Title

Prime editing efficiently generates W542L and S621I double mutations in two ALS genes of maize

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Abstract

A novel and universal CRISPR/Cas-derived precision genome editing technology named prime editing was developed. However, low prime editing efficiency was shown in transgenic rice lines. We reasoned that enhancing pegRNA expression would be able to improve prime editing efficiency. In this report, we used two strategies to enhance pegRNA expression and constructed a prime editing vector harboring two pegRNA variants for W542L and S621I double mutations in *ZmALS1* and *ZmALS2*. As compared with previous reports in rice, we achieved much higher prime editing efficiency in maize. Our results are inspiring and provide a direction for optimization of plant prime editors.

Keywords: prime editing, *PE2*, pegRNA, CRISPR/Cas9, maize, *ALS*, herbicide-resistance

Background

Despite rapid advances in genome editing technologies, precision genome editing remains challenging [1]. Recently, a novel and universal precision genome editing technology named prime editing was developed and tested in mammalian cells [1]. More recently, seven groups reported applications of prime editors (PE) in rice and wheat, and six groups achieved prime-edited rice lines [2-8]. Excluding the data based on enriching strategies or unsuitable for comparison, total 164 prime-edited rice lines transformed with 39 prime editors targeting 11 endogenous rice genes were generated in these previous reports [2, 3, 5, 6, 8]. The highest editing efficiencies achieved by the five groups are from 2.22% to 31.3% [2, 3, 5, 6, 8]. Out of the total 164 prime-edited rice lines, only one line achieved by Xu et al. [6] harbors homozygous mutations whereas all the other lines mainly harbor chimeric mutations. These results indicate that the reported prime editing tools require significant optimization before they are adopted for use by plant researchers. In this paper, in comparison with the previous reports in rice, we achieved much higher prime editing efficiency in maize possibly owing to the optimized pegRNA expression, clean pegRNA sequences trimmed by tRNA and ribozyme processing systems, or both.

52 **Results and discussion**

53 We selected two maize acetolactate synthase (*ALS*) genes to test prime editing efficiency in maize
54 and attempted to generate maize herbicide-resistance lines harboring P165S mutation or
55 W542L/S621I double mutations [9] in *ZmALS1* and *ZmALS2* by prime editing (Fig. 1a). We used
56 maize *Ubi1* promoter to drive the expression of maize codon-optimized *PE2* and used *OsU3* and
57 *TaU3* promoters to drive the expression of pegRNA and sgRNA, respectively. In this way, we
58 constructed two pGreen3 binary vectors [10, 11], pZ1PE3 and pZ1PE3b, for the same P165S
59 mutation but based on two different strategies, PE3 and PE3b [1], respectively (Fig. 1a,b). To
60 generate mutant maize lines harboring W542L/S621I double mutations, we based the W542L and
61 S621I mutations on PE3b and PE3 strategies, respectively, and assembled two pegRNA and two
62 nicking sgRNA variants into the same binary vectors (Fig. 1a,b). According to the structure of
63 pegRNAs, a hairpin can potentially form between the PBS and the protospacer of a pegRNA (Fig.
64 1a), and thus will possibly weaken the pegRNA activity. Based on this possibility, we reasoned that
65 enhancing pegRNA expression will be able to improve editing efficiency. We used two strategies to
66 enhance pegRNA expression: doubling pegRNA expression cassettes and using two promoter
67 systems to drive pegRNA expression, together with use of the tRNA, ribozyme, and Csy4 RNA
68 processing systems [12, 13] when appropriate (Fig. 1b). In this way, we generated two prime editors
69 named pZ1WS-Csy4 and pZ1WS (Fig. 1b). We also generate two additional prime editors including
70 p4xZ1PE3-Csy4 and p4xZ1PE3b-Csy4 for comparison with pZ1PE3 and pZ1PE3b, respectively. The
71 p4xZ1PE3-Csy4 and p4xZ1PE3b-Csy4 editors have similar structure to pZ1WS-Csy4 but harbor
72 redoubled pegRNA and sgRNA expression cassettes for the P165S mutation. The pZ1WS vector was
73 constructed as a control of pZ1WS-Csy4 for comparison of Csy4 and non-Csy4 systems. We
74 transformed maize with these six PE vectors via the Agrobacterium-mediated method. We obtained
75 no transgenic lines transformed with the three Csy4 vectors. Almost at the same time, we found
76 that transformations with 13 additional pGreen3 or pCambia binary vectors harboring the Csy4-
77 P2A-ZsGreen fusion gene, including 1 maize and 12 Arabidopsis transformations, were unsuccessful,
78 suggesting Csy4 protein severely affects Agrobacterium-mediated transformation of at least these
79 two plant species.
80

81 To detect targeted mutations, we amplified fragments spanning the targets from genomic DNA of
82 the transgenic lines and detected mutations by direct sequencing of PCR fragments. We observed
83 no editing for the 71 and 156 lines transformed with pZ1PE3 and pZ1PE3b, respectively. Fortunately,
84 we observed that 7 out of 16 lines transformed with pZ1WS harbor S621I edits (Fig. 1c,d).
85 Interestingly, one line displayed homozygous mutations in both *ZmALS1* and *ZmALS2*.
86 We cloned the PCR products from the 6 lines harboring chimeric or heterozygous mutations and
87 sequenced ~86 clones for each PCR fragment (Additional file 1: Table S1). Interestingly, we observed
88 the W542L edits in some cloned fragments, suggesting that low-frequency mutations were not able
89 to be displayed in sequencing chromatograms (Additional file 1: Table S1). To further analyze the
90 low-frequency W542L and/or S621I mutations, we cloned the PCR fragments from the line (#4)
91 harboring homozygous mutations and an additional line (#3) harboring no mutations according to
92 direct sequencing of PCR fragments. We observed the S621I edits in some cloned fragments from
93 #3, further demonstrating that low-frequency mutations could not be reflected by direct
94 sequencing of PCR products (Additional file 1: Table S1). Three cloned fragments harbor W542L and
95 S621I double mutations, suggesting that PE can simultaneously edit two non-allelic targets in a cell.
96 We also cloned 6 PCR fragments from three pZ1PE3 and three pZ1PE3b transgenic lines, sequenced
97 the cloned PCR fragments, but found no mutations in total 213 *ZmALS1* and 196 *ZmALS2* clones
98 (Additional file 1: Table S1).
99 We observed two types of undesired byproducts from the sequencing results of cloned PCR
100 fragments. One type is derived from the pegRNA scaffold, which acts as extended RT templates and
101 thus introduces unwanted edits; the other is only involved in editing of multiple nucleotides and is
102 derived from unbiased and well-balanced double-strand repair (rather than single-strand-biased
103 repair) of multiple mismatches in heteroduplex DNA, which leads to incomplete editing of multiple
104 nucleotides (Fig. 2a). In addition, the two mechanisms of forming these two types of byproducts
105 were sometimes overlapped (Fig. 2a). We observed high frequencies of byproducts at the S621
106 target: 23.8% and 7.2% derived from the pegRNA scaffold and the double-strand even DNA repair,
107 respectively (Fig. 2b). For the W542 target, frequencies of both types of byproducts remained to be
108 investigated because of limited number of cloned PCR fragments harboring W542L edits. Since the
109 T0 line #4 harbors homozygous mutations in the two *ALS* and the T0 line #15 harbors obvious
110 pegRNA scaffold-derived byproducts (Fig. 1d), we excluded these two lines for possibly unbiased

111 analysis of the two types of byproducts. The recalculated frequencies of the byproducts for the
112 S621I edits are 18.1% and 15.3% for the pegRNA scaffold-derived and double-strand even DNA
113 repair-derived byproducts, respectively (Additional file 1: Table S1), indicating that frequencies of
114 byproducts excluding the two lines are still high. Surprisingly, we observed no indel-type
115 byproducts, suggesting that indels induced by PE are infrequent in maize.

116 To further figure out the low-frequency mutations and byproducts, we analyzed all the 243 lines
117 transformed with the three PE vectors by next-generation sequencing (NGS) of PCR amplicons. Out
118 of the 227 T0 lines transformed with pZ1PE3 or pZ1PE3b, we revealed only one line transformed
119 with pZ1PE3 harboring the low frequency (0.07%, 21/30306) P165S edits. On the contrary, we found
120 that 12 out of the 16 T0 lines transformed with pZ1WS harbor the S621I edits, and 6 out of the 16
121 lines also harbor the W542L edits (Additional file 1: Table S2). For the S621 target, average
122 frequencies of pegRNA scaffold-derived and double-strand even DNA repair-derived byproducts
123 from NGS are 17.5% and 8.5%, respectively; those excluding the #4 and #15 lines are 12.1% and
124 16.2%, respectively (Additional file 1: Table S2). We observed no byproducts for the W542L edits
125 from NGS possibly owing to the low frequency of desired edits, which affected analysis of
126 byproducts with lower frequency.

127 We repeatedly transformed maize with pZ1WS after we observed that our first transformation
128 could not generate many transgenic lines. In the second transformation, we obtained 46 transgenic
129 lines. We detected targeted mutations induced by prime editing and the results demonstrated again
130 that the prime editing efficiency of pZ1WS was much higher than previous reports (Fig. 2c and
131 Additional file 1: Fig. S1). Interestingly, this time we observed the signals of the W542L edits in the
132 sequencing chromatograms (Fig. 2d and Additional file 1: Fig. S1). These results suggest that we will
133 be able to achieve homozygous mutants harboring W542L and S621I double mutations in both
134 *ZmALS1* and *ZmALS2* in the progeny of some lines, such as #38 (Additional file 1: Fig. S1). In
135 addition, we achieved two additional lines harboring the S621I homozygous mutations in the two
136 *ALS* genes (Additional file 1: Fig. S2), and these two lines, together with the above described line,
137 can be used to generate homozygous double mutants harboring W542L and S621I double mutations
138 in the progeny. Not surprisingly, we again observed pegRNA scaffold-derived byproducts in the
139 sequencing chromatograms (Additional file 1: Fig. S1). We cloned PCR products from the 4 lines
140 harboring strong W542L edit signals in chromatograms (Additional file 1: Fig. S1) and sequenced

141 ~106 clones for each PCR fragment (Fig. 2e,f and Additional file 1: Table S3). The results indicated
142 that 11.5% cloned fragments from the line #41 harbor S621I edits although direct sequencing of
143 PCR fragment from this line displayed weak S621I edit signals in chromatograms (Additional file 1:
144 Fig. S1 and Table S3). We detected no pegRNA scaffold-derived byproducts from the W542L edits
145 (Fig. 2e,f and Additional file 1: Table S3), suggesting low frequency of pegRNA scaffold-derived
146 byproducts was produced at this target and frequency of byproducts depends on targets. Out of
147 242 cloned PCR fragments harboring edits, 17.8% (43/242) harbor W542L and S621I double
148 mutations in *ZmALS1* or *ZmALS2* (Fig. 2f), demonstrating again that PE can simultaneously edit two
149 non-allelic targets in a cell. For the W542L edits, the protospacer of the sgRNA nicking non-edited
150 strand fully matches the edited *ZmALS1* target but has a mismatch adjacent to PAM of the edited
151 *ZmALS2* target (Figure 1a), therefore actually PE2 was used for editing *ZmALS2* although PE3b was
152 done for *ZmALS1*. Editing frequencies at the W542 target of *ZmALS1* and *ZmALS2* are 7.1% and
153 39.7%, respectively (Additional file 1: Table S3), suggesting that PE2 has much higher editing
154 efficiencies than PE3b.

155 Collectively, we achieved sufficiently high prime editing efficiency in maize possibly owing to the
156 optimized pegRNA expression, clean pegRNA sequences trimmed by tRNA and ribozyme processing
157 systems, or both. In addition, we observed high frequency the pegRNA scaffold-derived byproducts
158 and to reduce them we propose a rule of termination to design pegRNAs: one to a few nucleotides
159 (C, GC, or TGC, et al.) of the pegRNA scaffold adjoining RT template can be used as termination
160 signals at genomic DNA matching RT templates.

161 Although we achieved sufficient high prime editing efficiency, a few questions remain to be
162 answered. Is the pegRNA sequence, the optimized pegRNA expression, or both the decisive factor
163 for the high efficiency of the S621I edits? Are the Pol2-Pol3 fusion promoters (such as 35S-CmYLCV-
164 U6) or only Pol2 promoters (such as 35S-CmYLCV), together with the tRNA and ribozyme RNA
165 processing systems, better systems to drive pegRNA expression than the commonly used Pol3
166 promoters (such as OsU3 and TaU3)? Is doubling or redoubling pegRNA expression cassettes crucial
167 for high prime editing efficiency? Is clean pegRNA sequences trimmed by tRNA and ribozyme
168 processing systems, or both vital for high prime editing efficiency? Why does Csy4 system affect
169 maize transformation? To facilitate to answer these questions, we are developing additional user-
170 friendly tools for a variety of plant species and promise to share them with researchers as soon as

171 possible. Application of these tools to test more pegRNA variants in amenable plants, such as rice,
172 will help to clarify decisive factors for high prime editing efficiency in plants and thus facilitate the
173 development of more powerful prime editors for high throughput precise genome editing in plants.

174 **Conclusions**

175 As compared with previous reports in rice, we achieved much higher prime editing efficiency in
176 maize: 53.2% (33/62) and 6.5% (4/62) transgenic lines harbor S621I and W542L mutations,
177 respectively, in *ZmALS1* and/or *ZmALS2*; 4.8% (3/62) and 4.8% (3/62) lines harbor homozygous
178 S621I mutations and S621I/W542L double mutations, respectively, in the two *ALS* genes. We also
179 observed high frequency of the pegRNA scaffold-derived byproducts and to potentially overcome
180 this defect, we propose a rule to design pegRNAs. Our results are inspiring and provide a new
181 reference standard for future optimization of prime editors. In addition, the pZ1WS prime editor
182 generated in this study is useful for breeding transgene-free maize lines harboring W542L and S621I
183 double mutations in the two ALS genes, which confer resistance to multiple herbicides functioning
184 as ALS inhibitors.

185 **Methods**

186 All primers used in this study are listed in Additional file 1: Table S4 and sequences of PE2 and
187 pegRNA expression cassettes are listed in Additional file 1: Supplemental material. Vectors
188 described in this study together with their annotated sequences are available from Addgene and/or
189 MolecularCloud (GenScript).

190 **Vector construction**

191 We replaced the *Xba*I-*Sbf*I fragment of pUC57-dCas9 [14] with a synthetic fragment, resulting in
192 the generation of pUC57-PE2P1. We replaced the *Mlu*I and *Sac*I fragment of pUC57-PE2P1 with
193 another synthetic fragment, resulting in the generation of pUC57-PE2. We modified the maize Ubi1
194 promoter in pG3R23-U3Ub [11] so that the first of the two *Pst*I sites, the first of the four *Xba*I sites
195 (the last 3 *Xba*I sites are blocked by Dam methylation), the *Nco*I site, and the *Eco*RI site in the
196 promoter were disrupted. We replaced the *Xba*I and *Sac*I fragment of the modified pG3R23-U3Ub
197 with the *Xba*I and *Sac*I fragment of pUC57-PE2, resulting in the generation of pG3R23-PE2U3A. We
198 replaced the *Eco*RI-*Sac*II fragment of pG3R23-PE2U3A with the *Eco*RI-*Sac*II fragment of pG3GB411

199 [11], resulting in the generation of pG3GB-PE2U3A. To generate the final PE binary vectors
200 harboring a single pegRNA, we conducted the Golden Gate reaction to insert a synthetic pegRNA-
201 OsU3t-TaU3p-sgRNA fragment into the two *Bsa*I sites of pG3GB-PE2U3A. In this way, we generated
202 pZ1PE3 and pZ1PE3b.

203 We replaced the *Asc*I-*Pac*I fragment of pL21-iCre [11] with a synthetic fragment of 35S-CmYLCV-
204 Csy4-P2A-ZsGreen-polyA-Csy4-BB-HDV-HSPt, resulting in the generation of pL2L1-35Csy4. We
205 replaced the *Xba*I-*Eco*RI fragment of pL2L1-35Csy4 with a synthetic fragment of U6-tGly-BB-HDV-
206 HSPt, resulting in the generation of pL2L1-35C-U6. We replaced the *Hin*dIII-*Eco*RI fragment of pR14-
207 BWM [11] with an insert prepared by annealing two oligos oHEASmE-F/R, resulting in the
208 generation of pR1R4-HEAS. We replaced the *Eco*RI-*Aar*I fragment of pR1R4-HEAS with the *Eco*RI-
209 *Spe*I fragment of synthetic OsAct1p-CP4EPSPS-NOSt, resulting in the generation of pR1R4-Gly. We
210 amplified the U3p-BB-sgRNA cassette from pG3R23-PE2U3A with primers OsU3p-AsF and TaU3t-
211 EcR, purified the PCR products, digested them with *Asc*I and *Eco*RI, and allowed them to ligate with
212 *Asc*I and *Eco*RI-digested pR1R4-Gly and pL43-U3-Bar, resulting in the generation of pR1R4-U3G.0
213 and pL4L3-U3B.0, respectively.

214 We generated the two binary vectors harboring four pegRNA and four sgRNA cassettes in two
215 steps. First, we set up the Golden Gate reaction to clone synthetic fragments harboring pegRNAs
216 and/or sgRNAs into the destination vector pG3R23-PE2U3A and the three entry vectors including
217 pL2L1-35Csy4 or pL2L1-35C-U6, pR1R4-U3G.0, and pL4L3-U3B.0 (Additional file 1: Supplemental
218 material). Second, we set up the MultiSite Gateway reaction to assemble the final PE vectors. In this
219 way, we generated pZ1WS-Csy4 and pZ1WS. We constructed p4xZ1PE3-Csy4 and p4xZ1PE3b-Csy4
220 harboring redoubled pegRNA and sgRNA expression cassettes for the P165S mutation in a similar
221 way to the generation of pZ1WS-Csy4.

222 **Maize transformation and analysis of prime editing**

223 We separately transformed the six PE vectors, pZ1PE3, pZ1PE3b, p4xZ1PE3-Csy4, p4xZ1PE3b-
224 Csy4, pZ1WS-Csy4, and pZ1WS into the engineered Agrobacterium strain LBA4404/pVS1-VIR2 to
225 generate strains harboring the ternary vector system [11]. We separately used these strains to
226 transform maize ND73, a B73-derived inbred line, according to the published protocols [11].

227 To analyze the P165S mutations in *ZmALS1* and *ZmALS2*, we simultaneously amplified two
228 fragments spanning the target site in the two *ALS* genes from genomic DNA of transgenic lines by

229 PCR using primers ALS1&2P-F/R. We then submitted purified PCR products for direct sequencing
230 with the primer ALS1&2P-F. To analyze the W542L and S621I mutations in *ZmALS1* and *ZmALS2*, we
231 simultaneously amplified two fragments spanning the two target sites in the two *ALS* genes by PCR
232 using primers ALS1&2WS-F/R and submitted purified PCR products for direct sequencing with
233 primer ALS1&2WS-F2. Since we designed the primers according to homologous sequences of
234 *ZmALS1* and *ZmALS2*, the PCR fragments contain both *ZmALS1* and *ZmALS2* sequences. Since
235 *ZmALS1* and *ZmALS2* are highly homologous, the co-existence of two PCR fragments can be
236 determined according to the double peaks of the sequencing chromatograms. Similarly, chimeric
237 and heterozygous mutations can also be determined according to the double peaks. For sequencing
238 cloned PCR fragments, we picked ~86 or ~106 colonies from each transformation and submitted
239 them for Sanger sequencing. We also used Hi-TOM assay [15] with 0.5% threshold to analyze the
240 mutations in T0 transgenic plants. To analyze the P165S, W542L, and S621I mutations in *ZmALS1*
241 and *ZmALS2*, we simultaneously amplified two fragments spanning the target site in the two *ALS*
242 genes from genomic DNA of transgenic lines by PCR using primers ALS1&2P-NGSF/R, ALS1&2W-
243 NGSF/R, and ALS1&2S-NGSF/R, respectively.
244

245 **Supplementary information**

246 **Additional file 1**: **Figure S1.** Sequencing chromatograms from 7 prime-edited lines harboring W542L
247 edits. **Figure S2.** Sequencing chromatograms from 2 prime-edited lines harboring homozygous
248 S621I edits. **Table S1.** Edits and byproducts revealed from cloned PCR fragments. **Table S2.** Analysis
249 of mutations in T0 transgenic plants by NGS with 0.5% threshold. **Table S3.** Edits and byproducts
250 from the 4 additional lines. **Table S4.** Sequences of primers, targets, and rtT-PBS of pegRNAs.
251 **Supplemental material.** Sequences of PE2 and pegRNA expression cassettes.

252

253 **Acknowledgments**

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Authors' contributions

QJC, YZhou, and XCW conceived and designed the research. YYJ, YPC, MHL, XLH, YZ, and QZ conducted experiments and analyzed the data. QJC, YZhou, and XCW wrote the paper. All authors read and approved the final version.

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Availability of data and materials

Vectors described in this study together with their annotated sequences are available from Addgene and/or MolecularCloud (GenScript).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

Not applicable.

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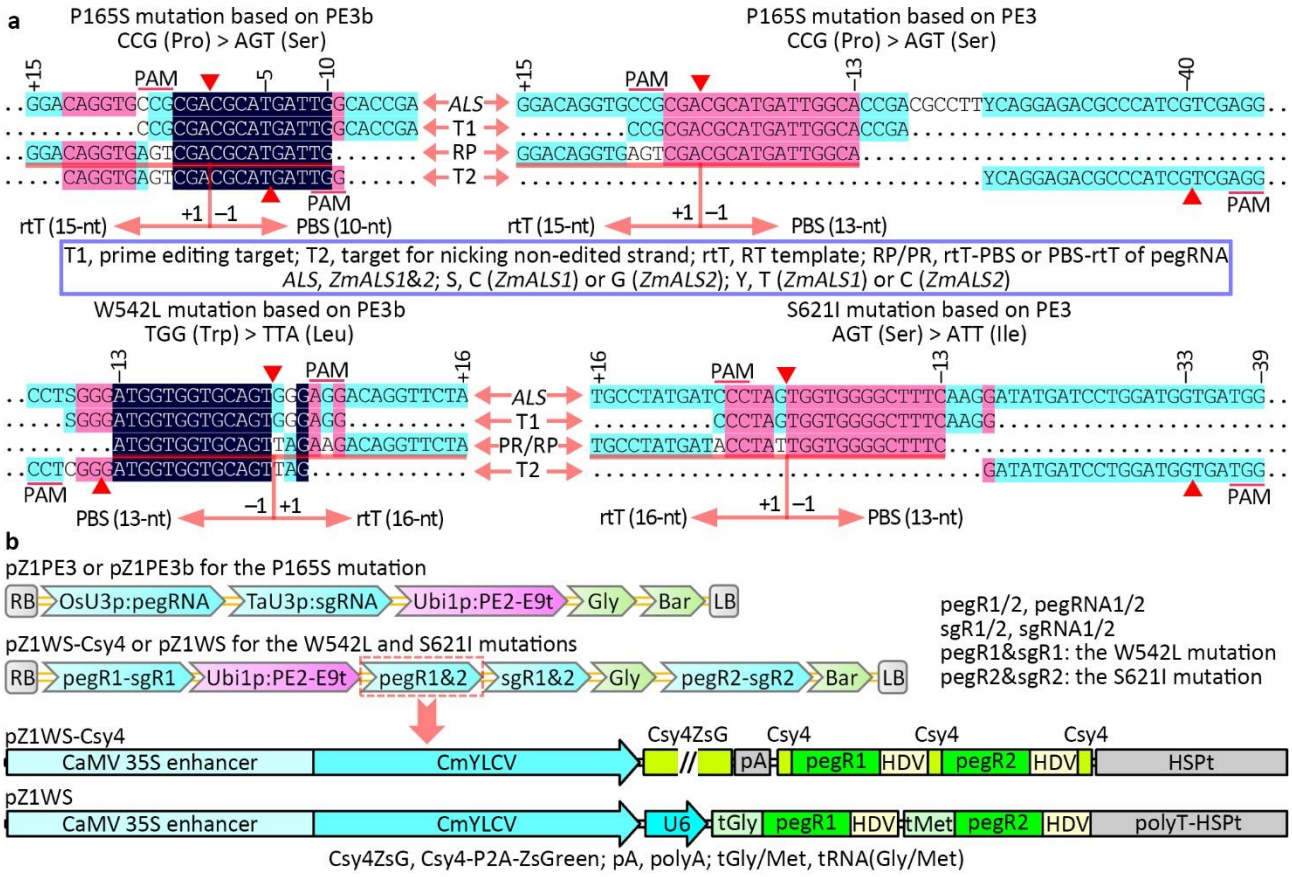
311 **Figure legends for Fig. 1**

312 **Fig. 1** Efficiency of prime editing in maize. **a** Sequences and their relationship of the two *ALS* genes,
313 the pegRNA targets, the pegRNA RT templates, the pegRNA primer binding sites (PBS), and the
314 sgRNA targets for nicking non-edited strands used for generation of 4 maize prime editing vectors.
315 The PAMs, nicking sites, RT template and PBS lengths, and introduced mutations are indicated. **b** T-
316 DNA structures of 4 PE vectors. The pZ1WS and pZ1WS-Csy4 vectors are designed for editing
317 double-targets. Each double-PE vector includes two expression cassettes for each of two pegRNAs
318 or sgRNAs. Two strategies are designed in the peg1&2: one is based on Csy4 RNA processing system
319 and the other is based on tRNA and HDV ribozyme-based RNA processing systems integrated with
320 double-drives of polymerase II (35S enhancer-CmYLCV) and III (shortened U6-26) promoters. The
321 structure of pegRNA and sgRNA expression cassettes in pegR1-sgR1 and pegR2-sgR2 is the same as
322 that in pZ1PE3/3b. The expressions of two sgRNAs in sgR1-sgR2 are driven by OsU3p and TaU3p,
323 respectively. **c** Prime editing efficiencies based on direct sequencing of PCR products. The transgenic
324 lines transformed with pZ1WS-Csy4 were not able to be achieved. **d** The sequencing
325 chromatograms of PCR fragments from the prime-edited plants transformed with pZ1WS. Double
326 peaks represent heterozygous or chimeric mutations and an asterisk indicates a mutation induced
327 by PE. Note that the first mutation in #15 is the unwanted mutation introduced by pegRNA scaffold.
328
329

330 **Figure legends for Fig. 2**

331 **Fig. 2** Desired edits and unwanted byproducts in the two transformations with pZ1WS. **a** Desired
332 edits and unwanted byproducts at the two target sites in the first transformation. Only partial
333 sgRNA sequence is shown. The homologous sgRNA-rtT sequences shared by all the aligned
334 sequences are shaded with yellow and mutated nucleotides are indicated with red letters. Number
335 of cloned PCR fragments harboring the same edits is indicated in parentheses and a vertical line
336 indicates the same type of byproducts. For convenience, the byproducts derived from the two
337 mechanisms were assorted into the pegRNA scaffold-derived byproducts. **b** Summary of the desired
338 edits and byproducts in the first transformation according to sequences of cloned PCR fragments. **c**
339 Prime editing efficiency achieved in the second transformation. Strong and Weak, T0 lines harboring
340 strong and weak peak signals for edits, respectively, in the sequencing chromatograms. Both, total
341 No. of lines harboring strong and weak edits. **d** Sequencing chromatograms from a prime-edited line
342 harboring W542L edits and obtained in the second transformation. Double peaks represent
343 heterozygous or chimeric mutations and an asterisk indicates a mutation induced by PE. Note that
344 the first mutation in the S621 target is the pegRNA scaffold-derived byproducts. **e** Desired edits and
345 unwanted byproducts at the two target sites in the second transformation. **f** Summary of the
346 desired edits and byproducts in the second transformation.
347

348 **Figure 1**



Vector	Mutation	No. of double mutants		No. of single mutants		Total	Ratio of mutants
		Ho	He/Ch	Ho	He/Ch		
pZ1PE3b	**P165S**	0	0	0	0	156	0
pZ1PE3	**P165S**	0	0	0	0	71	0
pZ1WS	**W542L**	0	0	0	0	16	0
	S621I	1	6	0	0	16	43.8%

Ho, homozygous; He/Ch, heterozygous or chimeric; Double mutants, T0 lines harboring mutations in *ZmALS1* and *ZmALS2*; Single mutants, T0 lines harboring mutations in *ZmALS1* or *ZmALS2*; Total, total No. of T0 lines.

(The sequencing chromatograms of #5, #6, #14, and #16 are similar to that of #8)

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351 **Figure 2**

a

S621I edits

```
CACCAGGAGCATGTGTTGCCTATGATCCCTAGTGGTGGGGCTTTC Ref 1
GGCACCGAGTCGGTGCTGCCTATGATACCTATTGGTGGGGCTTTC Ref 2
CACCAGGAGCATGTGTTGCCTATGATACCTATTGGTGGGGCTTTC Seq (×154)
CACCAGGAGCATGTGTTGCCTATGATACCTAGTGGTGGGGCTTTC Seq (×10)
CACCAGGAGCATGTGTTGCCTATGATCCCTATTGGTGGGGCTTTC Seq (×6)
CACCAGGAGCATGTGCTGCCTATGATACCTATTGGTGGGGCTTTC Seq (×41)
CACCAGGAGCATGTGCTGCCTATGATCCCTAGTGGTGGGGCTTTC Seq (×10)
CACCAGGAGCATGTG.TGCCTATGATACCTATTGGTGGGGCTTTC Seq (×1)
CACACCGAGTCGGTGCTGCCTATGATCCCTAGTGGTGGGGCTTTC Seq (×1)
```

W542L edits

```
ATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGC Ref 1
ATGGTGGTGCAGTTAGAAGACAGGTTCTAGCACCG Ref 3
ATGGTGGTGCAGTTAGAAGACAGGTTCTATAAGGC Seq (×7)
ATGGTGGTGCAGTGGGAAGACAGGTTCTATAAGGC Seq (×1)
ATGGTGGTGCAGTTAGAAGACAGGTTCTAGCAGGC Seq (×1)
```

Ref 1/2/3, reference sequence 1/2/3; Ref 1, ZmALS1 & 2
Ref 2, sgRNA-rtT-PBS; Ref 3, PBS-rtT-sgRNA
Seq, sequence of cloned PCR fragments

b

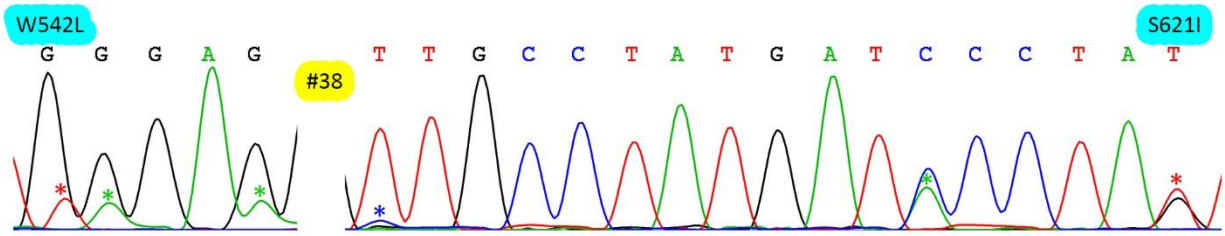
Mutation	De	Sc	Re	To	De/To	Sc/To	Re/To
S621I	154	53	16	223	69.1%	23.8%	7.2%
W542L	7	1	1	9	77.8%	11.1%	11.1%

De, Sc, or Re, No. of clones harboring specified mutations
De, desired edits
Sc, pegRNA scaffold-derived byproducts
Re, double-strand even DNA repair-derived byproducts
To, total No. of cloned fragments harboring mutations

c

Mutation	No. of prime-edited lines			Total No. of T0 lines	Ratio of prime-edited lines		
	Strong	Weak	Both		Strong	Weak	Both
W542L	4	3	7	46	8.7%	6.5%	15.2%
S621I	26	7	33	46	56.5%	15.2%	71.7%
W542L & S621I	3	4	7	46	6.5%	8.7%	15.2%

d



e

S621I edits

```
CACCAGGAGCATGTGTTGCCTATGATCCCTAGTGGTGGGGCTTTC Ref 1
GGCACCGAGTCGGTGCTGCCTATGATACCTATTGGTGGGGCTTTC Ref 2
CACCAGGAGCATGTGTTGCCTATGATACCTATTGGTGGGGCTTTC Seq (x117)
CACCAGGAGCATGTGTTGCCTATGATCCCTATTGGTGGGGCTTTC Seq (x15)
CACCAGGAGCATGTGTTGCCTATGATACCTAGTGGTGGGGCTTTC Seq (x11)
CACCAGGAGCATGTGCTGCCTATGATACCTATTGGTGGGGCTTTC Seq (x34)
CACCAGGAGCATGTGCTGCCTATGATCCCTAGTGGTGGGGCTTTC Seq (x4)
CACCAGGAGCATGTGCTGCCTATGATCCCTATTGGTGGGGCTTTC Seq (x2)
CACCAGGAGCATGTGCTGCCTATGATACCTAGTGGTGGGGCTTTC Seq (x1)
CACCAGGAGTCGGTGCTGCCTATGATACCTATTGGTGGGGCTTTC Seq (x1)
```

W542L edits

```
ATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGC Ref 1
ATGGTGGTGCAGTTAGAAGACAGGTTCTAGCACCG Ref 3
ATGGTGGTGCAGTTAGAAGACAGGTTCTATAAGGC Seq (×93)
ATGGTGGTGCAGTTAGAGGACAGGTTCTATAAGGC Seq (×4)
ATGGTGGTGCAGTGGGAAGACAGGTTCTATAAGGC Seq (×3)
```

Ref 1/2/3, reference sequence 1/2/3; Ref 1, *ZmALS1 & 2*
Ref 2, sgRNA-rtT-PBS; Ref 3, PBS-rtT-sgRNA
Seq, sequence of cloned PCR fragments

f

Mutation	De	Sc	Re	To	De/To	Sc/To	Re/To
W542L	93	0	7	100	93.0%	0.0%	7.0%
S621I	117	42	26	185	63.2%	22.7%	14.1%
WS	27	16		43	62.8%	37.2%	

De, Sc, or Re, No. of clones harboring specified mutations
De, desired edits
Sc, pegRNA scaffold-derived byproducts
Re, double-strand even DNA repair-derived byproducts
To, total No. of cloned fragments harboring mutations
WS, W542L and S621I double mutations

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