected only low levels of PE alleles in several plants transformed with
134 the SlALC and SlALS1 PE constructs (Figure 2c and d). Sanger sequencing data revealed that only
135 1% of the PE alleles were successfully fixed in the plant genomes. Nevertheless, the PE
136 efficiency obtained at the plant stage in our work is comparable to that of recently published
137 data in tomato (Lu et al., 2020). Moreover, in one event at SlALC, two byproducts were also
138 formed, with frequencies of 1 and 3% (Figure 2d).

139 The very low PE efficiency obtained for 10 tomato loci based on various editing types under our
140 experimental conditions indicates that many critical factors affected the overall performance of
141 PE in tomato and other dicot plants, as also concluded previously (Veillet et al., 2020). To exclude
142 the possibility that our tobacco codon-optimized RT coding sequence may trigger aberrant
143 transcription or cryptic splicing, thereby affecting nCas9-RT activity, next, we used the nCas9-PPE
144 coding sequence that showed good PE activities in rice and wheat (Lin et al., 2020). The nCas9-
145 PPE prime editor is a rice codon-optimized RT that performed the best among the RTs tested by
146 Lin and coworkers. We constructed PE2 tools using nCas9-PPE with all 10 tomato loci tested
147 earlier (Figure 2b). However, targeted deep sequencing data collected for two replicates showed
148 similar levels of PE activity across all 10 loci. The PE efficiency was even reduced in the case of
149 the SlWH9 and SlKD1 loci but improved at the SlALC locus (Table 1), indicating that our tobacco
150 codon-optimized RT activity was not worse than the reported version. These data illustrate that
151 the PE approach needs improvement for further applications in tomato.

152 **The PE components negatively affect dsDNA cleavage**

153 In an attempt to find solutions for improving the PE performance, we sought to investigate the
154 roles of the PE components (i.e., the nCas9-RT and the pegRNAs) in the activation of the PE
155 nuclease complex and nicking the template strand. We constructed binary plasmids with fully
156 functional SpCas9 fused with RT or SpCas9 alone in combination with either pegRNAs or normal
157 sgRNAs for four loci (SlBMP21, SlALC, SlDMR6-1, and SlALS1) (Supplemental Figure 4) and
158 assessed their ability to generate indel mutations by targeted deep sequencing. Unexpectedly,
159 the Cas9-RT fusion combined with the pegRNAs did not show indel mutation efficiencies above

160 background levels at three (SlBPM21, SlDMR6-1, and SlALS1) out of the four loci (Figure 2e),
161 while the conventional Cas9-sgRNA complexes worked well at all the tested loci and
162 significantly higher at the SlDMR6-1 and SlALS1 loci (Figure 2e and Supplemental Table 4). Cas9-
163 RT combined with normal sgRNAs generated indel mutations at 2 of the 4 tested loci (SlBMP21
164 and SlALC), with indel mutation efficiencies similar to those of Cas9 and normal gRNAs (Figure
165 2e and Supplemental Table 4). The pegRNAs also supported the formation of indel mutations
166 when combined with the Cas9 nuclease at 3 out of the 4 tested loci (SlBMP21, SlALC, and
167 SlDMR6-1), but the indel mutation efficiencies were much low compared to that of the
168 Cas9/sgRNA complexes (Figure 2e and Supplemental Table 4). Either of the PE components
169 alone worked at extremely lower efficiencies compared to Cas9 with normal gRNA at the ALS1
170 locus. These data indicate that the PE components negatively affected the cleaving activity of
171 the Cas9 nuclease and that the use of pegRNAs led to reduced activity of the Cas9 enzyme
172 (Figure 2e). We reason that the Cas9-RT fusion might reduce the accessibility of the larger
173 active complexes (Cas9-RT is approximately 236 kDa, compared to ~158 kDa for SpCas9 alone)
174 to some genomic contexts, such as SlDMR6-1 and SlALS1, thereby blocking their cleavage
175 activity. Moreover, the pegRNAs shared intramolecular complementarity between PBS and
176 spacer sequences that potentially altered the secondary structure of the sgRNAs (Supplemental
177 Figure 5), leading to possible interference of SpCas9 activation and/or the ability of gRNA to
178 bind to targeted sequences and cleave them. The failure of the Cas9-RT/pegRNA complexes to
179 produce indel mutations at three out four loci and very low efficiency at the other locus might
180 have resulted from the combined negative impacts of both components. Based on these data,
181 we hypothesize that the activity of nCas9-RT/pegRNA complexes was affected by the negative
182 impacts of both RT fusion and pegRNA structure, which led to extremely low PE efficiency, and
183 by some unknown factor, resulting in especially low PE efficiencies in tomato and other dicot
184 plants (Lu et al., 2020; Veillet et al., 2020).

185 **Modifications of the PBS sequences did not significantly improve PE efficiency at three tested**
186 **loci**

187 If the above assumption is true, then we expect that when the PBS is shortened, the PE
188 efficiency will be improved. However, the PE data in plants suggested optimal ranges of PBS
189 lengths that could not be too short to prime RT polymerization (Lin et al., 2020). Higher
190 incubation temperatures may help to destabilize the PBS-spacer intramolecular interactions,
191 but they may also affect the activity of nuclease complexes or the activity of its target binding,
192 as observed with the transient BE data (Figure 1). That is also why Lu and coworkers could not
193 obtain a significant improvement in PE activity when they incubated explants at high
194 temperatures (Lu et al., 2020). During the preparation of this manuscript, Lin and coworkers
195 reported that the optimal melting temperature (Tm) of the PBS sequence was around 30°C for
196 PE in rice (Lin et al., 2021) reasoning that the PBS Tm affects the priming activity. Thus, we
197 hypothesize that a range of optimized melting temperature exists for an active PBS of pegRNA
198 in tomato. Alternatively, introducing several mismatches between the PBS and spacer
199 sequences may significantly weaken the interaction and improve PE activity in plants.

200 To test the hypothesis, we modified the pegRNAs of the three loci (SlBMP21, SlALC, and SlALS1)
201 by introducing mismatches to the original or shortened PBS sequences (Fig. 3a). Total four
202 different modifications to an original PBS were made and binary plasmids with replicon were
203 similarly constructed (Fig. 3a, b) to the original PE vectors (Fig. 2a) using the tobacco codon-
204 optimized RT. The PE efficiency was assessed by targeted deep sequencing. In three replicates,
205 the PE efficiencies at all the three tested loci were not significantly improved compared to that
206 of the original pegRNAs (Fig. 3c and Supplemental Table 5). These data were revealed even with
207 the modified PBS with Tm 30, 32, or 34°C compared to the original 38°C Tm (Fig. 3c and
208 Supplemental Table 5). It is still unknown if the way of introducing the mismatches was not
209 perfectly matched with the criteria of the PBS Tm optimization in the case of the data shown by
210 Lin and coworkers (Lin et al., 2021) or the mismatch itself was not sufficient to improve the PE
211 performance in tomato.

212 Since both the pegRNA and Cas9-RT fusions affected the cleaving activity of the complex, we
213 can also use a two-component PE complex that separates the nCas9 and RT polypeptides to
214 reduce the space constraint caused by the nCas9-RT fusion. However, the two-component

215 system may pose another risk for the proper assembly of the two components in a targeted
216 genomic context. Taken together, the PE approach offers an excellent alternative to precision
217 gene editing in plants. However, we could not obtain a better editing efficiency than that of the
218 BE tools in a transient assay in tobacco. Similar results were also recorded for 10 tomato loci
219 using stable *Agrobacterium*-mediated transformations. We revealed low but comparable PE
220 efficiency at the plant stage compared with recent data in plants. Further analyses showed that
221 the PE components (nCas9-RT fusion and pegRNA) might largely contribute to its low activity in
222 tomato. However, our PBS sequence modifications for reducing Tm were failed to improve the
223 PE efficiency. Significant improvement to the PE approach needs to be made for further
224 applications in tomato, and our data shed light on potential solutions for the improvement of
225 this editing tool.

226 **METHODS**

227 **System design for PE experiments in tobacco and tomato**

228 For the transient experiment, PE constructs were designed to modify two nucleotides of the (CA)n
229 substrate sequence using the 3' extension pegRNA (Figure 1a and 1b; Supplemental Data S1). A tobacco
230 codon-optimized RT DNA sequence was fused with a human codon-optimized SpCas9 nickase (H840A)
231 (nCas9-RT, Supplemental data S1) cloned using SpCas9 of the Addgene plasmid (#49771) that worked
232 well in plants (Belhaj et al., 2013). The PE experiment was performed by using dual agroinfiltration
233 (Figure 1c). For comparison, CBE constructs were designed to convert Cs to Ts, including the PE-targeted
234 C at position 17 counted from the PAM of the CBE gRNA (Figure 1b).

235 For PE of the selected tomato loci using *Agrobacterium*-mediated stable transformation (Supplemental
236 Figure 1), PE2 and PE3b approaches were applied for SlHKT1;2, SlEPSPS1, and SlOr (Supplemental
237 Figures 2 and 3). Further applications of the nCas9-RT (tobacco codon-optimized RT (PE2) (Anzalone et
238 al., 2019)),nCas9-PPE (rice codon-optimized RT (Lin et al., 2020)) systems using the original or modified
239 PBS sequences on the other seven loci (Supplemental Table 3) used a PE2 strategy (Figure 2a and 2b;
240 Supplemental Data S1).

241 In all binary vectors, the transcription of both CBE and nCas9-RT was driven by a long CaMV35S inserted
242 with a UBQ10 intron 1 (Vu et al., 2020). The CBE gRNA and PE pegRNA were cloned downstream of an

243 AtU6 promoter (Belhaj et al., 2013). The CBE and PE systems were delivered by either T-DNAs or
244 geminiviral replicons (Vu et al., 2020).

245 ***Agrobacterium*-mediated infiltration and sample analysis**

246 Leaves of tobacco (*Nicotiana benthamiana*) at the 6-leaf stage were infiltrated with *Agrobacteria*
247 containing binary plasmids. *Agrobacteria* (GV3101::pMP90 strain) were prepared by an overnight
248 primary culture with a subsequent 6-8 h secondary culture using liquid LB medium containing 75 mg/L
249 kanamycin, 30 mg/L rifampicin, and 30 mg/L streptomycin. *Agrobacterium* cells were collected by
250 centrifugation at 4,000 rpm for 10 minutes, resuspended in MS buffer containing 100 µM
251 acetosyringone to an OD_{600nm} of 0.8, and incubated for one hour before infiltration. At three days post-
252 infiltration, the infiltrated zones were cut, and total genomic DNA (gDNA) was isolated using the CTAB
253 method. PCRs were then conducted using primers flanking the targeted sequences (Supplemental Table
254 1), and the gDNA templates and PCR products were purified and sequenced by the Sanger method. The
255 editing efficiency was calculated based on the level of peaks of C and T at the targeted positions
256 revealed by the chromatograms (Figure 1 d, 1e, 1g, and 1h).

257 ***Agrobacterium*-mediated tomato transformation and assessment of PE efficiency**

258 *Agrobacterium*-mediated tomato transformation was performed using our in-house protocol
259 (Supplemental Figure 1) (Vu et al., 2020). Briefly, seven-day-old cotyledons were cut and used for
260 transformation. For targeted deep sequencing, thin cotyledon explants (0.1 x 0.3 cm) were used to
261 reduce the area containing untransformed cells beyond the cut edges of the explants. The *Agrobacteria*
262 containing the PE constructs were cultured and harvested as in the transient assay but were
263 resuspended in an ABM-MS solution (Vu et al., 2020) containing 100 µM acetosyringone to an OD_{600nm} of
264 0.8. The *Agrobacteria* were then incubated for one hour before the transformation. Regenerated shoots
265 were selected on a medium containing 80 mg/L kanamycin. Subsequently, hardened plants were
266 checked for PE activity.

267 For the assessment of PE efficiency, samples were collected at 10 dpt and analyzed by targeted deep
268 sequencing. At the plant stage, plant leaves were collected and screened for PE alleles by Sanger
269 sequencing, and targeted deep sequencing analysis was conducted for the events carrying PE alleles.

270 **Targeted deep sequencing**

271 Genomic DNA was isolated from cotyledon explants or plant leaves using the CTAB method. The
272 MiniSeq sequencing service (MiniSeqTM System, Illumina, USA) was used. MiniSeq samples were
273 prepared in three PCRs according to the manufacturer's guidelines, with genomic DNAs as templates for
274 the first PCR. The first and second PCRs used the primers listed in Supplemental Table 1, whereas the
275 third PCR was conducted with the manufacturer's primers to assign sample IDs. The MiniSeq raw data
276 FASTQ files were analyzed by the Cas-Analyzer tool (Park et al., 2016) and PE-Analyzer
277 (http://www.rgenome.net/pe-analyzer). The PE efficiency was assessed using the RT template
278 sequences as the input HDR donor sequence.

279 **Data analysis and presentation**

280 Most of the experiments analyzed by targeted deep sequencing were conducted in two to three
281 replicates. The editing data, statistical analysis if applicable, and scattered plots were further processed
282 by the MS Excel and Graphpad Prism 9.0 program. The multiple comparisons were conducted with the
283 uncorrected Fisher's LSD test. The data are appropriately explained in detail in the legends of figures
284 and/or tables.

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291 **COMPETING INTERESTS**

292 The authors declare no competing interests.

293 **AUTHOR CONTRIBUTIONS**

294 T.V.V. and J.Y.K. conceived and designed the research. T.V.V., J.K., and S.D. conducted the
295 experiments. T.V.V. and J.Y.K. analyzed the data. T.V.V. wrote the manuscript. T.V.V. and J.Y.K.
296 finalized the manuscript. All authors read and approved the manuscript.

297 **SHORT LEGENDS FOR SUPPORTING INFORMATION**

298 **List of Supplemental Tables**

299 Supplemental Table 1. Sequences and primers used in this study.

300 Supplemental Table 2. Base editing efficiency obtained in the tobacco leaf infiltration assay.

301 Supplemental Table 3. PE targets in tomato used in the study.

302 Supplemental Table 4. Comparisons of indel mutation efficiency of the Cas9 complexes
303 containing the PE components compared to the normal complex.

304 Supplemental Table 5. Comparisons of indel mutation efficiency of the Cas9 complexes
305 containing the PE components compared to the normal complex.

306 **List of Supplemental Figures**

307 Supplemental Figure 1: *Agrobacterium*-mediated transformation protocol used in this work.

308 Supplemental Figure 2: PE targeting maps of the first three loci. (A) SlHKT1;2. (B) SlEPSPS1. (C)
309 SlOr.

310 Supplemental Figure 3: PE constructs for editing the SlHKT1;2, SlEPSPS1 and SlOr loci by PE2
311 and PE3 approaches.

312 Supplemental Figure 4: Constructs used for the assessment of indel mutation formation by
313 different combinations of either Cas9-RT or Cas9 with either sgRNAs or pegRNAs at the
314 SlBMP21, SlALC, SlDMR6 and SlALS1 loci.

315 Supplemental Figure 5: MFOLD-predicted secondary structures of pegRNAs and sgRNAs of
316 SlBMP21, SlALC, SlDMR6-1 and SlALS1.

317 **List of Supplemental data file**

318 Supplemental data S1

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363 **Table 1. Prime editing efficiency in tomato using tobacco codon-optimized RT and rice codon-optimized PPE**

No.	Locus	Targeted change	PBS length (nt)	RT length (nt)	Second nick position	Mock control		nCas9-RT		nCas9-PPE	
						Total reads	PE-mediated precise editing efficiency (%)	Total reads	PE-mediated precise editing efficiency (%)	Total reads	PE-mediated precise editing efficiency (%)
1	SlHKT1;2	N217D: +21 T to C; +10 A to G (reverse complement)	12	26	+76 (PE3)	321183	0.007	104610	0.000	-	-
			12	26	PE2			151412	0.000	312118	0.000
2	SlEPSPS1	T178I: +16 TG to GA; P182S: +4 CGG to AGA (reverse complement)	15	20	-55 (PE3)	2526	0.000	1357	0.000	-	-
			15	20	PE2			1380	0.000	351540	0.000
3	SlOr	R95H: +1 AGG to CAT	12	14	+61 (PE3)	449607	0.000	140325	0.000	-	-
			12	14	PE2			155826	0.000	322070	0.000
4	SlMBP21	Premature Q108stop: +1 C to T	13	13	PE2	49811	0.018	57943	0.022	148426	0.019
5	WH9	G69D (s-classic): +6 G to A	13	14	PE2	59484	0.018	54735	0.110	202599	0.032
6	SlKD1	1 bp deletion at 1266 bp upstream of an open reading frame (ORF): +21 A del	13	26	PE2	44911	0.002	60765	0.018	169327	0.008
7	SlPRD	A46 deletion: DELLAVLG to DELLVLG: +18 CTG del	13	22	PE2	46727	0.000	50399	0.000	130662	0.000
8	SlALC	ALC to alc allele: T to A; V106D: +2 A to T (reverse complement)	13	12	PE2	51754	0.015	45543	0.042	159889	0.064
9	SlDMR6-1	Premature truncation of the protein; E140stop: +1 G to T	13	13	PE2	37532	0.128	50231	0.020	170289	0.021
10	SlALS1	P186S: +1 C to T	13	13	PE2	56496	0.057	63128	0.095	201599	0.095

364

365 **FIGURE LEGENDS**

366 **Figure 1. Failure of prime editing in tobacco cells in transient assays. (a)** Schematic processes
367 of primed editing. The nontargeted strand is nicked by CRISPR/Cas9 nickase (H840A), and the 3'
368 nicked end binds to a complementary RNA (priming binding site, PBS, 13-15 nt) introduced by
369 extending the 3' end of the sgRNA (RT template) and primes reverse transcription by the nCas9-
370 fused RT using its free OH group. RT copies the genetic information from the RNA template that
371 is complementary to the sequence downstream from the nicked site and includes intentionally
372 introduced base modifications (indicated by red and bright orange lines). The RT product
373 appears as a 3'-flap sequence that competes with the full complement of the original 5'-flap
374 during the repair process, and a precisely edited sequence could be fixed to the targeted site
375 after 5'-flap removal and subsequent replication of the DNA. A second nick is introduced during
376 the improvement of the prime editor for supporting 3'-edited flap fixation. **(b)** Selected
377 synthetic target and pegRNA design. pegRNA and its 3' extension and second nick spacer
378 sequences are indicated. A PmCDA1-based C->T editing tool is designed in parallel for
379 comparison, and its spacer sequence is also shown. Intended base modifications are denoted
380 and explained. **(c)** Vector arrangements for the study. The nCas9(D10A)-PmCDA1 base editing
381 system is designed with the control plasmid (pCEc, -gRNA), T-DNA (pCE1), and replicon (pCE2)-
382 based editors. The prime editors are designed with a dual vector system for T-DNA (pPEsubc1 +
383 pPEsub1) and replicon (pPEsubc2 + pPEsub2)-based tools for editing C->T and C->A (shown in
384 **b**). **(d-e)** Sequencing data showing C->T editing by T-DNA- (**d**) and replicon-based (**e**) tools
385 (Supplemental Table 2, (**e**)). The –sgRNA (pCEc) chromatogram represents all the temperature
386 tested. **(f)** Editing efficiency of the base editors. The T-DNA construct represents the pCE1
387 plasmid, and the replicon construct is for the pCE2 plasmid. The editing efficiencies at different
388 temperatures are plotted for the 15th, 17th, and 19th bases counted from the PAM site to its
389 upstream spacer sequence. **(g-h)** Failure of the T-DNA- (**g**) and replicon-based (**g**) prime editors
390 to edit the intended bases. The -pegRNA (pPEsubc1) and -pegRNA (pPEsubc2) chromatograms
391 represent for all the temperature tested. Discontinuous red boxes denote the intended
392 modifications in each of the editing conditions (25, 31, and 37°C) for the editors and the control.

393 **Figure 2. Prime editing performance in tomato. (a-b).** PE2 prime editing constructs using the
394 geminiviral replicon in tomato with synthetic tobacco codon-optimized RT (**a**, 7 loci) or rice
395 codon-optimized PPE (**b**, 10 loci). The NptII expression cassette (Addgene plasmid #51144) was
396 used for the kanamycin selection of transformed cells, tissues, and plants. The expression of the
397 prime editors was driven by the p35S promoter with the UBQ10 intron 1 and CaMV 35S
398 terminator (Data S1). The transcription of the pegRNAs was controlled by the AtU6 core
399 promoter sequence (Data S1). **(c)** PE performance data obtained from the analysis of the plant-
400 stage transformants of SlWH9, SlALC, and SlALS1 by Sanger sequencing and ICE Synthego. **(d)**
401 The traces of PE-edited alleles were obtained for SlALC (upper two panels) and SlALS1 (bottom
402 panel). The precisely edited alleles are shown at the bottom of each panel and are denoted by
403 "HDR" labels. The PE-mediated replaced bases are highlighted with an orange background. **(e)**
404 Scatter dot-bar plots showing the pairwise comparisons between the indel mutation efficiencies
405 of the Cas9-gRNA complexes with the PE components and that of the normal construction at
406 the SlBMP21, SlALC, SlDMR6-1, and SlALS1. The comparison at each locus is presented with the
407 locus name at the bottom of the plot. The combinations (denoted as "+") of nuclease complexes
408 are shown at the bottom of each scattered bar with Cas proteins (Cas9 and Cas9-RT fusion) and
409 guide RNAs (normal gRNA with gR prefix and pegRNA with pegR prefix, followed by the locus
410 name). The sequences flanking the targeted sites were amplified at 10 dpt and analyzed by
411 targeted deep sequencing in three replicates. The indel mutation efficiencies were revealed by
412 RGEN Cas-Analyzer. Multiple comparisons of the means and plotting were conducted by
413 GraphPad Prism version 9 using uncorrected Fisher's LSD test. The p-values of each compared
414 mean pair are shown on the top of the bars.

415 **Figure 3. Assessment of prime editing efficiency using modified PBS sequences. (a)**
416 Modifications of the previously tested pegRNAs by shortened and/or introducing mismatches
417 to their PBS sequences**.** There are four modifications that introduce mismatches to the original
418 PBS sequences (13 nt in length, pegR.BMPm1, pegR.BMPm2, pegR.ALCm1, pegR.ALCm2,
419 pegR.ALS1m1 and pegR.ALS1m2 for the BMP21, ALC and ALS1) or shortened PBS (10nt,
420 pegR.BMPm3, pegR.BMPm4, pegR.ALCm3, pegR.ALCm4, pegR.ALS1m3 and pegR.ALS1m4 for
421 the BMP21, ALC and ALS1) with mismatches**. (b)** PE2 prime editing constructs using the

422 modified pegRNAs. **(c)** Scatter dot-bar plots showing the pairwise comparisons between the PE
423 efficiencies with the original and modified pegRNAs at the SlBMP21, SlALC, and SlALS1. The
424 comparison at each locus is presented with the locus name at the bottom of the plot. The PBS
425 sequences are shown on the bottom of each bar plot with the red font letters represent
426 mismatches between the PBS and spacer sequences. The sequences flanking the targeted sites
427 were amplified at 10 dpt and analyzed by targeted deep sequencing in three replicates. The PE
428 efficiencies were revealed by RGEN Cas-Analyzer. Multiple comparisons of the means and
429 plotting were conducted by GraphPad Prism version 9 using uncorrected Fisher's LSD test. The
430 p-values of each compared mean pair are shown on the top of the bars.

Figure 1

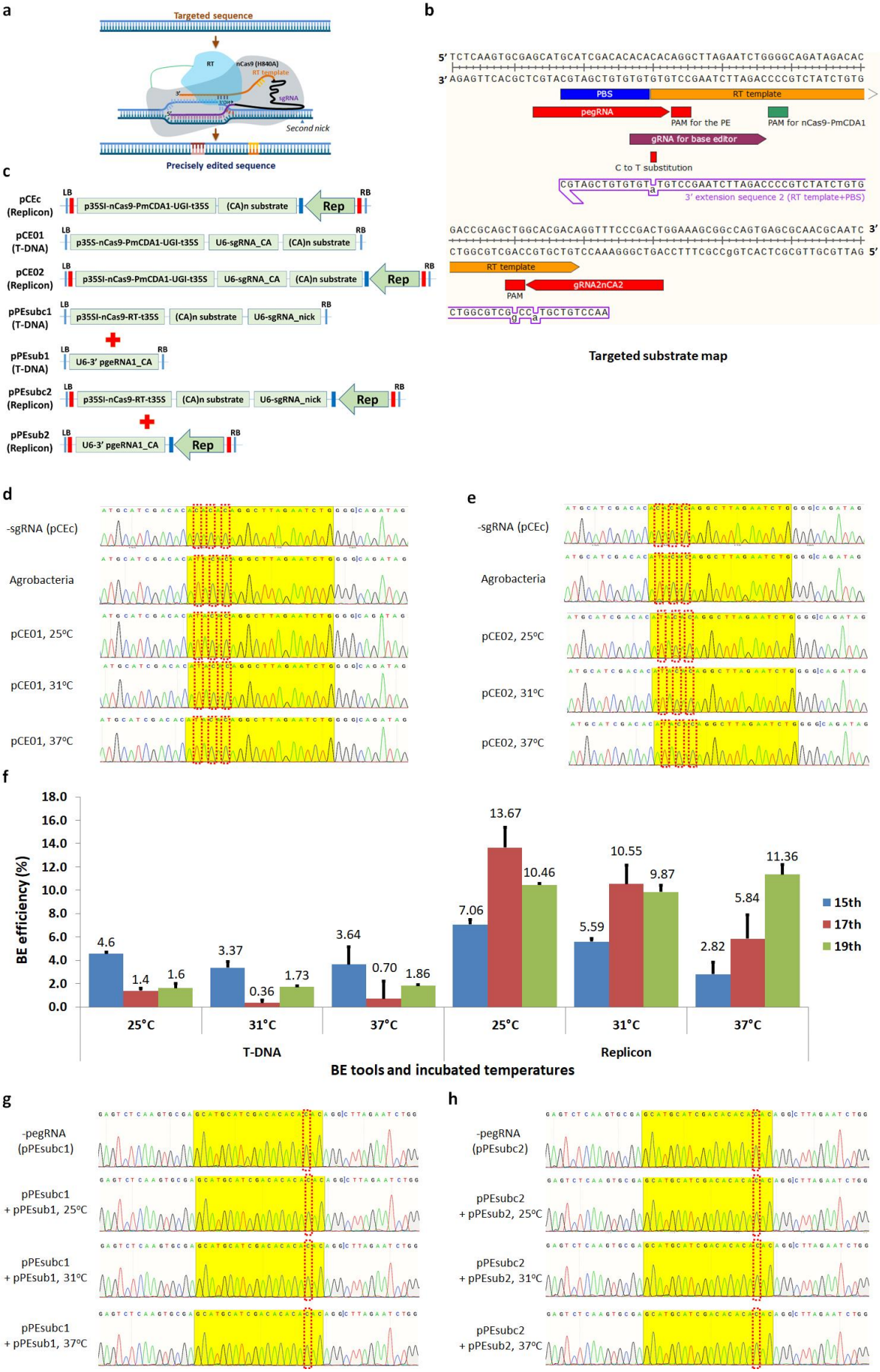
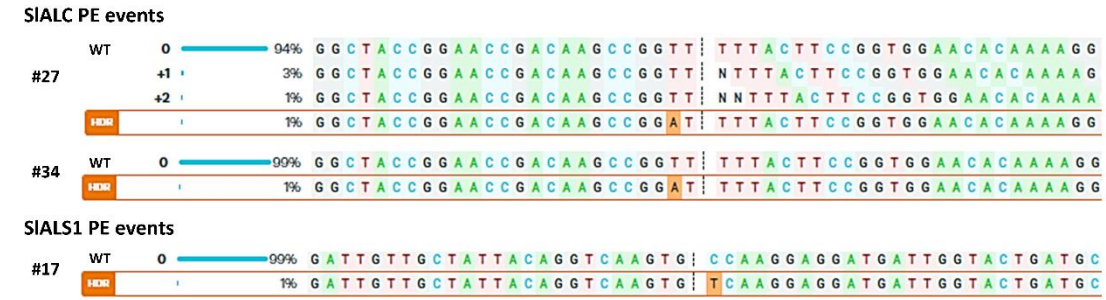

a
Targeted sequence
RT
nCas9 (H840A)
RT template
sgRNA
Second nick
Precisely edited sequence
b
5' TCTCAAGTGCGAGCATGCATCGACACACACACAGGCTTAGAATCTGGGGCAGATAGACAC
3' AGAGTTCACGCTCGTACGTAGCTGTGTGTGTGTCCGAATCTTAGACCCCGTCTATCTGTG
PBS
RT template
pegRNA
PAM for the PE
PAM for nCas9-PmCDA1
gRNA for base editor
C to T substitution
CGTAGCTGTGTGTGTGTCCGAATCTTAGACCCCGTCTATCTGTG
3' extension sequence 2 (RT template+PBS)
GACCGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGcCAGTGAGCGCAACGCAATC 3'
CTGGCGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCGCCgGTCACTCGCGTTGCGTTAG 5'
RT template
gRNA2nCA2
PAM
CTGGCGTCGCCATGCTGTCCAA
Targeted substrate map
c
pCEc (Replicon)
LB
p35SI-nCas9-PmCDA1-UGI-t35S
(CA)n substrate
Rep
RB
pCE01 (T-DNA)
p35S-nCas9-PmCDA1-UGI-t35S
U6-sgRNA_CA
pCE02 (Replicon)
pPEsubc1 (T-DNA)
p35SI-nCas9-RT-t35S
U6-sgRNA_nick
pPEsub1 (T-DNA)
U6-3' pgeRNA1_CA
pPEsubc2 (Replicon)
pPEsub2 (Replicon)
d
-sgRNA (pCEc)
Agrobacteria
pCE01, 25ºC
pCE01, 31ºC
pCE01, 37ºC
e
pCE02, 25ºC
pCE02, 31ºC
pCE02, 37ºC
f
BE efficiency (%)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
4.6
1.4
1.6
3.37
0.36
1.73
3.64
0.70
1.86
7.06
13.67
10.46
5.59
10.55
9.87
2.82
5.84
11.36
15th
17th
19th
25°C
31°C
37°C
T-DNA
Replicon
BE tools and incubated temperatures
g
-pegRNA (pPEsubc1)
pPEsubc1 + pPEsub1, 25ºC
pPEsubc1 + pPEsub1, 31ºC
pPEsubc1 + pPEsub1, 37ºC
h
-pegRNA (pPEsubc2)
pPEsubc2 + pPEsub2, 25ºC
pPEsubc2 + pPEsub2, 31ºC
pPEsubc2 + pPEsub2, 37ºC

Figure 1. Failure of prime editing in tobacco cells in transient assays. (a) Schematic processes of primed editing. The nontargeted strand is nicked by CRISPR/Cas9 nickase (H840A), and the 3' nicked end binds to a complementary RNA (priming binding site, PBS, 13-15 nt) introduced by extending the 3' end of the sgRNA (RT template) and primes reverse transcription by the nCas9-fused RT using its free OH group. RT copies the genetic information from the RNA template that is complementary to the sequence downstream from the nicked site and includes intentionally introduced base modifications (indicated by red and bright orange lines). The RT product appears as a 3'-flap sequence that competes with the full complement of the original 5'-flap during the repair process, and a precisely edited sequence could be fixed to the targeted site after 5'-flap removal and subsequent replication of the DNA. A second nick is introduced during the improvement of the prime editor for supporting 3'-edited flap fixation. **(b)** Selected synthetic target and pegRNA design. pegRNA and its 3' extension and second nick spacer sequences are indicated. A PmCDA1-based C->T editing tool is designed in parallel for comparison, and its spacer sequence is also shown. Intended base modifications are denoted and explained. **(c)** Vector arrangements for the study. The nCas9(D10A)-PmCDA1 base editing system is designed with the control plasmid (pCEc, -gRNA), T-DNA (pCE1), and replicon (pCE2)-based editors. The prime editors are designed with a dual vector system for T-DNA (pPEsubc1 + pPEsub1) and replicon (pPEsubc2 + pPEsub2)-based tools for editing C->T and C->A (shown in **b**). **(d-e)** Sequencing data showing C->T editing by T-DNA- (**d**) and replicon-based (**e**) tools (Supplemental Table 2, (**e**)). The –sgRNA (pCEc) chromatogram represents all the temperature tested. **(f)** Editing efficiency of the base editors. The T-DNA construct represents the pCE1 plasmid, and the replicon construct is for the pCE2 plasmid. The editing efficiencies at different temperatures are plotted for the 15th, 17th, and 19th bases counted from the PAM site to its upstream spacer sequence. **(g-h)** Failure of the T-DNA- (**g**) and replicon-based (**g**) prime editors to edit the intended bases. The -pegRNA (pPEsubc1) and -pegRNA (pPEsubc2) chromatograms represent for all the temperature tested. Discontinuous red boxes denote the intended modifications in each of the editing conditions (25, 31, and 37°C) for the editors and the control.

Figure 2

c

Locus	Total analyzed events	Event carrying PE allele	Event carrying unintended allele	The highest PE allele level (%)
SlHKT1;2	17	0	3	0
SlEPSPS1	15	0	1	0
SlWH9	17	0	0	0
SlALC	42	2	1	1
SlALS1	22	1	0	1



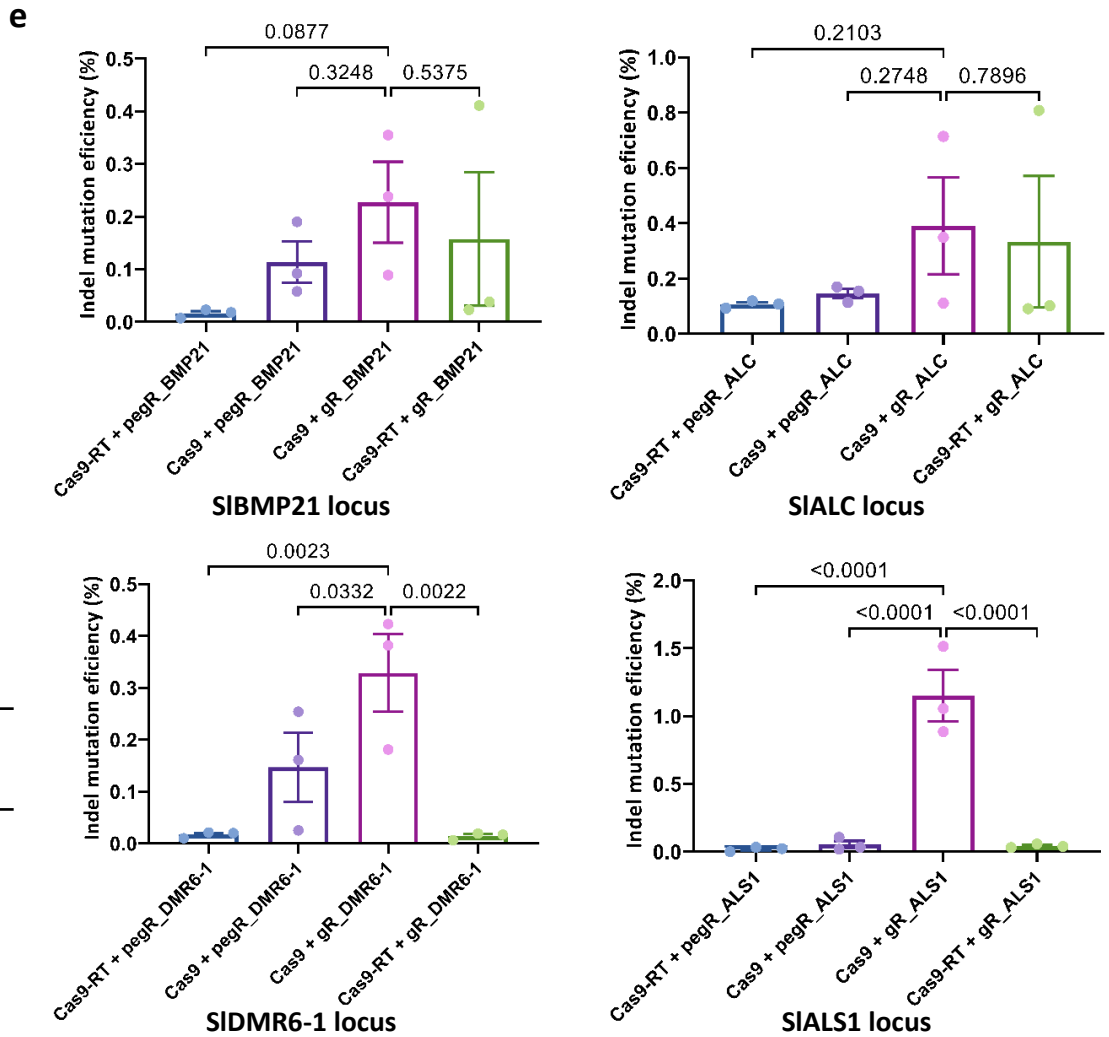


Figure 2. Prime editing performance in tomato. (a-b). PE2 prime editing constructs using the geminiviral replicon in tomato with synthetic tobacco codon-optimized RT (**a**, 7 loci) or rice codon-optimized PPE (**b**, 10 loci). The NptII expression cassette (Addgene plasmid #51144) was used for the kanamycin selection of transformed cells, tissues, and plants. The expression of the prime editors was driven by the p35S promoter with the UBQ10 intron 1 and CaMV 35S terminator (Data S1). The transcription of the pegRNAs was controlled by the AtU6 core promoter sequence (Data S1). **(c)** PE performance data obtained from the analysis of the plant-stage transformants of SlWH9, SlALC, and SlALS1 by Sanger sequencing and ICE Synthego. **(d)** The traces of PE-edited alleles were obtained for SlALC (upper two panels) and SlALS1 (bottom panel). The precisely edited alleles are shown at the bottom of each panel and are denoted by "HDR" labels. The PE-mediated replaced bases are highlighted with an orange background. **(e)** Scatter dot-bar plots showing the pairwise comparisons between the indel mutation efficiencies of the Cas9-gRNA complexes with the PE components and that of the normal construction at the SlBMP21, SlALC, SlDMR6-1, and SlALS1. The comparison at each locus is presented with the locus name at the bottom of the plot. The combinations (denoted as "+") of nuclease complexes are shown at the bottom of each scattered bar with Cas proteins (Cas9 and Cas9-RT fusion) and guide RNAs (normal gRNA with gR prefix and pegRNA with pegR prefix, followed by the locus name). The sequences flanking the targeted sites were amplified at 10 dpt and analyzed by targeted deep sequencing in three replicates. The indel mutation efficiencies were revealed by RGEN Cas-Analyzer. Multiple comparisons of the means and plotting were conducted by GraphPad Prism version 9 using uncorrected Fisher's LSD test. The p-values of each compared mean pair are shown on the top of the bars.

Figure 3

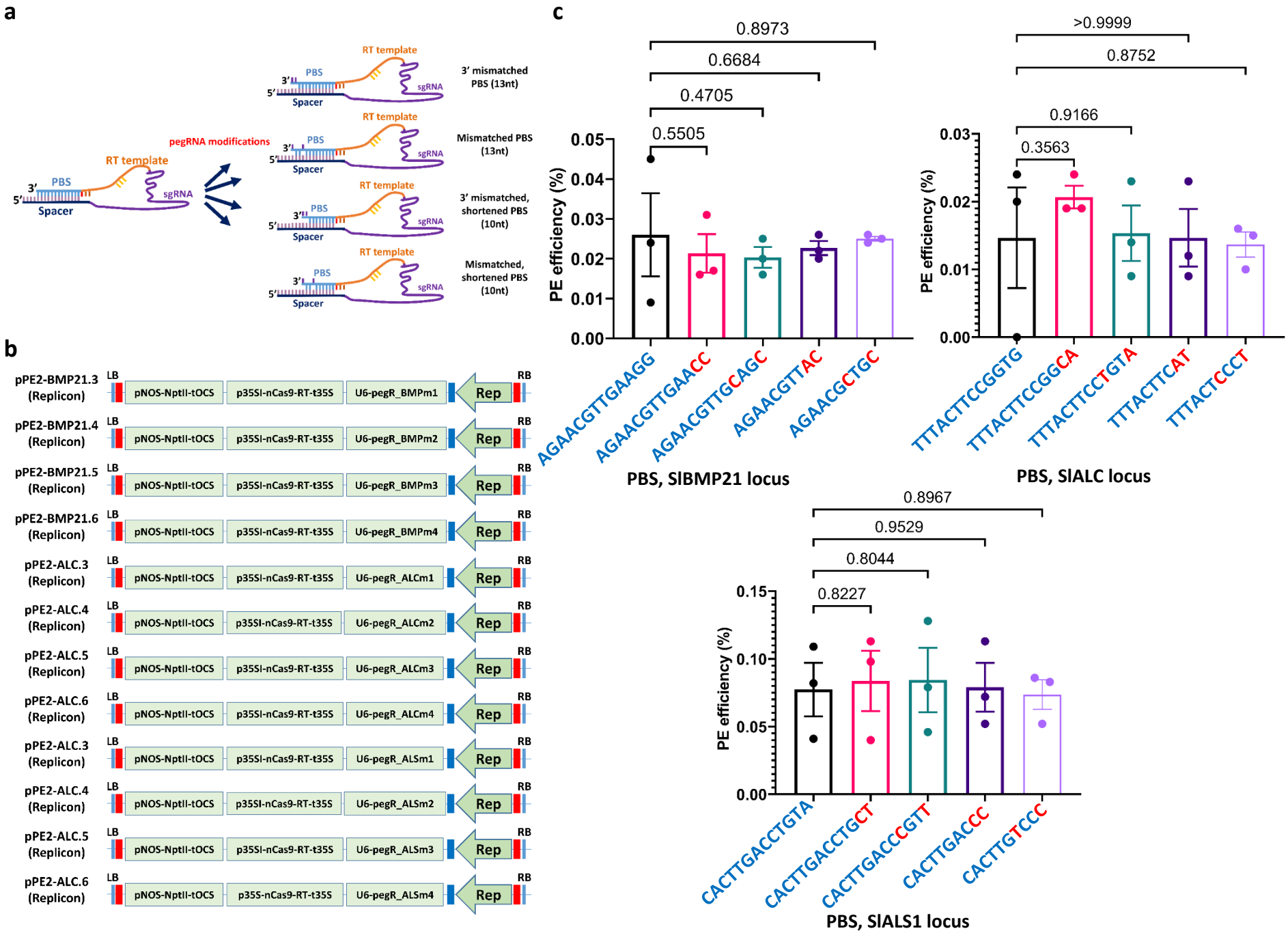

a
RT template
PBS
3'
5'
sgRNA
Spacer
pegRNA modifications
3' mismatched PBS (13nt)
Mismatched PBS (13nt)
3' mismatched, shortened PBS (10nt)
Mismatched, shortened PBS (10nt)
b
pPE2-BMP21.3 (Replicon)
pPE2-BMP21.4 (Replicon)
pPE2-BMP21.5 (Replicon)
pPE2-BMP21.6 (Replicon)
pPE2-ALC.3 (Replicon)
pPE2-ALC.4 (Replicon)
pPE2-ALC.5 (Replicon)
pPE2-ALC.6 (Replicon)
pPE2-ALC.3 (Replicon)
pPE2-ALC.4 (Replicon)
pPE2-ALC.5 (Replicon)
pPE2-ALC.6 (Replicon)
LB
RB
pNOS-NptII-tOCS
p35SI-nCas9-RT-t35S
p35S-nCas9-RT-t35S
U6-pegR_BMPm1
U6-pegR_BMPm2
U6-pegR_BMPm3
U6-pegR_BMPm4
U6-pegR_ALCm1
U6-pegR_ALCm2
U6-pegR_ALCm3
U6-pegR_ALCm4
U6-pegR_ALSm1
U6-pegR_ALSm2
U6-pegR_ALSm3
U6-pegR_ALSm4
Rep
c
PE efficiency (%)
0.8973
0.6684
0.4705
0.5505
AGAACGTTGAAGG
AGAACGTTGAACC
AGAACGTTGCAGC
AGAACGTTAC
AGAACGCTGC
PBS, SlBMP21 locus
>0.9999
0.8752
0.9166
0.3563
TTTACTTCCGGTG
TTTACTTCCGGCA
TTTACTTCCTGTA
TTTACTTCAT
TTTACTCCCT
PBS, SlALC locus
0.8967
0.9529
0.8044
0.8227
CACTTGACCTGTA
CACTTGACCTGCT
CACTTGACCCGTT
CACTTGACCC
CACTTGTCCC
PBS, SlALS1 locus

Figure 3. Assessment of prime editing efficiency using modified PBS sequences. (a) Modifications of the previously tested pegRNAs by shortened and/or introducing mismatches to their PBS sequences**.** There are four modifications that introduce mismatches to the original PBS sequences (13 nt in length, pegR.BMPm1, pegR.BMPm2, pegR.ALCm1, pegR.ALCm2, pegR.ALS1m1 and pegR.ALS1m2 for the BMP21, ALC and ALS1) or shortened PBS (10nt, pegR.BMPm3, pegR.BMPm4, pegR.ALCm3, pegR.ALCm4, pegR.ALS1m3 and pegR.ALS1m4 for the BMP21, ALC and ALS1) with mismatches**. (b)** PE2 prime editing constructs using the modified pegRNAs. **(c)** Scatter dot-bar plots showing the pairwise comparisons between the PE efficiencies with the original and modified pegRNAs at the SlBMP21, SlALC, and SlALS1. The comparison at each locus is presented with the locus name at the bottom of the plot. The PBS sequences are shown on the bottom of each bar plot with the red font letters represent mismatches between the PBS and spacer sequences. The sequences flanking the targeted sites were amplified at 10 dpt and analyzed by targeted deep sequencing in three replicates. The PE efficiencies were revealed by RGEN Cas-Analyzer. Multiple comparisons of the means and plotting were conducted by GraphPad Prism version 9 using uncorrected Fisher's LSD test. The p-values of each compared mean pair are shown on the top of the bars.

The obstacles and potential clues of prime editing applications in tomato, a dicot plant

Tien Van Vu[1,2,*], Jihae Kim[1], Swati Das[1], Jinsu Lee[1], Jae-Yean Kim[1,3,*]

[1]Division of Applied Life Science (BK21 FOUR Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea.

[2]National Key Laboratory for Plant Cell Biotechnology, Agricultural Genetics Institute, Km 02, Pham Van Dong Road, Co Nhue 1, Bac Tu Liem, Hanoi 11917, Vietnam.

[3]Division of Life Science, Gyeongsang National University, 501 Jinju-daero, Jinju 52828, Republic of Korea.

***Correspondence: Tien Van Vu (tienvu.agi@gmail.com; ORCID: 0000-0002-6369-7664). Jae-Yean Kim (kimjy@gnu.ac.kr; ORCID ID: 0000-0002-1180-6232)**.

Keywords: Prime editor; CRISPR/SpCas9; Precision gene editing; Prime editing; DSB-independent gene editing, Geminiviral replicon.

SUPPLEMENTAL FIGURES, TABLES AND REFERENCES

Supplemental Figures, Tables and References

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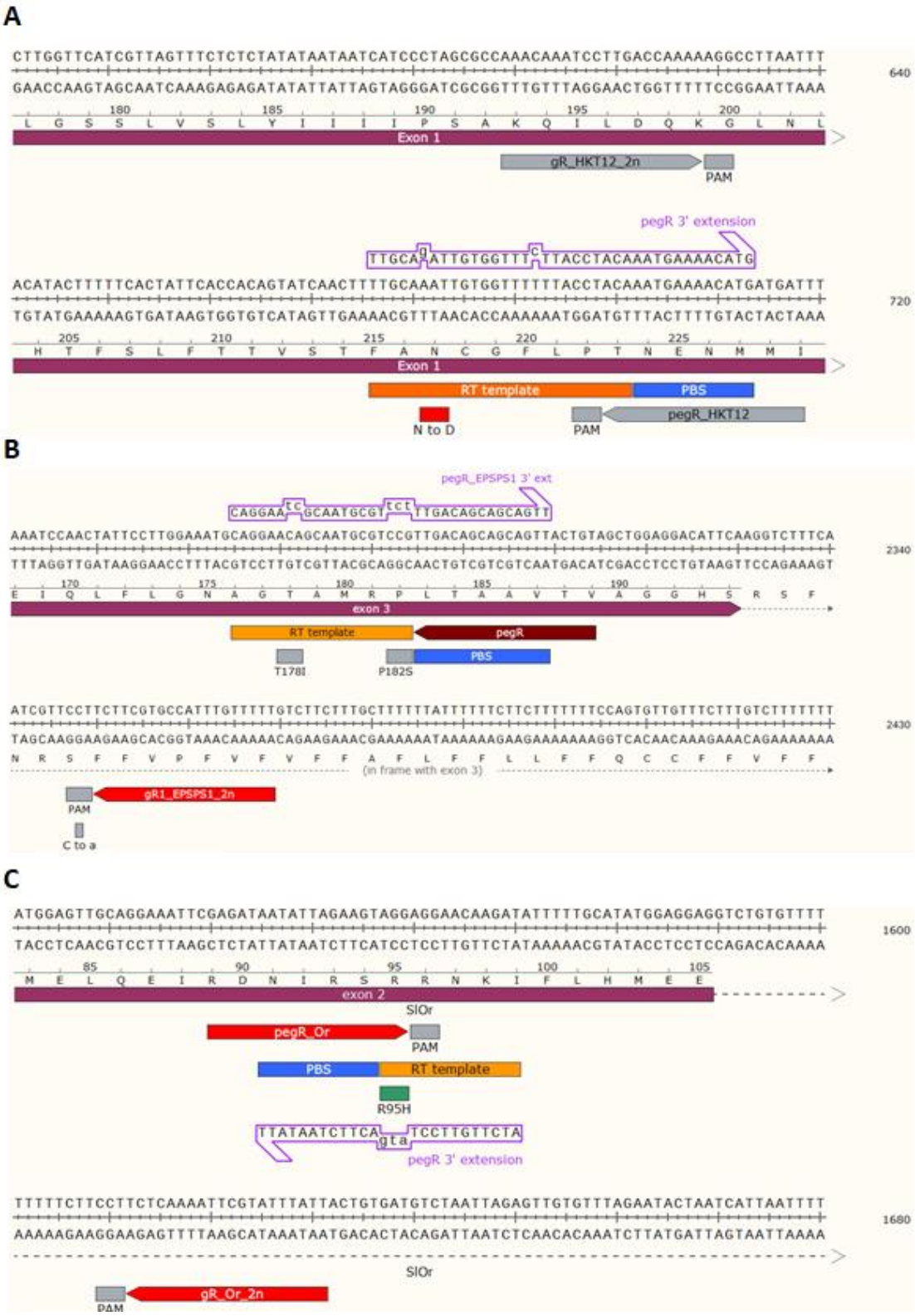
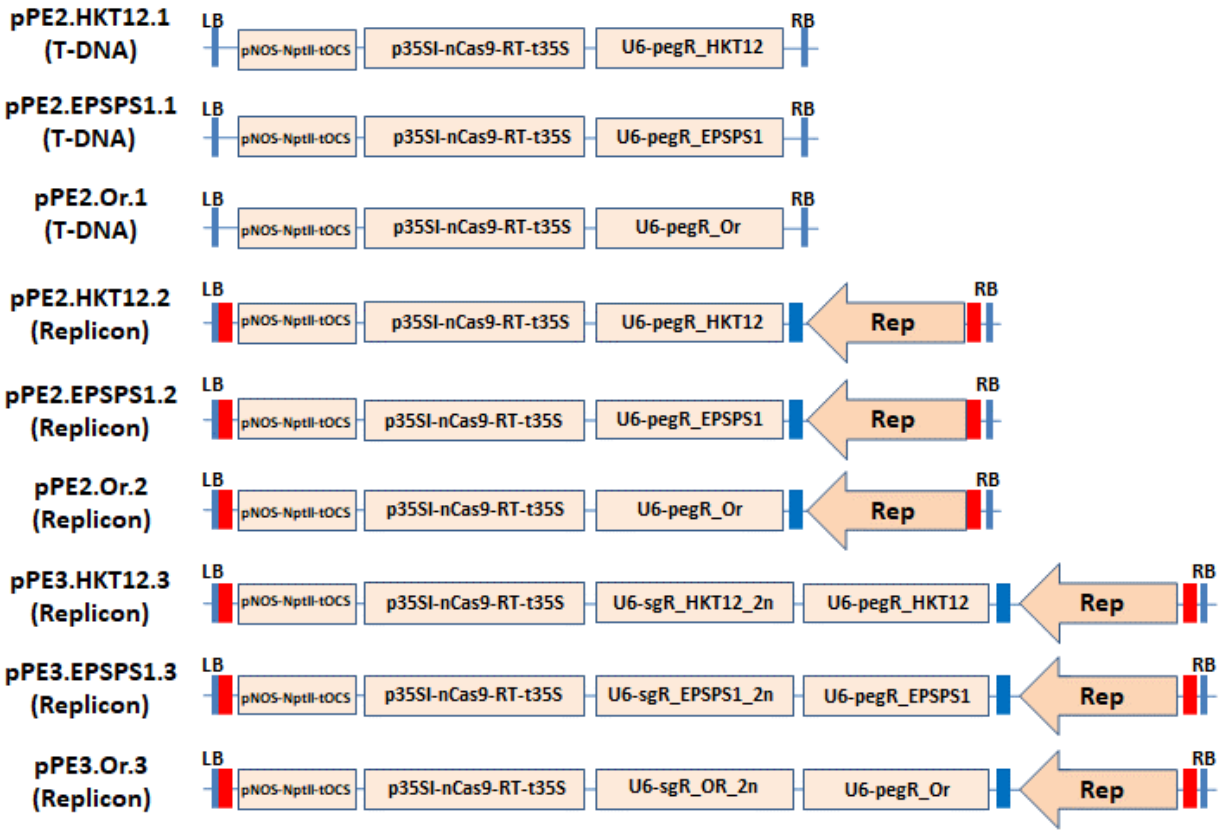
Stage	Germination	Pre-culture, callus induction	Co-cultivation, callus induction	Selection, callus induction and shoot regeneration	Selection, callus induction and shoot regeneration	Selection, shoot formation and elongation	Selection, root formation
Medium	1/2MSO	PREMC	ABM-MS+AS	SEL4-80	SEL4-80	SEL4-80	RIM
Temperature (°C)	25±2	25±1	25±1	28±1 for 5 days and then 25±2	25±2	25±2	25±2
Photoperiod	3 days-dark and then 4days 16L/8D	1 day dark	2 days-dark	5 days-8L/16D and then 9 days-16L/8D	14 days 16L/8D	14 days 16L/8D	10-14 days 16L/8D
Data collection (if needed)	-	-			Sampling at 10dpt		Sampling at plant stage

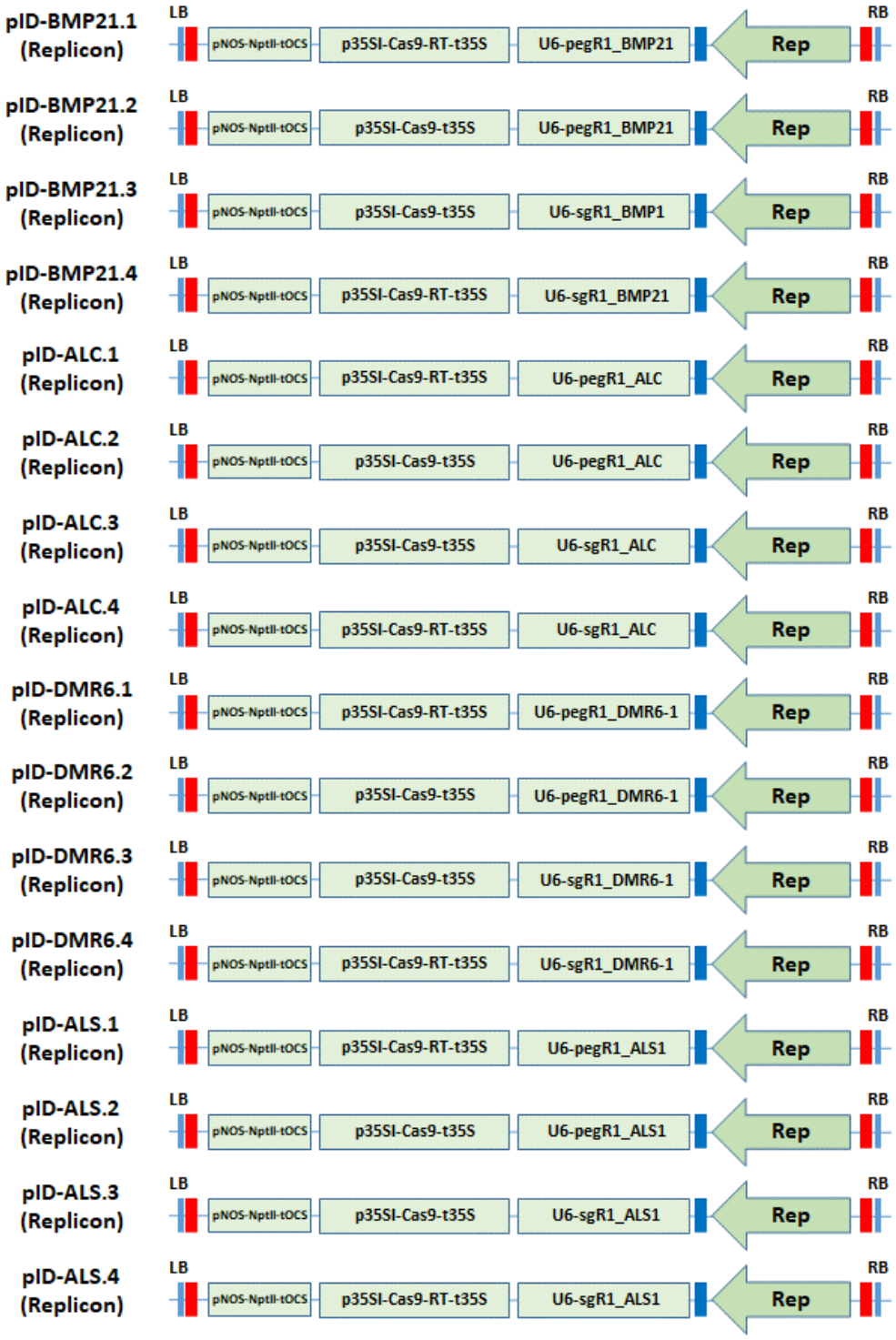
Supplemental Figure 1: *Agrobacterium*-mediated transformation protocol used in this work. The step-by-step protocol is presented with each number in the circles indicating the number of days after seed sowing (upper panel), and the treatments used in each step are shown in the lower panel.



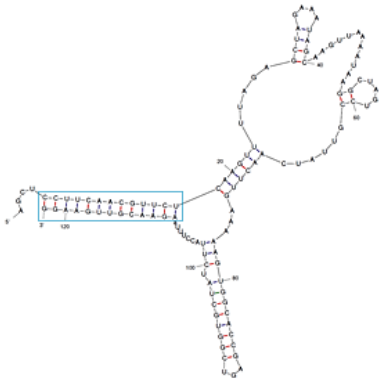
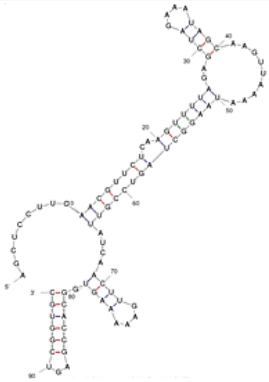
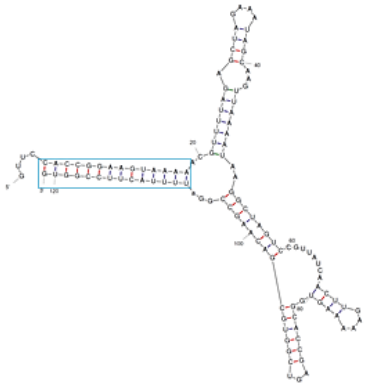
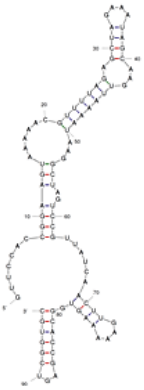
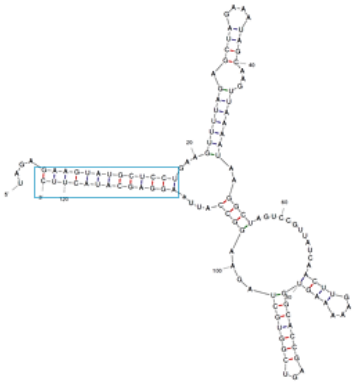
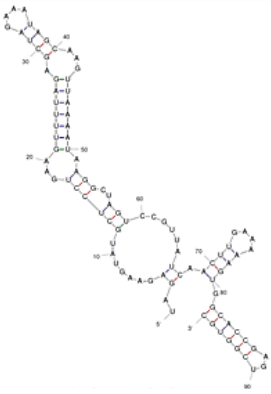
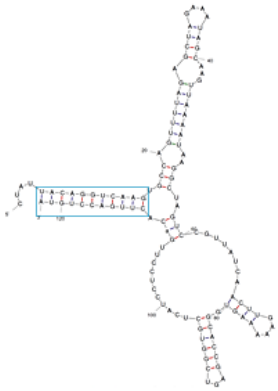
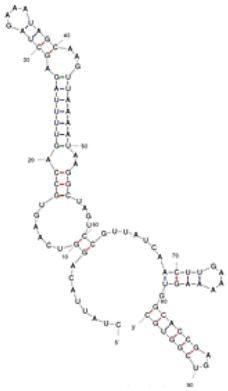
Supplemental Figure 2: PE targeting maps of the first three loci. (A) SlHKT1;2. (B) SlEPSPS1. (C) SlOr.



Supplemental Figure 3: PE constructs for editing the SlHKT1;2, SlEPSPS1 and SlOr loci by PE2 and PE3 approaches.



Supplemental Figure 4: Constructs used for the assessment of indel mutation formation by different combinations of either Cas9-RT or Cas9 with either sgRNAs or pegRNAs at the SlBMP21, SlALC, SlDMR6 and SlALS1 loci. All the component were expressed with the geminiviral replicon system.

	pegRNA secondary structure	sgRNA secondary structure
SlBMP21	 dG=-36.96 kcal/mol	 dG=-22.70 kcal/mol
SlALC	 dG=-44.98 kcal/mol	 dG=-23.20 kcal/mol
SlDMR6-1	 dG=-41.57 kcal/mol	 dG=-21.50 kcal/mol
SlALS1	 dG=-37.28 kcal/mol	 dG=-24.20 kcal/mol

Supplemental Figure 5: MFOLD-predicted secondary structures of pegRNAs and sgRNAs of SlBMP21, SlALC, SlDMR6-1 and SlALS1. The pegRNA structures (left panel) and sgRNA structures (right panel) were predicted using MFOLD software. The deltaG (dG) of each structure is denoted at the bottom of it. The discontinuous light blue boxes indicate the intermolecular annealing of the PBS and spacer sequences.

Supplemental Table 1. Sequences and primers used in this study

No.	Name	Sequence (5'-3')	Note
Primers for PCR amplification of sequences flanking targeted sites			
1	t35S-F1	TGTGTGAGTAGTTCCCAGATAAG	Sequences flanking the (CA)n substrate of pCEc and pCE02
2	RRA-R8	CTGACGAGGACTGGATGTTATC	
3	RB-qF2	CTCTTAGGTTTACCCGCCAATA	Sequences flanking the (CA)n substrate of pCE01
4	PmCDA1-R1	GATGGTTTCCGAGCACTATCA	
5	RT-F1	AGGCTAGAGGTAACCGGATG	Sequences flanking the (CA)n substrate of pEsubc1
6	PE-R1	TCGGTCACATGTGCATCCTC	
7	RT-F1	AGGCTAGAGGTAACCGGATG	Sequences flanking the (CA)n substrate of pEsubc2
8	PE-R2	CTGCGATATTACCGAGGACTAC	
9	t35S-sF1	TAGGGTTTCGCTCATGTGTTG	sequencing
Primers for targeted deep sequencing sample preparation			
9	HKPE-F1	ACAAAGATTATGAGCTAGGGAATGT	First PCR for SlHKT1;2
10	HKPE-R1	CCTTCAGAATTCCACTCCAATGA	
11	HKPE-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT CGCCAAACAAATCCTTGACC	Second PCR for SlHKT1;2
12	HKPE-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTTGAGGGATAAGAATGAGAAGAAGAC	
13	EPPE-F1	TGAGTTACTTACAACCTTGTGCT	First PCR for SlEPSPS1
14	EPPE-R1	GTAATTTATCACCAGGGCAACTATTATC	
15	EPPE-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGCAGTTTCCTGTCGGTAA	Second PCR for SlEPSPS1
16	EPPE-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTAAACAAATGGCACGAAGAAGG	
17	ORPE-F1	ATGCGAATCGTAGAAATGGAATTAG	First PCR for SlOr
18	ORPE-R1	ACTCGTTTCTTCGGTCCTTAAT	
19	ORPE-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AGGACCTGAGACAGTAGA	Second PCR for SlOr
20	ORPE-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTAGACATCACAGTAATAAATACGA	
21	BMP-F1	CCGGTTAGTGATACTCAGGTATTG	First PCR for SlBMP21
22	BMP -R1	TGACTGCTTCCTAAGATGCTAAA	
23	BMP-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT CTCGACGTTTTTCTCTTTCAACAGAT	Second PCR for SlBMP21
24	BMP-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT CACTTATTAAAAACTTATGTAAATCTAAC	
25	WH9-F1	GGAGTCTATATAAGAAGTGTGTTAGGG	First PCR for SlWH9
26	WH9-R1	TCATTAAGAAGAAGTCCAGAAGAAGA	
27	WH9-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT CAGAGCCAAAGCCTCGAT	Second PCR for SlWH9

28	WH9-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CTAGCATCACCTACTTGACCATATT	
29	KD1-F1	TTTGGTTACAACGTGTGAGATAAC	First PCR for SlKD1
30	KD1-R1	ACCATGTATAGTAGTCGTGTATGTT	
31	KD1-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT ACACACAATCTGTAACAATGATAGTAAT	Second PCR for SlKD1
32	KD1-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT CCTGACTAGTTTGAAGGGTATGT	
33	PRD-F1	GTGAATATTATTGCTGGCCTGTT	First PCR for SlPRD
34	PRD-R1	CAACTAGTACAACAGGTCTCGT	
35	PRD-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGTGCTGTTTCTTCAGGGAA	Second PCR for SlPRD
36	PRD-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT TAGCCATCTCAAGCTGTTCAAG	
37	ALC-F1	AAGTAGTGGACAAACATAAAGTAGTGG	First PCR for SlALC
38	ALC-R1	AATAATCTATGTCGAACCTCTTTCGG	
39	ALC-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATATCCTAACGGGGCGAGGCCAAAT	Second PCR for SlALC
40	ALC-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT GCATGATCCAATTAGTTTTTACCCCT	
41	DMR-F1	CTTTCAGGTTCTTATCGTTGGATTC	First PCR for SlDMR6-1
42	DMR-R1	AGGTTAGCTAGGTAGATTAGGTAGT	
43	DMR-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGAGACAGTTCATAATTGGAGAGATTAT	Second PCR for SlDMR6-1
44	DMR-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT TTGAAGTATGACTCAGATAGCA	
45	ALS-F1	CCTCACCATCTCCATGTTTCTC	First PCR for SlALS1
46	ALS-R1	GTCTCAGCTCCTCACTTGATTG	
47	ALS-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT GCTACAAATCTTGTTAGTGGTCTTG	Second PCR for SlALS1
48	ALS-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CT GCTTCGTAATAGATCTCGTTACCTC	
49	HKT12-sF1	CAAAGATTATGAGCTAGGGAATGT	Sequencing primer for the SlHKT1;2 targeted site
50	EPSPS1-sF3	GGAGATATTTGTTAGAGTACCATCA	Sequencing primer for the SlEPSPS1 targeted site
51	WH9-sR1	CACCGAAGAAGTAGGAGAATTGA	Sequencing primer for the SlWH9 targeted site
52	ALC-sF1	CATGTGTTTAACGCAGCTAAGG	Sequencing primer for the SlALC targeted site
53	ALS1-sF1	CCCAGAAAGGGTTGTGATGT	Sequencing primer for the SlALS1targeted site

Supplemental Table 2. Base editing efficiency obtained in the tobacco leaf infiltration assay

Position counted from PAM	T-DNA			Replicon		
	25°C	31°C	37°C	25°C	31°C	37°C
15th	4.57 ± 0.14	3.37 ± 0.55	3.64 ± 1.5	7.06 ± 0.41	5.59 ± 0.25	2.82 ± 1.02
17th	1.38 ± 0.21	0.36 ± 0.22	0.70 ± 1.5	13.67 ± 1.74	10.55 ± 1.63	5.84 ± 2.03
19th	1.63 ± 0.41	1.73 ± 0.10	1.86 ± 0.03	10.46 ± 0.10	9.87 ± 0.57	11.36 ± 0.83

Supplemental Table 3. PE targets in tomato used in the study

No.	Gene/allele name	Accession no.	Genotype vs WT	Phenotype	Reference
1	*jointless-2/*SlMBP21	Solyc12g038510	L169Stop (T->A)	Absence of abscission layer in Pedicels/short pedicels	Roldan et al., 2017
2	WUSCHEL HOMEOBOX 9 (SlWH9)/*compound inflorescence (s)*	Solyc02g077390.1	G69D (s-classic)	Mutant alleles of s dramatically increase branch and flower number	Lippman et al., 2008
3	*Petroselinum (Pts) allele/KNOTTED 1-LIKE HOMEOBOX (KNOX)/SlKD1 (TOMATO KNOX-LIKE HOMEODOMAIN PROTEIN 1)*	*AF375969/* Solyc06g072480.1	1 bp deletion at 1266 bp upstream of the open reading frame (ORF)	A semidominant KD1 mutant in tomato with multiply compound leaves. TKD1 silencing delay absicion.	Kimura et al., 2008
4	PROCERA D/DELLA D allele (SlPRD)	Solyc11g011260	A46 deletion: DELLAVLG to DELLVLG	Dwarf/gibberellin-responsive dominant dwarf DELLA allele	Nir et al., 2017; Tomlinson et al., 2019
5	SlALC (Alcobaca)	FJ404469	ALC to alc allele: T to A; V106D	Long-shelf life	Garg et al., 2008
6	SlDMR6-1	Solyc03g080190.2	small deletions in the *SlDMR6-1* gene that result in frameshift and premature truncation of the protein	No significant detrimental effects in terms of growth and development under greenhouse conditions and show disease resistance against different pathogens, including *P. syringae*, *P. capsici* and *Xanthomonas* spp.	Thomazella et al., 2016
7	SlALS1 gene	Solyc03g044330	Any substitution to change Pro186 amino acid to Ser, Ala or Thr residues	Strong resistance to chlorsulfuron. After two weeks, explants were transferred onto a fresh culture medium with 40 ng/mL chlorsulfuron.	Yu et al., 2009; Veillet et al., 2019

Supplemental Table 4. Comparisons of indel mutation efficiency of the Cas9 complexes containing the PE components compared to the normal complex.

Gene/allele name	Accession no.	Construction	PBS length (nt)	RT length (nt)	Total reads	Indel mutation efficiency (%)					p value** (Uncorrected Fisher's LSD)
						Replicate 1	Replicate 2	Replicate 3	Average	SEM	
SlBMP21	Solyc12g038510	-*	-	-	49819	0.026	-	-	0.026	-	-
		Cas9-RT + pegR_BMP21	13	13	132955	0.018	0.007	0.023	0.016	0.005	0.0877
		Cas9 + pegR_BMP21	13	13	139784	0.092	0.190	0.058	0.113	0.040	0.3248
		Cas9 + gR_BMP21	0	0	127696	0.355	0.238	0.089	0.227	0.077	
		Cas9-RT + gR_BMP21	0	0	128919	0.411	0.023	0.038	0.158	0.127	0.5375
SlALC	FJ404469	-	-	-	51907	0.075	-	-	0.075	-	-
		Cas9-RT + pegR_ALC	13	12	140946	0.119	0.108	0.093	0.107	0.007	0.2103
		Cas9 + pegR_ALC	13	12	140852	0.170	0.155	0.114	0.146	0.017	0.2748
		Cas9 + gR_ALC	0	0	129357	0.714	0.111	0.349	0.391	0.175	
		Cas9-RT + gR_ALC	0	0	121087	0.091	0.808	0.102	0.334	0.237	0.7896
SlDMR6-1	Solyc03g080190	-	-	-	37540	0.019	-	-	0.019	-	-
		Cas9-RT + pegR_DMR6-1	13	13	108382	0.010	0.021	0.020	0.017	0.004	0.0023
		Cas9 + pegR_DMR6-1	13	13	106969	0.254	0.161	0.025	0.147	0.067	0.0332
		Cas9 + gR_DMR6-1	0	0	124113	0.423	0.382	0.181	0.329	0.075	
		Cas9-RT + gR_DMR6-1	0	0	106991	0.006	0.017	0.019	0.014	0.004	0.0022
SlALS1	Solyc03g080190	-	-	-	56500	0.000	-	-	0.000	-	-
		Cas9-RT + pegR_ALS1	13	13	131194	0.000	0.032	0.023	0.018	0.009	<0.0001
		Cas9 + pegR_ALS1	13	13	145331	0.033	0.106	0.018	0.052	0.027	<0.0001
		Cas9 + gR_ALS1	0	0	144979	1.055	1.513	0.884	1.151	0.188	
		Cas9-RT + gR_ALS1	0	0	129222	0.039	0.057	0.031	0.042	0.008	<0.0001

*WT sample. **Multiple comparisons were conducted between the normal Cas9+gRNA combination and the complex containing either or both the PE components with the Uncorrected Fisher's LSD test using the Graphpad 9.0 software.

Supplemental Table 5. Comparisons of indel mutation efficiency of the Cas9 complexes containing the PE components compared to the normal complex.

Locus	PBS sequence (5'-3')	Tm of PBS (oC)	PE efficiency (%)				SEM	p value* (Uncorrected Fisher's LSD)
			Replicate 1	Replicate 2	Replicate 3	Average		
SlBMP21	AGAACGTTGAAGG	38	0.024	0.009	0.045	0.026	0.010	
	AGAACGTTGAACC	30	0.017	0.031	0.016	0.021	0.005	0.5505
	AGAACGTTGCAGC	32	0.020	0.016	0.025	0.020	0.003	0.4705
	AGAACGTTAC	22	0.022	0.020	0.026	0.023	0.002	0.6684
	AGAACGCTGC	24	0.026	0.024	0.025	0.025	0.001	0.8973
SlALC	TTTACTTCCGGTG	38	0.020	0.024	0.000	0.015	0.007	
	TTTACTTCCGGCA	32	0.019	0.024	0.019	0.020	0.002	0.3563
	TTTACTTCCTGTA	30	0.014	0.009	0.023	0.015	0.004	0.9166
	TTTACTTCAT	20	0.012	0.009	0.023	0.015	0.004	>0.9999
	TTTACTCCCT	22	0.010	0.015	0.016	0.014	0.002	0.8752
SlALS	CACTTGACCTGTA	38	0.109	0.082	0.041	0.077	0.020	
	CACTTGACCTGCT	34	0.098	0.113	0.040	0.084	0.022	0.8227
	CACTTGACCCGTT	34	0.128	0.079	0.046	0.084	0.024	0.8044
	CACTTGACCC	24	0.113	0.072	0.052	0.079	0.018	0.9529
	CACTTGTCCC	26	0.086	0.083	0.052	0.074	0.011	0.8967

*Multiple comparisons were conducted between PE complexes with the original PBS containing pegRNAs and that of the complexes containing modified PBS sequences with the Uncorrected Fisher's LSD test using the Graphpad 9.0 software.

Supplemental References

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SEQUENCES USED IN THE STUDY

- **Moloney Murine Leukemia Virus reverse transcriptase (MMLVrt) coding sequence (tobacco codon-optimized)**

  TCTGGAGGATCTAGCGGAGGATCCTCTGGGTCGGAAACACCAGGTACTAGCGAGTCAGCTACACCAGAGTCT
  AGTGGAGGCAGCAGCGGCGGGAGTAGCACACTAAATATAGAAGATGAATATCGGCTACATGAAACTTCAAA
  AGAGCCAGATGTTTCTCTAGGGAGCACATGGCTATCTGATTTTCCTCAAGCCTGGGCGGAAACCGGCGGCAT
  GGGACTGGCAGTTAGACAAGCTCCTCTGATTATACCACTGAAAGCAACATCTACTCCCGTTTCCATAAAACAAT
  ATCCCATGTCACAAGAAGCCAGATTAGGAATCAAGCCTCATATACAGAGACTGTTGGACCAGGGAATACTGG
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  CAAGATCTGAGAGAAGTCAATAAGCGTGTGGAAGATATTCACCCTACCGTGCCAAACCCTTACAACCTCTTGT
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  CCCACAAGTCAGCCTCTTTTCGCCTTTGAATGGAGAGATCCAGAAATGGGGATTTCAGGACAATTGACCTGGA
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  AGAATCCAGCACCCAGACTTGATTCTGCTACAGTACGTGGATGATTTACTGTTAGCCGCAACTTCTGAACTAG
  ACTGTCAACAGGGTACTCGAGCCCTGTTACAAACCTTGGGGAACCTTGGGTATCGGGCTTCAGCAAAGAAAG
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  GTACTCTGTTTAATTGGGGCCCAGATCAACAAAAGGCTTATCAAGAAATTAAGCAAGCTTTGCTCACTGCCCC
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  GAAGCACACGGAACCCGACCCGATCTAACGGACCAGCCGCTCCCAGATGCCGATCATACCTGGTACACTGAT
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  GGCAGAAGGTAAGAAGCTAAACGTTTATACTGATAGCAGATATGCTTTTGCTACTGCTCATATCCATGGAGAA
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  AGGCTAGAGGTAACCGGATGGCTGATCAAGCTGCCCGAAAGGCAGCTATTACTGAGACTCCAGATACCTCTA
  CACTCCTCATTGAAAATTCATCACCTTCTGGCGGATCAAAAAGAACCGCCGACGGTTCGGAATTCGAACCAAA
  GAAGAAGAGGAAAGTGTAA

  Blue font: linker; purple font: MMLVrt; green font: SV40 NLS; black font: spacer sequence; red font: stop codon.

- **Moloney Murine Leukemia Virus reverse transcriptase (MMLVrt) coding sequence (rice codon-optimized) from Addgene Plasmid #140445.**
- **(CA)n substrate sequence**

TCTCAAGTGCGAGCATGCATCGACACACACACAGGCTTAGAATCTGGGGCAGATAGACACGACCGCAGCTGG
CACGACAGGTTTCCCGACTGGAAAGCGGcCAGTGAGCGCAACGCAATCCACCAGCGcC

- **p35SI-nCas9-PmCDA1-UGI-t35S**

GAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTCAACA
CCCTCATATCAACTACTACGTTGTGTATAACGGTCCACATGCCGGTATATACGATGACTGGGGTTGTACAAAG
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Blue font: CaMV 35S promoter with UBQ10 intron; black font: nCas9 (D10A, <u>underlined</u>); purple font: PmCDA1; orange font: UGI; red font: CaMV 35S terminator.

- **p35SI-nCas9-RT-t35S**

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Blue font: CaMV 35S promoter with UBQ10 intron; orange font: nCas9 (H840A, underlined); purple font: linker; dark blue font: MMLVrt; green font: SV40 NLSs; red font: CaMV 35S terminator.

- **pegRNA1_CA**

5'-
GCATGCATCGACACACACACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGCTGTCGTGCCAGCTGCtgtcgTGTCTATCTGCCCCAGATTCTAAGCCTGTAT
GTGTGTCGATGCAT-3'
Red font sequence: gRNA; Purple sequence: 5' pgeRNA linker; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution; black font: spCas9 scaffold.

- **sgRNA_nick for second nick on the (CA)n substrate**

GAAACCTGTCGTGCCAGCTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGC
Red font sequence: gRNA; black font: spCas9 scaffold

- **sgRNA_CA for base editing on the (CA)n substrate**

ACACACAGGCTTAGAATCTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGC
Red font sequence: gRNA; black font: spCas9 scaffold

- **pegR_HKT12**

5'-
ATCATCATGTTTTCATTTGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGCTTGCAgATTGTGGTTTcTTACCTACAAATGAAAACATG -3'

Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT template; green nucleotide: base substitution.

- **sgR_HKT12_2n for second nick at the SlHKT1;2**

  AAACAAATCCTTGACCAAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

  Red font sequence: gRNA; black font: spCas9 scaffold

- **pegR_EPSPS1**

  5'-ACAGTAACTGCTGCTGTCAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAGGAAtcGCAATGCGTtctTTGACAGCAGCAGTT-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT template; green nucleotide: base substitution.

- **sgR_EPSPS1_2n for second nick at the SlEPSPS1**

  AATAAATACGAATTTTGAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

  Red font sequence: gRNA; black font: spCas9 scaffold

- **pegR_Or**

  GAGATAATATTAGAAGTAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCATCTTGTTCCTatgACTTCTAATATT

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT template; green nucleotide: base substitution.

- **sgR_Or_2n for second nick at the SlOr**

  AAAAACAAATGGCACGAAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

  Red font sequence: gRNA; black font: spCas9 scaffold

- **pegR1_ MBP21**

  5'-AGCTCCTTCAACGTTCTCAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTATCTTACCTTTaAGAACGTTGAAGG-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution.

- **pegR1_WH9**

  5'-
  CTTGAAGCAATCTTTAATTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
  AAAAGTGGCACCGAGTCGGTGCTCACCATGtCTGAATTAAAGATTGCTT-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution.

- **pegR1_KD1**

  5'-
  TTTGACAAAGACACATTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
  AAAAGTGGCACCGAGTCGGTGCACTGTAAAAAATGTTCTTTTCCAAAAATGTGTCTTTGTC-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution.

- **pegR1_PRD**

  5'-
  AGAAAAACCAGATGCTGGAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT
  GAAAAAGTGGCACCGAGTCGGTGCAAAACTAAAAGCTCATCCATTCCAGCATCTGGTTT-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution.

- **pegR1_ALC**

  5'-
  GTTCCACCGGAAGTAAAAACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
  AAAAAGTGGCACCGAGTCGGTGCGACAAGCCGGaTTTTACTTCCGGTG-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT tem plate; green nucleotide: base substitution.

- **pegR1_DMR6**

  5'-
  TAGAGAAGTATGCTCCTGAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
  AAAAAGTGGCACCGAGTCGGTGCTAGAAGGCCATTaAGGAGCATACTTC-3'

  Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT template; green nucleotide: base substitution.

- **pegR1_ALS1**

5'-
CTATTACAGGTCAAGTGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGCTCATCCTCCTTGaCACTTGACCTGTA-3'

Red font sequence: gRNA; Blue sequence: PBS; orange sequence: RT template; green nucleotide: base substitution.

- **sgR1_ MBP21**

5'-
AGCTCCTTCAACGTTCTCAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_WH9**

5'-
CTTGAAGCAATCTTTAATTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_KD1**

5'-
TTTGACAAAGACACATTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_PRD**

5'-
AGAAAAACCAGATGCTGGAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_ALC**

5'-
GTTCCACCGGAAGTAAAAACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_DMR6**

5'-
TAGAGAAGTATGCTCCTGAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **sgR1_ALS1**

5'-
CTATTACAGGTCAAGTGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGC-3'

Red font sequence: gRNA; black font: SpCas9 scaffold.

- **p35SI-Cas9-RT-t35S**

GAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTCAACA
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Blue font: CaMV 35S promoter with UBQ10 intron; orange font: full function Cas9 (H840, underlined); purple font: linker; dark blue font: MMLVrt; green font: SV40 NLSs; red font: CaMV 35S terminator.

- **p35SI-Cas9-t35S**

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GCCGACGGTTCGGAATTCGAACCAAAGAAGAAGAGGAAAGTGTAAGCTTCTCTAGCTAGAGTCGATCGACAA
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ATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATT
CCTAAAACCAAAATCCAGTACTAAAATCCAGATC

Blue font: CaMV 35S promoter with UBQ10 intron; orange font: full function Cas9 (H840, underlined; green font: SV40 NLSs; red font: CaMV 35S terminator.

- **p35SI-nCas9-PPE-t35S expression cassette**:

GGAGGAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTC
AACACCCTCATATCAACTACTACGTTGTGTATAACGGTCCACATGCCGGTATATACGATGACTGGGGTTGTAC
AAAGGCGGCAACAAACGGCGTTCCCGGAGTTGCACACAAGAAATTTGCCACTATTACAGAGGCAAGAGCAG
CAGCTGACGCGTACACAACAAGTCAGCAAACAGACAGGTTGAACTTCATCCCCAAAGGAGAAGCTCAACTCA
AGCCCAAGAGCTTTGCTAAGGCCCTAACAAGCCCACCAAAGCAAAAAGCCCACTGGCTCACGCTAGGAACCA
AAAGGCCCAGCAGTGATCCAGCCCCAAAAGAGATCTCCTTTGCCCCGGAGATTACAATGGACGATTTCCTCTA
TCTTTACGATCTAGGAAGGAAGTTCGAAGGTGAAGGTGACGACACTATGTTCACCACTGATAATGAGAAGGT
TAGCCTCTTCAATTTCAGAAAGAATGCTGACCCACAGATGGTTAGAGAGGCCTACGCAGCAAGTCTCATCAAG
ACGATCTACCCGAGTAACAATCTCCAGGAGATCAAATACCTTCCCAAGAAGGTTAAAGATGCAGTCAAAAGAT
TCAGGACTAATTGCATCAAGAACACAGAGAAAGACATATTTCTCAAGATCAGAAGTACTATTCCAGTATGGAC
GATTCAAGGCTTGCTTCATAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCTACTGAA
TCTAAGGCCATGCATGGAGTCTAAGATTCAAATCGAGGATCTAACAGAACTCGCCGTCAAGACTGGCGAACA
GTTCATACAGAGTCTTTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCACGACACTCTG
GTCTACTCCAAAAATGTCAAAGATACAGTCTCAGAAGATCAAAGGGCTATTGAGACTTTTCAACAAAGGATAA
TTTCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAGG
TGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCATTCAAGATCTCTCTGCCGACAGTGGTCCC
AAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGAGGTTCCAACCACGTCTACAAAGCAAGT
GGATTGATGTGACATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTA
TATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTCGAGTATAAGGTAAATTTCTGTGTTCCTTATTCTCTCA
AAATCTTCGATTTTGTTTTCGTTCGATCCCAATTTCGTATATGTTCTTTGGTTTAGATTCTGTTAATCTTAGATCG
AAGATGATTTTCTGGGTTTGATCGTTAGATATCATCTTAATTCTCGATTAGGGTTTCATAGATATCATCCGATTT
GTTCAAATAATTTGAGTTTTGTCGAATAATTACTCTTCGATTTGTGATTTCTATCTAGATCTGGTGTTAGTTTCT
AGTTTGTGCGATCGAATTTGTCGATTAATCTGAGTTTTTCTGATTAACAGGAGCTCATTTTTACAACAATTACCA
ACAACAACAAACAACAAACAACATTACAATTACATTTACAATTATCGATACAATGCCTAAGAAAAAGAGAAAA
GTAAGACGATCCTCGACTTCCTGAAGAGCGATGGCTTCGCGAACCGCAATTTCATGCAGCTGATTCACGATGA
CAGCCTCACATTCAAGGAGGATATCCAGAAGGCTCAGGTGAGCGGCCAGGGGGACTCGCTGCACGAGCATA
TCGCGAACCTCGCTGGCTCGCCAGCTATCAAGAAGGGGATTCTGCAGACCGTGAAGGTTGTGGACGAGCTGG
TGAAGGTCATGGGCAGGCACAAGCCTGAGAACATCGTCATTGAGATGGCCCGGGAGAATCAGACCACGCAG

AAGGGCCAGAAGAACTCACGCGAGAGGATGAAGAGGATCGAGGAGGGCATTAAGGAGCTGGGGTCCCAGA
TCCTCAAGGAGCACCCGGTGGAGAACACGCAGCTGCAGAATGAGAAGCTCTACCTGTACTACCTCCAGAATG
GCCGCGATATGTATGTGGACCAGGAGCTGGATATTAACAGGCTCAGCGATTACGACGTCGATGCCATCGTTC
CACAGTCATTCCTGAAGGATGACTCCATTGACAACAAGGTCCTCACCAGGTCGGACAAGAACCGGGGCAAGT
CTGATAATGTTCCTTCAGAGGAGGTCGTTAAGAAGATGAAGAACTACTGGCGCCAGCTCCTGAATGCCAAGC
TGATCACGCAGCGGAAGTTCGATAACCTCACAAAGGCTGAGAGGGGCGGGCTCTCTGAGCTGGACAAGGCG
GGCTTCATCAAGAGGCAGCTGGTCGAGACACGGCAGATCACTAAGCACGTTGCGCAGATTCTCGACTCACGG
ATGAACACTAAGTACGATGAGAATGACAAGCTGATCCGCGAGGTGAAGGTCATCACCCTGAAGTCAAAGCTC
GTCTCCGACTTCAGGAAGGATTTCCAGTTCTACAAGGTTCGGGAGATCAACAATTACCACCATGCCCATGACG
CGTACCTGAACGCGGTGGTCGGCACAGCTCTGATCAAGAAGTACCCAAAGCTCGAGAGCGAGTTCGTGTACG
GGGACTACAAGGTTTACGATGTGAGGAAGATGATCGCCAAGTCGGAGCAGGAGATTGGCAAGGCTACCGCC
AAGTACTTCTTCTACTCTAACATTATGAATTTCTTCAAGACAGAGATCACTCTGGCCAATGGCGAGATCCGGAA
GCGCCCCCTCATCGAGACGAACGGCGAGACGGGGGAGATCGTGTGGGACAAGGGCAGGGATTTCGCGACC
GTCAGGAAGGTTCTCTCCATGCCACAAGTGAATATCGTCAAAAAGACAGAGGTCCAGACTGGCGGGTTCTCT
AAGGAGTCAATTCTGCCTAAGCGGAACAGCGACAAGCTCATCGCCCGCAAGAAGGACTGGGATCCGAAGAA
GTACGGCGGGTTCGACAGCCCCACTGTGGCCTACTCGGTCCTGGTTGTGGCGAAGGTTGAGAAGGGCAAGTC
CAAGAAGCTCAAGAGCGTGAAGGAGCTGCTGGGGATCACGATTATGGAGCGCTCCAGCTTCGAGAAGAACC
CGATCGATTTCCTGGAGGCGAAGGGCTACAAGGAGGTGAAGAAGGACCTGATCATTAAGCTCCCCAAGTACT
CACTCTTCGAGCTGGAGAACGGCAGGAAGCGGATGCTGGCTTCCGCTGGCGAGCTGCAGAAGGGGAACGAG
CTGGCTCTGCCGTCCAAGTATGTGAACTTCCTCTACCTGGCCTCCCACTACGAGAAGCTCAAGGGCAGCCCCG
AGGACAACGAGCAGAAGCAGCTGTTCGTCGAGCAGCACAAGCATTACCTCGACGAGATCATTGAGCAGATTT
CCGAGTTCTCCAAGCGCGTGATCCTGGCCGACGCGAATCTGGATAAGGTCCTCTCCGCGTACAACAAGCACC
GCGACAAGCCAATCAGGGAGCAGGCTGAGAATATCATTCATCTCTTCACCCTGACGAACCTCGGCGCCCCTGC
TGCTTTCAAGTACTTCGACACAACTATCGATCGCAAGAGGTACACAAGCACTAAGGAGGTCCTGGACGCGAC
CCTCATCCACCAGTCGATTACCGGCCTCTACGAGACGCGCATCGACCTGTCTCAGCTCGGGGGCGACGAATTC
CCAAAGAAGAAGCGGAAGGTGGAGCTCAGCGGAGGATCTTCCGGAGGATCTAGCGGCTCCGAGACACCAG
GAACATCCGAAAGCGCTACACCAGAATCTAGCGGAGGCTCTTCCGGAGGATCTAGGCCTACCCTCAACATCG
AGGATGAGTATCGCCTCCACGAAACCTCCAAAGAACCGGACGTGTCCCTCGGCAGCACATGGCTCAGCGACT
TCCCACAAGCGTGGGCCGAAACCGGCGGCATGGGCCTCGCCGTCCGCCAAGCCCCACTCATTATCCCGCTGA
AGGCGACCTCCACACCGGTGTCCATCAAGCAGTACCCGATGAGCCAAGAGGCGAGGCTCGGGATTAAGCCG
CACATTCAGCGCCTCCTCGATCAAGGCATTCTCGTGCCGTGCCAATCCCCGTGGAATACACCACTCCTCCCGGT
CAAAAAGCCGGGCACCAACGACTATCGCCCGGTCCAAGATCTCCGCGAGGTCAACAAGCGCGTGGAAGATAT
CCACCCGACCGTCCCGAACCCGTATAATCTGCTCTCCGGGCTCCCACCATCCCACCAGTGGTATACAGTGCTG
GACCTCAAAGACGCCTTCTTCTGTCTCCGCCTCCACCCAACAAGCCAGCCGCTCTTCGCCTTCGAGTGGCGCGA
CCCGGAGATGGGCATCTCCGGCCAACTGACATGGACACGCCTCCCGCAAGGCTTCAAGAACAGCCCGACACT
CTTCAACGAGGCGCTCCATAGGGACCTCGCGGATTTTCGCATCCAGCATCCGGACCTCATCCTCCTCCAGTATG
TGGATGATCTCCTCCTCGCCGCGACCTCCGAGCTGGATTGTCAACAAGGCACACGCGCGCTCCTCCAAACACT
CGGGAACCTCGGCTATCGCGCGTCCGCGAAAAAGGCCCAAATCTGCCAGAAGCAAGTGAAGTACCTCGGGTA
TCTGCTCAAGGAAGGCCAACGCTGGCTCACCGAAGCGCGCAAAGAAACAGTGATGGGGCAACCGACACCGA
AAACACCACGCCAGCTGCGCGAGTTTCTCGGCAAAGCCGGCTTCTGTCGCCTCTTCATCCCGGGCTTTGCCGA
GATGGCCGCGCCACTCTACCCACTCACCAAGCCGGGCACACTGTTTAACTGGGGGCCGGATCAGCAGAAAGC
CTACCAAGAGATCAAACAAGCGCTCCTCACCGCCCCAGCGCTCGGGCTCCCAGATCTCACAAAGCCGTTCGAG

CTGTTCGTCGATGAGAAGCAAGGCTACGCGAAGGGCGTGCTCACACAGAAGCTCGGCCCGTGGAGGAGGCC
AGTGGCCTATCTCTCCAAAAAACTCGATCCAGTGGCCGCCGGCTGGCCACCGTGTCTGCGCATGGTCGCCGCG
ATTGCCGTGCTCACAAAGGATGCCGGCAAACTCACAATGGGCCAGCCGCTGGTGATCCTCGCGCCACATGCC
GTGGAAGCCCTCGTCAAACAGCCGCCGGATAGGTGGCTCTCCAATGCGCGCATGACCCATTACCAAGCGCTC
CTCCTCGACACCGATCGCGTCCAGTTCGGCCCAGTGGTCGCCCTCAATCCGGCGACACTGCTGCCACTCCCAG
AGGAGGGCCTCCAACACAACTGTCTGGATATTCTCGCGGAAGCGCATGGCACAAGGCCAGACCTCACAGATC
AACCGCTCCCGGATGCGGATCACACATGGTATACCGACGGCTCCTCTCTGCTCCAAGAGGGCCAAAGGAAAG
CCGGCGCCGCGGTGACCACAGAAACAGAAGTGATCTGGGCCAAGGCCCTCCCAGCCGGCACATCCGCGCAA
AGGGCGGAACTCATCGCGCTCACACAAGCCCTCAAGATGGCCGAGGGCAAGAAGCTCAACGTCTACACAGAC
TCCCGCTATGCCTTCGCCACCGCCCACATTCACGGCGAAATCTATAGGAGGCGCGGCTGGCTCACAAGCGAG
GGGAAGGAGATCAAGAACAAGGATGAGATCCTCGCGCTGCTCAAGGCCCTCTTTCTCCCGAAGCGCCTCTCC
ATCATCCACTGTCCGGGCCACCAAAAGGGGCACTCCGCGGAAGCGAGGGGCAATAGGATGGCCGATCAAGC
CGCGCGCAAAGCCGCGATTACCGAAACCCCAGACACATCCACCCTCCTCATCGAAAACTCCTCCCCAAGCGGC
GGCAGCCCGAAGAAGAAAAGGAAGGTGTAAGCTTCTCTAGCTAGAGTCGATCGACAAGCTCGAGTTTCTCCA
TAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATAT
AAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATC
CAGTACTAAAATCCAGAT

Blue font: CaMV 35S promoter with UBQ10 intron; orange font: nCas9 (H840A, underlined); purple font: linker; dark blue font: MMLVrt; green font: SV40 NLSs; red font: CaMV 35S terminator.

- **Plant selection marker**: pNOS-NptII-tOCS cloned from pICSL11024 (pICH47732::NOSp-NPTII-OCST) (Addgene Plasmid #51144).