A potential clue for the application of prime editing in tomato, a dicot plant

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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. However, 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. However, our data obtained by targeted deep sequencing also revealed inefficient PE activity 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. These data could also help explain the recent successes of some prime editors in plants using improved expression systems. Our work provides an important clue for the application of the PE approach in crop improvement.

Introduction

RNAs have been used for gene correction in human and yeast cells[1]. CRISPR/Cas-mediated precision editing using a chimeric single guide RNA (sgRNA) and a donor template was shown in rice protoplasts[2, 3]. However, the gene targeting (GT) frequency recorded using the RNA donor was much lower than that of a single-stranded DNA (ssDNA) donor[3]. Further work needs to be done on the use of RNA as a template for precision plant gene editing. Recently, prime editing (PE) using guide RNA extensions for priming reverse transcription-mediated precise editing appeared to be an excellent precision genome editing technique in mammalian cell lines[4]. The best version of the prime editor used a CRISPR/Cas complex developed by fusing a reverse transcriptase (RT) to the C-terminus of a Cas9 nickase (H840A) and a PE gRNA (pegRNA) with a 3' extension that could bind to the 3' nicked strands produced by nCas9. When bound, the nicked strand's free 3'-OH is used as the substrate for the RT to copy genetic information from the pegRNA's 3' extension. If pegRNAs were designed to produce modified nucleotides, these nucleotides would be inserted into the genome during downstream repair processes[4]. A second nick site present downstream of the first nick site would support the retention of the introduced nucleotides. The PE approach may also be an excellent alternative to GT with shorter editing sequence coverage[5]. Prime editors have been shown to work well in monocot plants by Lin and

54 coworkers[6]. In the same year, other reports also showed the activities of PE complexes in
55 monocots such as rice[7-11] and maize[12]. However, the majority of the data showed successful PE
56 at the acetolactate synthase (ALS) locus. Very limited data regarding the applications of PE in
57 dicots were released, with relatively low efficacy in potato[13] and tomato[14], and this process would
58 need to be improved for further applications. Therefore, we sought to investigate the activity of
59 PE complexes in plants using a transient assay with a synthetic substrate in tobacco and stable
60 transformation for editing ten loci in tomato. Our data showed low PE efficiency at both the
61 somatic cell and plant levels. Additional analyses revealed the possible negative effects of the PE
62 components on PE performance. Our work provides an important clue for the further
63 improvement of the PE approach in plants.

64 **Results and Discussion**

65 **The PE complexes did not perform better than the BE tool in a transient assay.** The PE tool
66 includes an nCas9 fused with an RT enzyme and a pegRNA for binding to the targeted sequence,
67 and its 3' extension primes an RNA-dependent DNA polymerization reaction by annealing to the
68 sequence located upstream of the nicked site on the nontargeted strand (Fig. 1a). If a second
69 nick is simultaneously added, we have a PE3 or PE3b approach, depending on the position of
70 the second nick outside or inside the area covered by the RT, respectively[4]. To quickly validate
71 the PE activity in plants, we started our PE experiments with a transient *Agrobacterium*-
72 mediated infiltration system in tobacco leaves that was designed to modify a single nucleotide
73 of a synthetic substrate ((CA)n substrate) (Fig. 1b,c). A cytidine base editor (CBE) system using
74 PmCDA1 cytosine deaminase was used for comparison of the editing efficiency. The CBE design
75 included a "-sgRNA" control (pCEC1) and two test vectors, one with the T-DNA (pCE01) and one
76 with a geminiviral replicon system (pCE02)[15], for amplifying the editing tools (Fig. 1c). The PE3b
77 tools were designed with both T-DNA and replicon cargos but also included dual vector systems
78 to avoid possible activity in bacterial cells. One vector carried the nCas9-RT expression cassette,
79 the synthetic substrate, and an sgRNA expression cassette for generating a second nick
80 (pPEsubc1 and pPEsubc2), and the other vector contained the pegRNA expression cassette
81 (pPEsub1 and pPEsub2) (Fig. 1b, c). RT DNA was chemically synthesized as a tobacco codon-

82 optimized Moloney murine leukemia virus (M-MLV) RT based on the PE2 approach used in the
83 work of Anzalone and coworkers[4]. pPEsubc1 and pPEsubc2 were also used alone as controls for
84 the PE experiments. Three temperature conditions were applied to the infiltrated plants during
85 the incubation stage to study the impacts of temperature on the activities of the BE and PE
86 complexes. Surprisingly, we observed a very strong C to T transition at the 19th nucleotide
87 counted from the PAM by BE in *Agrobacterium* cells (Fig. 1d, e, *Agrobacteria* panel) that led to
88 indistinguishable BE activity at that position in tobacco leaves (Fig. 1d, e). By contrast, we
89 observed BE activities in converting C to T at the 15th and 17th nucleotides in the tobacco leaves
90 (Fig. 1d, e). Interestingly, the BE activities were reduced when the temperature increased from
91 25°C to 37°C (Fig. 1e, f and Supplemental Table 2). Our data also indicate that the replicon-
92 based BE tool worked much more efficiently than the T-DNA tool under transient expression
93 conditions (Fig. 1e, f and Supplemental Table 2). Unexpectedly, we failed to show any PE
94 activity with the transient system using both the T-DNA and replicon systems. Furthermore, no
95 improvement was obtained using the temperature treatments (Fig. 1g, h). One of the reasons
96 for the failure of the PE3b constructs might be due to the inefficiency of the dual
97 *Agrobacterium*-mediated infiltration that required simultaneous transfer of the two T-DNAs
98 carrying PE components into one cell.

99 **The PE tools showed low precise editing efficiencies at 10 tomato loci.** We decided to evaluate
100 the ability of the PE system to edit genomic sites in tomato by Agrobacterium-mediated stable
101 transformations. The tomato transformation system was efficiently used for GT experiments in
102 our lab (Supplemental Fig. 1)[15]. We first attempted to edit three tomato loci, SlHKT1;2,
103 SlEPSPS1, and SlOr, using PE2 and PE3 approaches with the T-DNA and replicon systems
104 (Supplemental Figs. 2-3 and Data S1). Transformed explants collected at 10 days post-
105 transformation (dpt) were subjected to targeted deep sequencing of the targeted sites.
106 However, in two replicates, we could not reveal any evidence of PE activity that was higher than
107 the background level of the targeted sequencing method (Table 1). PE activity was shown to be
108 locus-specific, and several parameters, such as PBS and RT template lengths, may also affect PE
109 efficiency in plants[6]. During our study, several PE data were reported that showed comparable
110 PE efficiencies among the PE2, PE3, and PE3b constructs[6-8, 10], and in some cases, the PE2

111 approach performed better than the PE3 approach[12, 13]. We then extended the PE experiment
112 to 7 more loci using the PE2 approach and single replicon-based vectors (Fig. 2a and
113 Supplemental Table 3) to check whether the PE system would work in tomato. Most of the loci
114 were selected to require only single nucleotide changes, and the RT template lengths were
115 designed within the optimal ranges shown in reports on monocots. The PBS lengths were also
116 appropriately selected (Table 1). Using targeted deep sequencing analysis, we found a higher PE
117 performance at SlWH9, SlKD1, SlALC and SlALS1 than at the mock control, albeit at low absolute
118 values (Table 1). The low PE efficiency might be partially explained by the sample type
119 (cotyledon explants) we collected for targeted deep sequencing. Since the cotyledon explants
120 (0.1 x 0.3 cm in size) contained many untransformed cells due to the nature of the
121 Agrobacterium-mediated transformation, they confer much lower editing efficiency than the
122 protoplast system. Therefore, we also analyzed plants regenerated from the transformed
123 cotyledon explants. We detected only low levels of PE alleles in several plants transformed with
124 the SlALC and SlALS1 PE constructs (Fig. 2c, d). Sanger sequencing data revealed that only 1% of
125 the PE alleles were successfully fixed in the plant genomes. Nevertheless, the PE efficiency
126 obtained at the plant stage in our work is comparable to that of recently published data in
127 tomato[14]. Moreover, in one event at SlALC, two byproducts were also formed, with frequencies
128 of 1 and 3% (Fig. 2d).

129 The very low PE efficiency obtained for 10 tomato loci based on various editing types under our
130 experimental conditions indicates that there are many critical factors that affect the overall
131 performance of PE in tomato and other dicot plants, as concluded previously[13]. To exclude the
132 possibility that our tobacco codon-optimized RT coding sequence may trigger aberrant
133 transcription or cryptic splicing, thereby affecting nCas9-RT activity, next, we used the nCas9-PPE
134 coding sequence that showed good PE activities in rice and wheat[6]. The nCas9-PPE prime editor
135 is a rice codon-optimized RT that performed the best among the RTs tested by Lin and coworkers.
136 We constructed PE2 tools using nCas9-PPE with all 10 tomato loci tested earlier (Fig. 2b).
137 However, targeted deep sequencing data collected for two replicates showed similar levels of PE
138 activity across all 10 loci. The PE efficiency was even reduced in the case of the SlWH9 and SlKD1
139 loci but improved at the SlALC locus (Table 1), indicating that our tobacco codon-optimized RT

140 activity was not worse than the reported version. These data illustrate that the PE approach
141 needs improvement for further applications in tomato.

142 **The PE components negatively affect dsDNA cleavage.** In an attempt to improve the PE
143 performance, we sought to investigate the roles of the PE components (i.e., the nCas9-RT and
144 the pegRNAs) in the activation of the PE nuclease complex and nicking the template strand. We
145 constructed binary plasmids with fully functional SpCas9 fused with RT or SpCas9 alone in
146 combination with either pegRNAs or normal sgRNAs for four loci (SlBMP21, SlALC, SlDMR6-1,
147 and SlALS1) (Supplemental Fig. 4) and assessed their ability to generate indel mutations by
148 targeted deep sequencing. Unexpectedly, the Cas9-RT fusion combined with the pegRNAs did
149 not show indel mutation efficiencies above background levels, while the conventional Cas9-
150 sgRNA complexes worked well at all the tested loci (Fig. 2d). Cas9-RT combined with normal
151 sgRNAs generated indel mutations at 2 of the 4 tested loci (SlBMP21 and SlALC), with indel
152 mutation efficiencies similar to those of Cas9 (Fig. 2d). The pegRNAs also supported the
153 formation of indel mutations when combined with the Cas9 nuclease at 2 of the 4 tested loci
154 (SlBMP21 and SlDMR6-1), but the indel mutation efficiencies were only half of those generated
155 by Cas9/sgRNA complexes (Fig. 2d). Either of the PE components alone worked at extremely
156 low efficiencies compared to Cas9 with normal gRNA at the ALS1 locus. These data indicate that
157 the PE components negatively affected the cleaving activity of the Cas9 nuclease and that the
158 use of pegRNAs led to reduced activity of the Cas9 enzyme (Fig. 2d). We reason that the Cas9-
159 RT fusion might reduce the accessibility of the larger active complexes (Cas9-RT is
160 approximately 236 kDa, compared to ~158 kDa for SpCas9 alone) to some genomic contexts,
161 such as SlDMR6-1 and SlALS1, thereby blocking their cleavage activity. Moreover, the pegRNAs
162 shared intramolecular complementarity between PBS and spacer sequences that potentially
163 altered the secondary structure of the sgRNAs (Supplemental Fig. 5), leading to possible
164 interference of SpCas9 activation and/or the ability of gRNA to bind to targeted sequences and
165 cleave them. The failure of the Cas9-RT/pegRNA complexes to produce indel mutations at all
166 four loci might have resulted from the combined negative impacts of both components. Based
167 on these data, we hypothesize that the activity of nCas9-RT/pegRNA complexes was affected by
168 the negative impacts of both RT fusion and pegRNA structure, which led to extremely low

169 efficiency, and by some unknown factor, resulting in especially low efficiencies in tomato and
170 other dicot plants[13, 14]. If the above assumption is true, then we expect that when the PBS is
171 shortened, the PE efficiency will be improved. However, the PE data in plants suggested optimal
172 ranges of PBS lengths that could not be too short to prime RT polymerization[6]. Higher
173 incubation temperatures may help to destabilize the PBS-spacer intramolecular interactions,
174 but they may also affect the activity of nuclease complexes or the activity of its target binding,
175 as observed with the transient BE data (Fig. 1). That is also why Lu and coworkers could not
176 obtain a significant improvement in PE activity when they incubated explants at high
177 temperatures[14]. Thus, we hypothesize that a range of optimized melting temperature exists for
178 an active PBS of pegRNA. Alternatively, introducing several mismatches between the PBS and
179 spacer sequences may significantly weaken the interaction and improve PE activity in plants.
180 We can also use a two-component PE complex that separates the nCas9 and RT polypeptides to
181 reduce the space constraint caused by the nCas9-RT fusion. However, the two-component
182 system may pose another risk for the proper assembly of the two components in a targeted
183 genomic context.

184 Taken together, the PE approach offers an excellent alternative to precision gene editing in plants.
185 However, we could not obtain a better editing efficiency than that of the BE tools in a transient
186 assay in tobacco. Similar results were also recorded for 10 tomato loci using stable
187 *Agrobacterium*-mediated transformations. We revealed low but comparable PE efficiency at the
188 plant stage compared with recent data in plants. Further analyses showed that the PE
189 components (nCas9-RT fusion and pegRNA) might largely contribute to its low activity in tomato.
190 Significant improvement to the PE approach needs to be made for further applications in tomato,
191 and our data shed light on potential solutions for the improvement of this editing tool.

192 **Methods**

193 **System design for PE experiments in tobacco and tomato.** For the transient experiment, PE constructs
194 were designed to modify two nucleotides of the (CA)n substrate sequence using the 3' extension
195 pegRNA (Fig. 1a,b and Data S1). A tobacco codon-optimized RT DNA sequence was fused with a human
196 codon-optimized SpCas9 nickase (H840A) (nCas9-RT, Data S1) cloned using SpCas9 of the Addgene
197 plasmid (#49771) that worked well in plants[16]. The PE experiment was performed by using dual

198 agroinfiltration (Fig. 1c). For comparison, CBE constructs were designed to convert Cs to Ts, including the
199 PE-targeted C at position 17 counted from the PAM of the CBE gRNA (Fig. 1b).

200 For PE of the selected tomato loci using *Agrobacterium*-mediated stable transformation (Supplemental
201 Fig. 1), PE2 and PE3b approaches were applied for SlHKT1;2, SlEPSPS1, and SlOr (Supplemental Figs. 2-3).
202 Further applications of the nCas9-RT and nCas9-PPE systems on the other seven loci (Supplemental
203 Table 7) used a PE2 strategy (Fig. 2a, b and Data S1).

204 In all binary vectors, the transcription of both CBE and nCas9-RT was driven by a long CaMV35S inserted
205 with a UBQ10 intron 1[15]. The CBE gRNA and PE pegRNA were cloned downstream of an AtU6 promoter
206 [16]. The CBE and PE systems were delivered by either T-DNAs or geminiviral replicons[15].

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

218 ***Agrobacterium*-mediated tomato transformation and assessment of PE efficiency.** *Agrobacterium*-
219 mediated tomato transformation was performed using our in-house protocol (Supplemental Fig. 1)[15].
220 Briefly, seven-day-old cotyledons were cut and used for transformation. For targeted deep sequencing,
221 thin cotyledon explants (0.1 x 0.3 cm) were used to reduce the area containing untransformed cells
222 beyond the cut edges of the explants. The *Agrobacteria* containing the PE constructs were cultured and
223 harvested as in the transient assay but were resuspended in an ABM-MS solution[15] containing 100 µM
224 acetosyringone to an OD_{600nm} of 0.8. The *Agrobacteria* were then incubated for one hour before
225 transformation. Regenerated shoots were selected on medium containing 80 mg/L kanamycin.
226 Subsequently, hardened plants were checked for PE activity.

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

230 **Targeted deep sequencing.** Genomic DNA was isolated from cotyledon explants or plant leaves using
231 the CTAB method. The MiSeq sequencing service (MiniSeqTM System, Illumina, USA) was used. MiSeq
232 samples were prepared in three PCRs according to the manufacturer's guidelines, with genomic DNAs as
233 templates for the first PCR. The first and second PCRs used the primers listed in Supplemental Table 1,
234 whereas the third PCR was conducted with the manufacturer's primers to assign sample IDs. The MiSeq
235 raw data FASTQ files were analyzed by the Cas-Analyzer tool[17] and PE-Analyzer
236 (http://www.rgenome.net/pe-analyzer). The PE efficiency was assessed using the RT template
237 sequences as the input HDR donor sequence.

238 **Data analysis and presentation.** Most of the experiments analyzed by targeted deep sequencing were
239 conducted in two replicates. The editing data, statistical analysis if applicable, and plots were further
240 processed by the MS Excel program. The data are appropriately explained in detail in the legends of
241 figures and/or tables.

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249 **Author contributions**

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

253 **Competing interests**

254 The authors declare no competing interests.

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290 **Figure legends**

291 **Fig. 1: Failure of prime editing in tobacco cells in transient assays. a**. Schematic processes of
292 primed editing. The nontargeted strand is nicked by CRISPR/Cas9 nickase (H840A), and the 3'
293 nicked end binds to a complementary RNA (priming binding site, PBS, 13-15 nt) introduced by
294 extending the 3' end of the sgRNA (RT template) and primes reverse transcription by the nCas9-
295 fused RT using its free OH group. RT copies the genetic information from the RNA template that
296 is complementary to the sequence downstream from the nicked site and includes intentionally

297 introduced base modifications (indicated by red and bright orange lines). The RT product
298 appears as a 3'-flap sequence that competes with the full complement of the original 5'-flap
299 during the repair process, and a precisely edited sequence could be fixed to the targeted site
300 after 5'-flap removal and subsequent replication of the DNA. A second nick is introduced during
301 the improvement of the prime editor for supporting 3'-edited flap fixation. **b**. Selected synthetic
302 target and pegRNA design. pegRNA and its 3' extension and second nick spacer sequences are
303 indicated. A PmCDA1-based C->T editing tool is designed in parallel for comparison, and its
304 spacer sequence is also shown. Intended base modifications are denoted and explained. **c**.
305 Vector arrangements for the study. The nCas9(D10A)-PmCDA1 base editing system is designed
306 with control plasmid (pCEc, -gRNA), T-DNA (pCE1), and replicon (pCE2)-based editors. The prime
307 editors are designed with a dual vector system for T-DNA (pPEsubc1 + pPEsub1) and replicon
308 (pPEsubc2 + pPEsub2)-based tools for editing C->T and C->A (shown in **b**). **d-e**. Sequencing data
309 showing C->T editing by T-DNA- (**d**) and replicon-based (**e**) tools (Supplemental Table 2, (**e**)). The
310 –sgRNA (pCEc) chromatogram represents for all the temperature tested. **f**. Editing efficiency of
311 the base editors. The T-DNA construct represents the pCE1 plasmid, and the replicon construct
312 is for the pCE2 plasmid. The editing efficiencies at different temperatures are plotted for the
313 15th, 17th and 19th bases counted from the PAM site to its upstream spacer sequence. **g-h**.
314 Failure of the T-DNA- (**g**) and replicon-based (**h**) prime editors to edit the intended bases. The -
315 pegRNA (pPEsubc1) and -pegRNA (pPEsubc2) chromatograms represent for all the temperature
316 tested. Discontinuous red boxes denote the intended modifications in each of the editing
317 conditions (25, 31 and 37°C) for the editors and the control.

318 **Fig. 2: Prime editing performance in tomato. a-b.** PE2 prime editing constructs using the
319 geminiviral replicon in tomato with synthetic tobacco codon-optimized RT (**a**, 7 loci) or rice
320 codon-optimized PPE (**b**, 10 loci). The NptII expression cassette (Addgene plasmid #51144) was
321 used for the kanamycin selection of transformed cells, tissues and plants. The expression of the
322 prime editors was driven by the p35S promoter with the UBQ10 intron 1 and CaMV 35S
323 terminator (Data S1). The transcription of the pegRNAs was controlled by the AtU6 core
324 promoter sequence (Data S1). **c**. PE performance data obtained from the analysis of the plant-
325 stage transformants of SlWH9, SlALC and SlALS1 by Sanger sequencing and ICE Synthego. **d**. The

326 traces of PE-edited alleles obtained for SlALC (upper two panels) and SlALS1 (bottom panel).
327 The precisely edited alleles are shown at the bottom of each panel and are denoted by "HDR"
328 labels. The PE-mediated replaced bases are highlighted with an orange background. **e**. Targeted
329 deep sequencing data for the assessment of the activities of the PE components via indel
330 mutation formation. The SpCas9 or SpCas9-RT fusion was used in combination with either the
331 pegRNA or sgRNA to target 4 loci (SlBMP21, SlALC, SlDMR6 and SlALS1). The sequences flanking
332 the targeted sites were amplified at 10 dpt and analyzed by targeted deep sequencing in two
333 replicates. The mock control (WT) and the nCas9-RT + pegRNA complexes were analyzed once.

334 **List of Supplementary Tables**

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

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

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

338 **List of Supplementary Figures**

339 Supplemental Fig. 1: *Agrobacterium*-mediated transformation protocol used in this work.

340 Supplemental Fig. 2: PE targeting maps of the SlHKT1;2, SlEPSPS1 and SlOr loci.

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

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

346 Supplemental Fig. 5: MFOLD-predicted secondary structures of pegRNAs and sgRNAs of
347 SlBMP21, SlALC, SlDMR6-1 and SlALS1.

348 **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

349

Figure 1

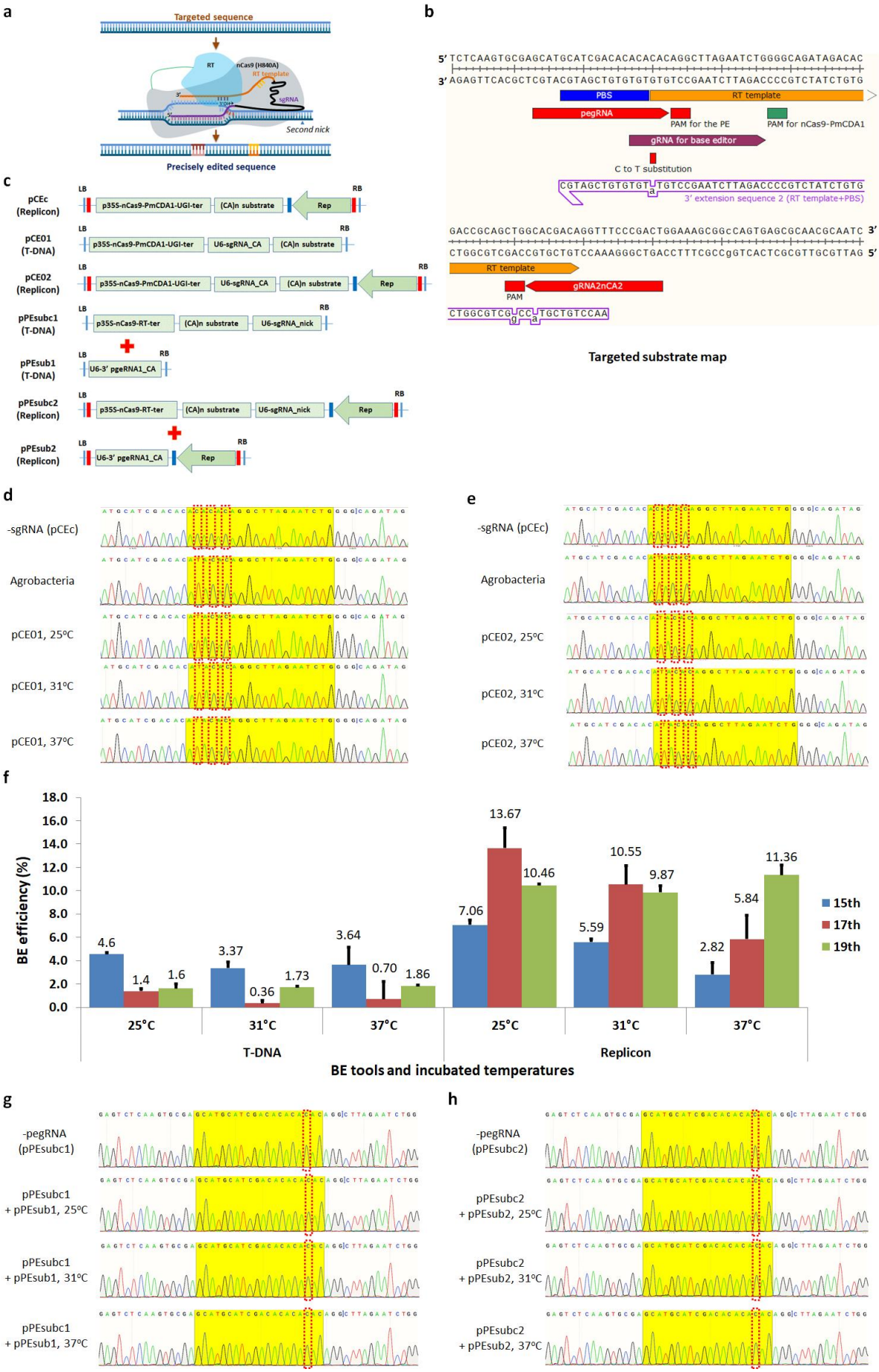
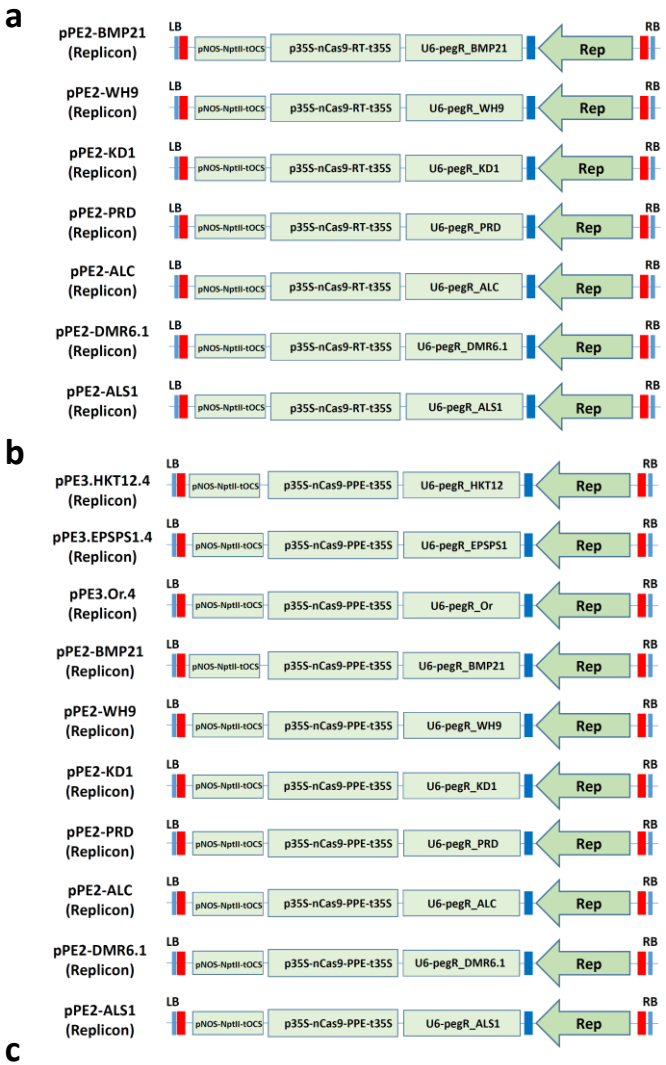

a
Targeted sequence
RT
nCas9 (H840A)
RT template
sgRNA
Second nick
Precisely edited sequence
b
5' TCTCAAGTGCGAGCATGCATCGACACACACACAGGCTTAGAATCTGGGGCAGATAGACAC
3' AGAGTTCACGCTCGTACGTAGCTGTGTGTGTGTCCGAATCTTAGACCCCGTCTATCTGTG
PBS
RT template
pegRNA
PAM for the PE
PAM for nCas9-PmCDA1
gRNA for base editor
C to T substitution
3' extension sequence 2 (RT template+PBS)
GACCGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGCCAGTGAGCGCAACGCAATC 3'
CTGGCGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCGCCgGTCACTCGCGTTGCGTTAG 5'
RT template
gRNA2nCA2
PAM
Targeted substrate map
c
pCEc (Replicon)
pCE01 (T-DNA)
pCE02 (Replicon)
pPEsubc1 (T-DNA)
pPEsub1 (T-DNA)
pPEsubc2 (Replicon)
pPEsub2 (Replicon)
LB
RB
p35S-nCas9-PmCDA1-UGI-ter
(CA)n substrate
Rep
U6-sgRNA_CA
p35S-nCas9-RT-ter
U6-sgRNA_nick
U6-3' pgeRNA1_CA
d
-sgRNA (pCEc)
Agrobacteria
pCE01, 25°C
pCE01, 31°C
pCE01, 37°C
e
-sgRNA (pCEc)
Agrobacteria
pCE02, 25°C
pCE02, 31°C
pCE02, 37°C
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
25°C
31°C
37°C
T-DNA
Replicon
BE tools and incubated temperatures
g
-pegRNA (pPEsubc1)
pPEsubc1 + pPEsub1, 25°C
pPEsubc1 + pPEsub1, 31°C
pPEsubc1 + pPEsub1, 37°C
h
-pegRNA (pPEsubc2)
pPEsubc2 + pPEsub2, 25°C
pPEsubc2 + pPEsub2, 31°C
pPEsubc2 + pPEsub2, 37°C

Fig. 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 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 for 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 (**h**) 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.

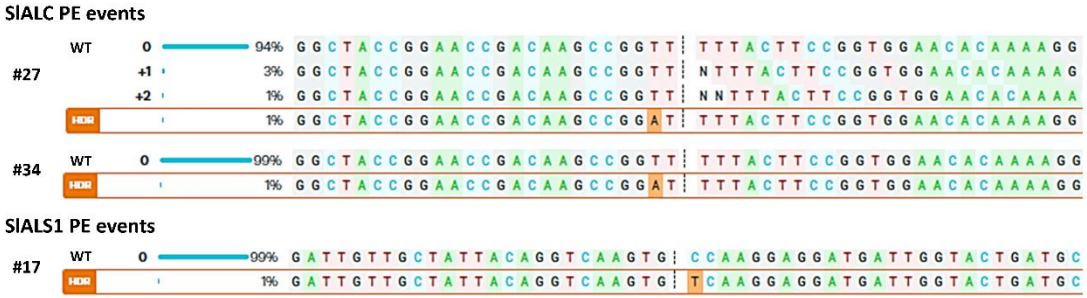
Figure 2



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

d



e

Locus	Cas protein	sgRNA	PBS length (nt)	RT length (nt)	Rep 1		Rep 2		Average (%)	
					Total reads	Indel rate (%)	Total reads	Indel rate (%)	Total reads	Indel rate (%)
SlBMP21	-	-	-	-	49819	0.026	-	-	49819	0.026
	nCas9-RT	pegR1_BMP21	13	13	19495	0.005	-	-	19495	0.005
	Cas9-RT	pegR1_BMP21	13	13	22542	0.018	70932	0.007	93474	0.012
	Cas9	pegR1_BMP21	13	13	26217	0.092	75370	0.190	101587	0.141
	Cas9	sgR1_BMP21	0	0	18044	0.355	72670	0.238	90714	0.296
	Cas9-RT	sgR1_BMP21	0	0	15569	0.411	76943	0.023	92512	0.217
SlALC	-	-	-	-	51907	0.075	-	-	51907	0.075
	nCas9-RT	pegR1_ALC	13	12	22256	0.103	-	-	22256	0.103
	Cas9-RT	pegR1_ALC	13	12	19379	0.119	76594	0.108	95973	0.114
	Cas9	pegR1_ALC	13	12	30042	0.170	69640	0.155	99682	0.162
	Cas9	sgR1_ALC	0	0	17929	0.714	69640	0.111	87569	0.412
	Cas9-RT	sgR1_ALC	0	0	22085	0.091	66793	0.808	88878	0.450
SlDMR6-1	-	-	-	-	37540	0.019	-	-	37540	0.019
	nCas9-RT	pegR1_DMR6	13	13	21227	0.000	-	-	21227	0.000
	Cas9-RT	pegR1_DMR6	13	13	20415	0.010	47207	0.021	67622	0.015
	Cas9	pegR1_DMR6	13	13	9434	0.254	61637	0.161	71071	0.208
	Cas9	sgR1_DMR6	0	0	20809	0.423	66265	0.382	87074	0.402
	Cas9-RT	sgR1_DMR6	0	0	16387	0.006	48261	0.017	64648	0.011
SlALS1	-	-	-	-	56500	0.000	-	-	56500	0.000
	nCas9-RT	pegR1_ALS1	13	13	21227	0.000	-	-	21227	0.000
	Cas9-RT	pegR1_ALS1	13	13	53738	0.000	-	-	53738	0.000
	Cas9	pegR1_ALS1	13	13	60067	0.033	40717	0.106	100784	0.069
	Cas9	sgR1_ALS1	0	0	63776	1.055	47391	1.513	111167	1.284
	Cas9-RT	sgR1_ALS1	0	0	56198	0.039	28000	0.057	84198	0.048

Fig. 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 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**. Targeted deep sequencing data for the assessment of the activities of the PE components via indel mutation formation. The SpCas9 or SpCas9-RT fusion was used in combination with either the pegRNA or sgRNA to target 4 loci (SlBMP21, SlALC, SlDMR6 and SlALS1). The sequences flanking the targeted sites were amplified at 10 dpt and analyzed by targeted deep sequencing in two replicates. The mock control (WT) and the nCas9-RT + pegRNA complexes were analyzed once.

SUPPLEMENTAL FIGURES, TABLES AND REFERENCES

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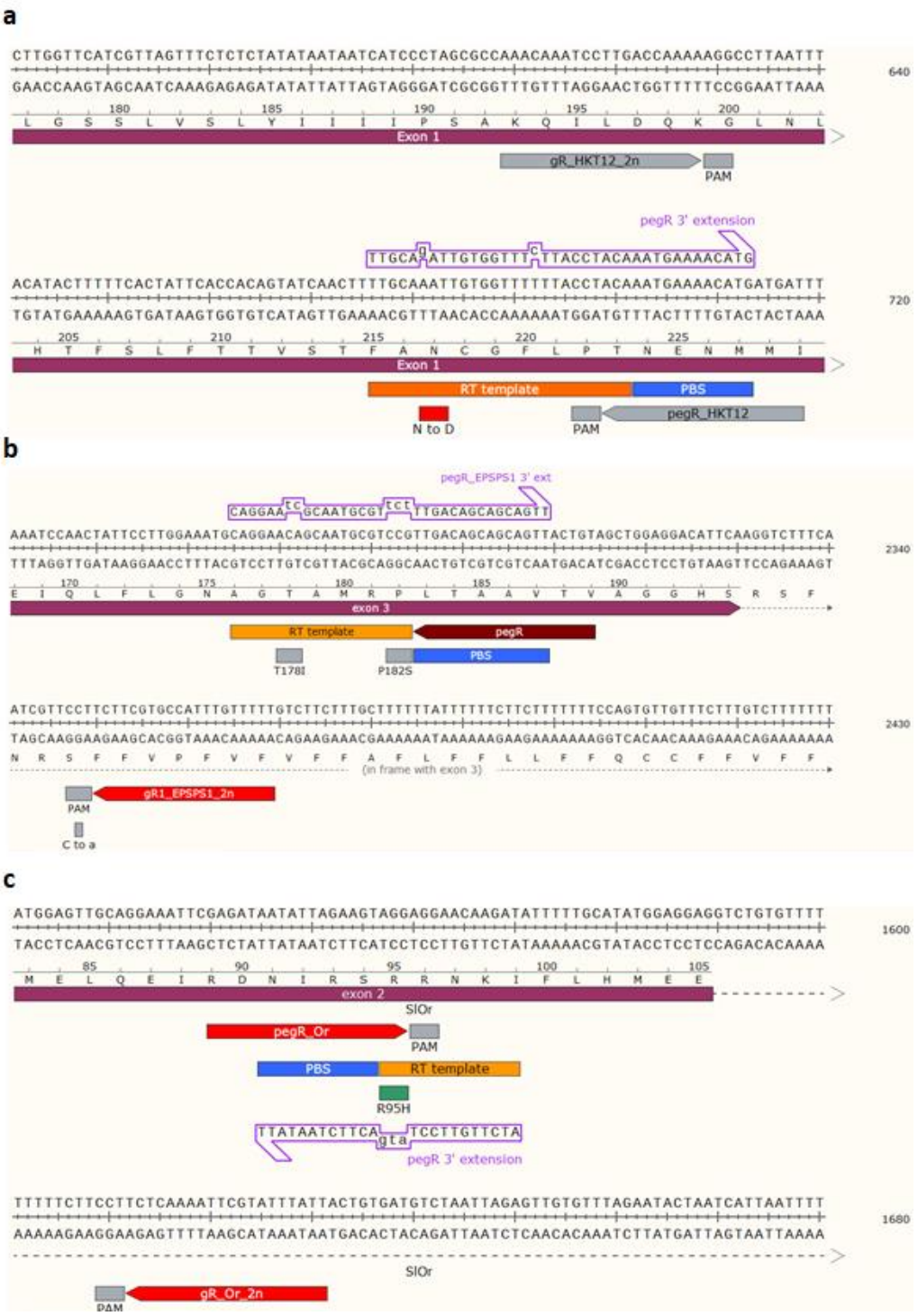
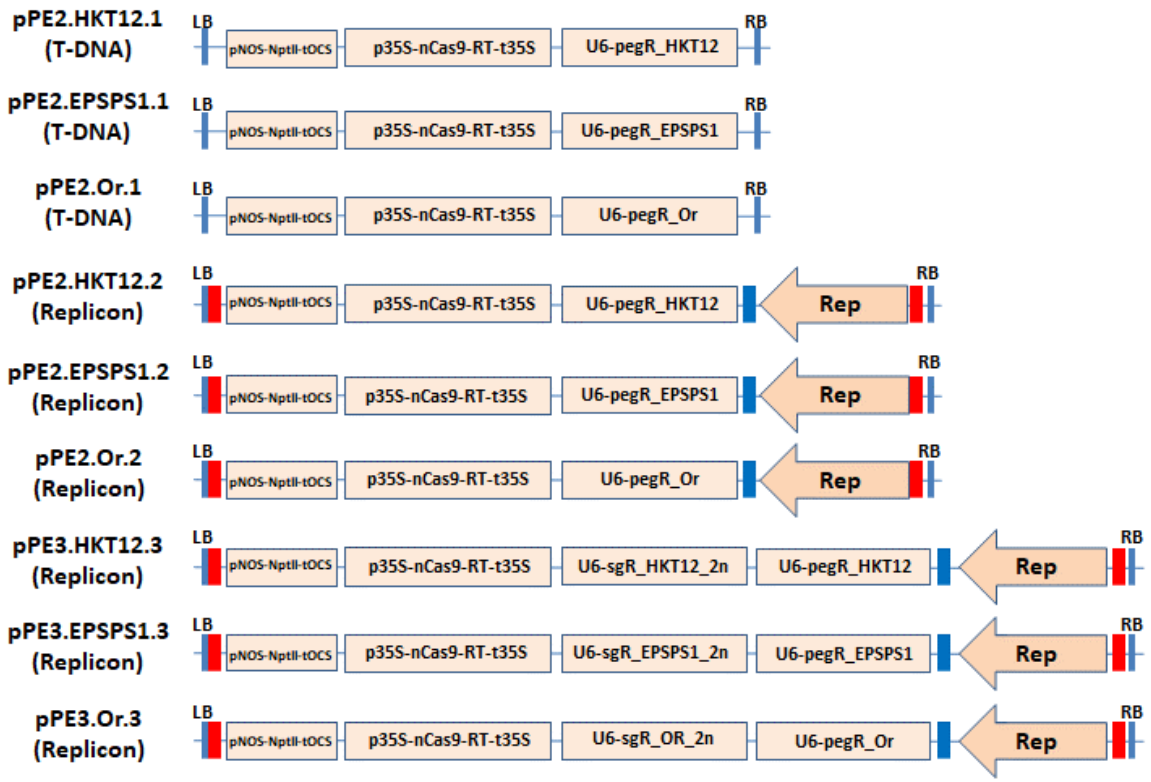
Stage	Germination	Pre-culture, callus induction	Co-cultivation, callus induction	Selection, callus induction and shoot regeneration	Selection, callus induction and shoot regeneration	Selection, shoot formation and elongation	Selection, root formation
Medium	1/2MSO	PREMC	ABM-MS+AS	SEL4-80	SEL4-80	SEL4-80	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 Fig. 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.

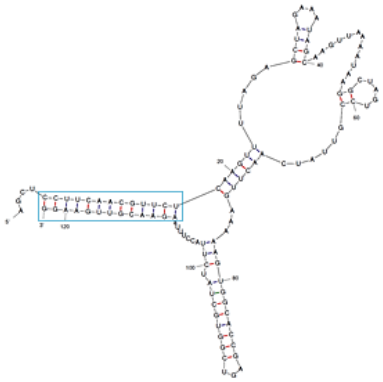
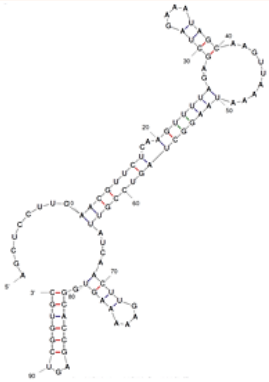
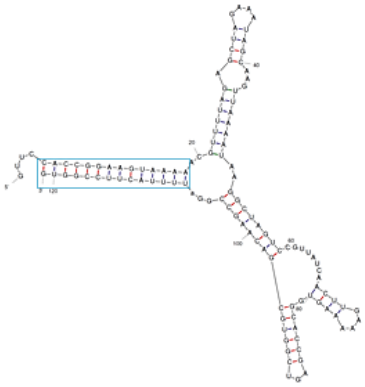
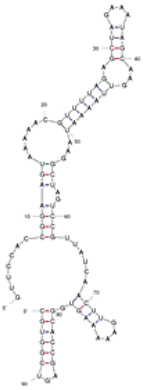
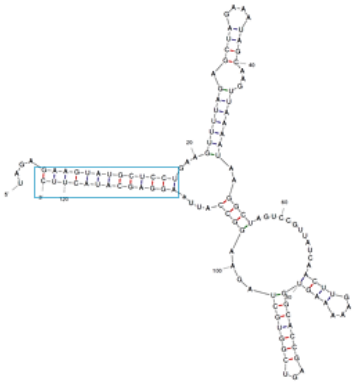
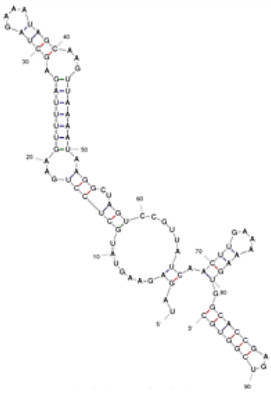
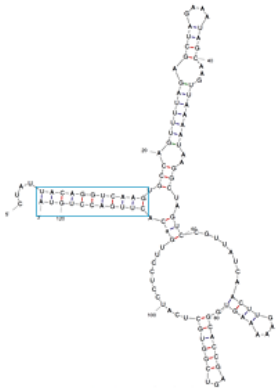
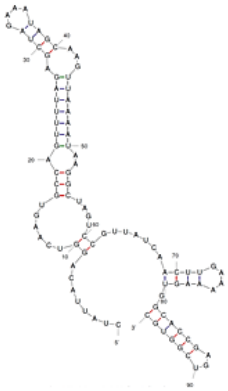


Supplemental Fig. 2: PE targeting maps of the SlHKT1;2, SlEPSPS1 and SlOr loci.



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

Supplemental Fig. 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.

	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 Fig. 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 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
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  CAAGATCTGAGAGAAGTCAATAAGCGTGTGGAAGATATTCACCCTACCGTGCCAAACCCTTACAACCTCTTGT
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  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
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- **p35S-nCas9-PmCDA1-UGI-t35S**

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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.

- **p35S-nCas9-RT-t35S**

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GAAGAACAGTAGGGAAAGGATGAAGAGGATTGAAGAGGGTATAAAAGAACTGGGGTCCCAAATCCTTAAG

GAACACCCAGTTGAAAACACCCAGCTTCAGAATGAGAAGCTCTACCTGTACTACCTGCAGAACGGCAGGGAC
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TCCCCTCAGAAGAAGTTGTCAAGAAAATGAAAAATTATTGGCGGCAGCTGCTGAACGCCAAACTGATCACAC
AACGGAAGTTCGATAATCTGACTAAGGCTGAACGAGGTGGCCTGTCTGAGTTGGATAAAGCCGGCTTCATCA
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AGTACGATGAAAATGACAAACTGATTCGAGAGGTGAAAGTTATTACTCTGAAGTCTAAGCTGGTTTCAGATTT
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TAAAAGATTAAGCATAATTCATTGTCCAGGACATCAAAAAGGACACAGCGCCGAGGCTAGAGGTAACCGGAT
GGCTGATCAAGCTGCCCGAAAGGCAGCTATTACTGAGACTCCAGATACCTCTACACTCCTCATTGAAAATTCAT
CACCTTCTGGCGGATCAAAAAGAACCGCCGACGGTTCGGAATTCGAACCAAAGAAGAAGAGGAAAGTGTAA
GCTTCTCTAGCTAGAGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGG
AATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAA
AATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATC

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.

- **p35S-Cas9-RT-t35S**

GAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTCAACA
CCCTCATATCAACTACTACGTTGTGTATAACGGTCCACATGCCGGTATATACGATGACTGGGGTTGTACAAAG
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GACGCGTACACAACAAGTCAGCAAACAGACAGGTTGAACTTCATCCCCAAAGGAGAAGCTCAACTCAAGCCC
AAGAGCTTTGCTAAGGCCCTAACAAGCCCACCAAAGCAAAAAGCCCACTGGCTCACGCTAGGAACCAAAAGG
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AATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAA
AATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATC

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.

- **p35S-Cas9-t35S**

GAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTCAACA
CCCTCATATCAACTACTACGTTGTGTATAACGGTCCACATGCCGGTATATACGATGACTGGGGTTGTACAAAG
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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.

- **p35S-nCas9-PPE-t35S expression cassette**:

GGAGGAATTCCAATCCCACAAAAATCTGAGCTTAACAGCACAGTTGCTCCTCTCAGAGCAGAATCGGGTATTC
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CAGTACTAAAATCCAGAT

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