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.
Results and discussion

We selected two maize acetolactate synthase (ALS) genes to test prime editing efficiency in maize and attempted to generate maize herbicide-resistance lines harboring P165S mutation or W542L/S621I double mutations [9] in ZmALS1 and ZmALS2 by prime editing (Fig. 1a). We used maize Ubi1 promoter to drive the expression of maize codon-optimized PE2 and used OsU3 and TaU3 promoters to drive the expression of pegRNA and sgRNA, respectively. In this way, we constructed two pGreen3 binary vectors [10, 11], pZ1PE3 and pZ1PE3b, for the same P165S mutation but based on two different strategies, PE3 and PE3b [1], respectively (Fig. 1a,b). To generate mutant maize lines harboring W542L/S621I double mutations, we based the W542L and S621I mutations on PE3b and PE3 strategies, respectively, and assembled two pegRNA and two nicking sgRNA variants into the same binary vectors (Fig. 1a,b). According to the structure of pegRNAs, a hairpin can potentially form between the PBS and the protospacer of a pegRNA (Fig. 1a), and thus will possibly weaken the pegRNA activity. Based on this possibility, we reasoned that enhancing pegRNA expression will be able to improve editing efficiency. We used two strategies to enhance pegRNA expression: doubling pegRNA expression cassettes and using two promoter systems to drive pegRNA expression, together with use of the tRNA, ribozyme, and Csy4 RNA processing systems [12, 13] when appropriate (Fig. 1b). In this way, we generated two prime editors named pZ1WS-Csy4 and pZ1WS (Fig. 1b). We also generate two additional prime editors including p4xZ1PE3-Csy4 and p4xZ1PE3b-Csy4 for comparison with pZ1PE3 and pZ1PE3b, respectively. The p4xZ1PE3-Csy4 and p4xZ1PE3b-Csy4 editors have similar structure to pZ1WS-Csy4 but harbor redoubled pegRNA and sgRNA expression cassettes for the P165S mutation. The pZ1WS vector was constructed as a control of pZ1WS-Csy4 for comparison of Csy4 and non-Csy4 systems. We transformed maize with these six PE vectors via the Agrobacterium-mediated method. We obtained no transgenic lines transformed with the three Csy4 vectors. Almost at the same time, we found that transformations with 13 additional pGreen3 or pCambia binary vectors harboring the Csy4-P2A-ZsGreen fusion gene, including 1 maize and 12 Arabidopsis transformations, were unsuccessful, suggesting Csy4 protein severely affects Agrobacterium-mediated transformation of at least these two plant species.
To detect targeted mutations, we amplified fragments spanning the targets from genomic DNA of the transgenic lines and detected mutations by direct sequencing of PCR fragments. We observed no editing for the 71 and 156 lines transformed with pZ1PE3 and pZ1PE3b, respectively. Fortunately, we observed that 7 out of 16 lines transformed with pZ1WS harbor S621I edits (Fig. 1c,d). Interestingly, one line displayed homozygous mutations in both ZmALS1 and ZmALS2.

We cloned the PCR products from the 6 lines harboring chimeric or heterozygous mutations and sequenced ~86 clones for each PCR fragment (Additional file 1: Table S1). Interestingly, we observed the W542L edits in some cloned fragments, suggesting that low-frequency mutations were not able to be displayed in sequencing chromatograms (Additional file 1: Table S1). To further analyze the low-frequency W542L and/or S621I mutations, we cloned the PCR fragments from the line (#4) harboring homozygous mutations and an additional line (#3) harboring no mutations according to direct sequencing of PCR fragments. We observed the S621I edits in some cloned fragments from #3, further demonstrating that low-frequency mutations could not be reflected by direct sequencing of PCR products (Additional file 1: Table S1). Three cloned fragments harbor W542L and S621I double mutations, suggesting that PE can simultaneously edit two non-allelic targets in a cell.

We also cloned 6 PCR fragments from three pZ1PE3 and three pZ1PE3b transgenic lines, sequenced the cloned PCR fragments, but found no mutations in total 213 ZmALS1 and 196 ZmALS2 clones (Additional file 1: Table S1).

We observed two types of undesired byproducts from the sequencing results of cloned PCR fragments. One type is derived from the pegRNA scaffold, which acts as extended RT templates and thus introduces unwanted edits; the other is only involved in editing of multiple nucleotides and is derived from unbiased and well-balanced double-strand repair (rather than single-strand-biased repair) of multiple mismatches in heteroduplex DNA, which leads to incomplete editing of multiple nucleotides (Fig. 2a). In addition, the two mechanisms of forming these two types of byproducts were sometimes overlapped (Fig. 2a). We observed high frequencies of byproducts at the S621 target: 23.8% and 7.2% derived from the pegRNA scaffold and the double-strand even DNA repair, respectively (Fig. 2b). For the W542 target, frequencies of both types of byproducts remained to be investigated because of limited number of cloned PCR fragments harboring W542L edits. Since the T0 line #4 harbors homozygous mutations in the two ALS and the T0 line #15 harbors obvious pegRNA scaffold-derived byproducts (Fig. 1d), we excluded these two lines for possibly unbiased
analysis of the two types of byproducts. The recalculated frequencies of the byproducts for the S621I edits are 18.1% and 15.3% for the pegRNA scaffold-derived and double-strand even DNA repair-derived byproducts, respectively (Additional file 1: Table S1), indicating that frequencies of byproducts excluding the two lines are still high. Surprisingly, we observed no indel-type byproducts, suggesting that indels induced by PE are infrequent in maize.

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

We repeatedly transformed maize with pZ1WS after we observed that our first transformation could not generate many transgenic lines. In the second transformation, we obtained 46 transgenic lines. We detected targeted mutations induced by prime editing and the results demonstrated again that the prime editing efficiency of pZ1WS was much higher than previous reports (Fig. 2c and Additional file 1: Fig. S1). Interestingly, this time we observed the signals of the W542L edits in the sequencing chromatograms (Fig. 2d and Additional file 1: Fig. S1). These results suggest that we will be able to achieve homozygous mutants harboring W542L and S621I double mutations in both ZmALS1 and ZmALS2 in the progeny of some lines, such as #38 (Additional file 1: Fig. S1). In addition, we achieved two additional lines harboring the S621I homozygous mutations in the two ALS genes (Additional file 1: Fig. S2), and these two lines, together with the above described line, can be used to generate homozygous double mutants harboring W542L and S621I double mutations in the progeny. Not surprisingly, we again observed pegRNA scaffold-derived byproducts in the sequencing chromatograms (Additional file 1: Fig. S1). We cloned PCR products from the 4 lines harboring strong W542L edit signals in chromatograms (Additional file 1: Fig. S1) and sequenced
~106 clones for each PCR fragment (Fig. 2e,f and Additional file 1: Table S3). The results indicated that 11.5% cloned fragments from the line #41 harbor S621I edits although direct sequencing of PCR fragment from this line displayed weak S621I edit signals in chromatograms (Additional file 1: Fig. S1 and Table S3). We detected no pegRNA scaffold-derived byproducts from the W542L edits (Fig. 2e,f and Additional file 1: Table S3), suggesting low frequency of pegRNA scaffold-derived byproducts was produced at this target and frequency of byproducts depends on targets. Out of 242 cloned PCR fragments harboring edits, 17.8% (43/242) harbor W542L and S621I double mutations in ZmALS1 or ZmALS2 (Fig. 2f), demonstrating again that PE can simultaneously edit two non-allelic targets in a cell. For the W542L edits, the protospacer of the sgRNA nicking non-edited strand fully matches the edited ZmALS1 target but has a mismatch adjacent to PAM of the edited ZmALS2 target (Figure 1a), therefore actually PE2 was used for editing ZmALS2 although PE3b was done for ZmALS1. Editing frequencies at the W542 target of ZmALS1 and ZmALS2 are 7.1% and 39.7%, respectively (Additional file 1: Table S3), suggesting that PE2 has much higher editing efficiencies than PE3b.

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

Although we achieved sufficient high prime editing efficiency, a few questions remain to be answered. Is the pegRNA sequence, the optimized pegRNA expression, or both the decisive factor for the high efficiency of the S621I edits? Are the Pol2-Pol3 fusion promoters (such as 35S-CmYLCV-U6) or only Pol2 promoters (such as 35S-CmYLCV), together with the tRNA and ribozyme RNA processing systems, better systems to drive pegRNA expression than the commonly used Pol3 promoters (such as OsU3 and TaU3)? Is doubling or redoubling pegRNA expression cassettes crucial for high prime editing efficiency? Is clean pegRNA sequences trimmed by tRNA and ribozyme processing systems, or both vital for high prime editing efficiency? Why does Csy4 system affect maize transformation? To facilitate to answer these questions, we are developing additional user-friendly tools for a variety of plant species and promise to share them with researchers as soon as
possible. Application of these tools to test more pegRNA variants in amenable plants, such as rice, will help to clarify decisive factors for high prime editing efficiency in plants and thus facilitate the development of more powerful prime editors for high throughput precise genome editing in plants.

Conclusions

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

Methods

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

Vector construction

We replaced the XbaI-SbfI fragment of pUC57-dCas9 [14] with a synthetic fragment, resulting in the generation of pUC57-PE2P1. We replaced the MluI and SacI fragment of pUC57-PE2P1 with another synthetic fragment, resulting in the generation of pUC57-PE2. We modified the maize Ubi1 promoter in pG3R23-U3Ub [11] so that the first of the two PstI sites, the first of the four XbaI sites (the last 3 XbaI sites are blocked by Dam methylation), the Ncol site, and the EcoRI site in the promoter were disrupted. We replaced the XbaI and SacI fragment of the modified pG3R23-U3Ub with the XbaI and SacI fragment of pUC57-PE2, resulting in the generation of pG3R23-PE2U3A. We replaced the EcoRI-SacII fragment of pG3R23-PE2U3A with the EcoRI-SacII fragment of pG3GB411.
[11], resulting in the generation of pG3GB-PE2U3A. To generate the final PE binary vectors harboring a single pegRNA, we conducted the Golden Gate reaction to insert a synthetic pegRNA-OsU3t-TaU3p-sgRNA fragment into the two BsaI sites of pG3GB-PE2U3A. In this way, we generated pZ1PE3 and pZ1PE3b.

We replaced the Ascl-Pacl fragment of pL21-iCre [11] with a synthetic fragment of 35S-CmYLCV-Csy4-P2A-ZsGreen-polyA-Csy4-BB-HDVsHpt, resulting in the generation of pL2L1-35Csy4. We replaced the XbaI-EcoRI fragment of pL2L1-35Csy4 with a synthetic fragment of U6-tGly-BB-HDv-HSpt, resulting in the generation of pL2L1-35C-U6. We replaced the HindIII-EcoRI fragment of pR14-BWM [11] with an insert prepared by annealing two oligos oHEASmE-F/R, resulting in the generation of pR1R4-HEAS. We replaced the EcoRI-AarI fragment of pR1R4-HEAS with the EcoRI-Spel fragment of synthetic OsAct1p-CP4EPSPS-NOST, resulting in the generation of pR1R4-Gly. We amplified the U3p-BB-sgRNA cassette from pG3R23-PE2U3A with primers OsU3p-AsF and TaU3t-EcR, purified the PCR products, digested them with Ascl and EcoRI, and allowed them to ligate with Ascl and EcoRI-digested pR1R4-Gly and pL43-U3-Bar, resulting in the generation of pR1R4-U3G.0 and pL4L3-U3B.0, respectively.

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

**Maize transformation and analysis of prime editing**

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

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

Supplementary information

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

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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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Figure legends for Fig. 1

**Fig. 1** Efficiency of prime editing in maize. 

- **a** Sequences and their relationship of the two ALS genes, the pegRNA targets, the pegRNA RT templates, the pegRNA primer binding sites (PBS), and the sgRNA targets for nicking non-edited strands used for generation of 4 maize prime editing vectors. The PAMs, nicking sites, RT template and PBS lengths, and introduced mutations are indicated.

- **b** T-DNA structures of 4 PE vectors. The pZ1WS and pZ1WS-Csy4 vectors are designed for editing double-targets. Each double-PE vector includes two expression cassettes for each of two pegRNAs or sgRNAs. Two strategies are designed in the peg1&2: one is based on Csy4 RNA processing system and the other is based on tRNA and HDV ribozyme-based RNA processing systems integrated with double-drives of polymerase II (35S enhancer-CmYLCV) and III (shortened U6-26) promoters. The structure of pegRNA and sgRNA expression cassettes in pegR1-sgR1 and pegR2-sgR2 is the same as that in pZ1PE3/3b. The expressions of two sgRNAs in sgR1-sgR2 are driven by OsU3p and TaU3p, respectively.

- **c** Prime editing efficiencies based on direct sequencing of PCR products. The transgenic lines transformed with pZ1WS-Csy4 were not able to be achieved.

- **d** The sequencing chromatograms of PCR fragments from the prime-edited plants transformed with pZ1WS. Double peaks represent heterozygous or chimeric mutations and an asterisk indicates a mutation induced by PE. Note that the first mutation in #15 is the unwanted mutation introduced by pegRNA scaffold.
Figure legends for Fig. 2

**Fig. 2** Desired edits and unwanted byproducts in the two transformations with pZ1WS. a Desired edits and unwanted byproducts at the two target sites in the first transformation. Only partial sgRNA sequence is shown. The homologous sgRNA-rtT sequences shared by all the aligned sequences are shaded with yellow and mutated nucleotides are indicated with red letters. Number of cloned PCR fragments harboring the same edits is indicated in parentheses and a vertical line indicates the same type of byproducts. For convenience, the byproducts derived from the two mechanisms were assorted into the pegRNA scaffold-derived byproducts. b Summary of the desired edits and byproducts in the first transformation according to sequences of cloned PCR fragments. c Prime editing efficiency achieved in the second transformation. Strong and Weak, T0 lines harboring strong and weak peak signals for edits, respectively, in the sequencing chromatograms. Both, total No. of lines harboring strong and weak edits. d Sequencing chromatograms from a prime-edited line harboring W542L edits and obtained in the second transformation. Double peaks represent heterozygous or chimeric mutations and an asterisk indicates a mutation induced by PE. Note that the first mutation in the S621 target is the pegRNA scaffold-derived byproducts. e Desired edits and unwanted byproducts at the two target sites in the second transformation. f Summary of the desired edits and byproducts in the second transformation.
Figure 1

**a**

P165S mutation based on PE3b
CCG (Pro) > AGT (Ser)

| RT (15-nt) +1 | PBS (10-nt) | PAM | RT (15-nt) +1 | PBS (13-nt) |
|--------------|-------------|-----|--------------|-------------|
| T1, prime editing target; T2, target for nicking non-edited strand; RT, RT template; RP/PR, RT-PBS or PBS-rtT of pegRNA ALS, ZmALS1&2, S, C (ZmALS2) or G (ZmALS2); Y, T (ZmALS1) or C (ZmALS2) |

W542L mutation based on PE3b
TGG (Trp) > TTA (Leu)

| PBS (13-nt) | RT (16-nt) | PAM | RT (16-nt) | PBS (13-nt) |
|-------------|-----------|-----|-----------|-------------|

S621I mutation based on PE3
AGT (Ser) > ATT (Ile)

| PBS (13-nt) | RT (16-nt) | PAM | RT (16-nt) | PBS (13-nt) |
|-------------|-----------|-----|-----------|-------------|

**b**

pZ1PE3 or pZ1PE3b for the P165S mutation

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RB > OsU3p:pegRNA > TaU3p:sgRNA > Ub1p:PE2-E9t > Gly > Bar > LB
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pZ1WS-Csy4 or pZ1WS for the W542L and S621I mutations

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RB > pegR1-sgr1 > Ub1p:PE2-E9t > pegR1&2 > sgr1&2 > Gly > pegR2-sgr2 > Bar > LB
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**c**

| Vector | Mutation | No. of double mutants | No. of single mutants | Total | Ratio of mutants |
|--------|----------|------------------------|-----------------------|-------|-----------------|
| pZ1PE3b | P165S | 0 | 0 | 0 | 0 |
| pZ1PE3 | P165S | 0 | 0 | 0 | 0 |
| pZ1WS | W542L | 0 | 0 | 0 | 0 |
| pZ1WS | S621I | 1 | 6 | 0 | 0 |

*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.*

**d**

#4

```
A C C T T
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#8

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A C C T T
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#15

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G C C T T
```

The sequencing chromatograms of #5, #6, #14, and #16 are similar to that of #8.
Figure 2

a) S621I edits

| 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% |

b) W542L edits

| 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, sgRNA scaffold-derived byproducts
Re, double-strand even DNA repair-derived byproducts
To, total No. of cloned fragments harboring mutations

Table ofprime-edited lines

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

Diagram of W542L and S621I edits