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Letter

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Optimized prime editing for precise gene modification conferring herbicide resistance in sorghum

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Dear editor,

Maximizing the yield of sorghum (*Sorghum bicolor*) is challenging due to competition from weeds, particularly wild grasses. Acetolactate synthase (ALS) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) are targets of broad-spectrum herbicides and precise orthologous residue mutations at specific positions each can confer herbicide resistance (Jin et al. 2022). Prime editing (PE) uses CRISPR/Cas9 nickase, enabling precise point mutations without double-strand breaks or DNA donor templates (Anzalone et al. 2019). However, PE application in plants is limited by low editing efficiency.

Introducing the R221K and N394K mutations into the nCas9 H840A improved PE efficiency in rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (Jiang et al. 2022; Ni et al. 2023; Qiao et al. 2023). Deleting the RNase H domain of the reverse transcriptase Moloney-murine leukemia virus (M-MLV$^{\Delta \text{RNase H}}$) and/or using its V223A mutant improved editing efficiency in wheat (Ni et al. 2023). In addition, adding a 5'–3' exonuclease directly to the prime editor increased editing efficiency in HEK293T cells (Fig. 1a) (Truong et al. 2024). Similarly, the 5'–3' exonucleases T5E from bacteriophage and UL12 from herpes simplex virus increased knockout and homology-directed repair efficiency in rice and *Nicotiana benthamiana* (Schreiber et al. 2024; Wang et al. 2025). The stability of prime editing guide RNAs (pegRNAs) also affects editing efficiency, adding evopreQ$_1$ motif engineered pegRNA (epegRNA) stability in rice, wheat, and maize (Xu et al. 2022; Ni et al. 2023).

Due to limited precise editing research and low transformation efficiency, PE has not been reported in sorghum. Here, we generated prime editors by fusing nCas9 H840A or nCas9 H840A/R221K/N394K to M-MLV and the T5E or UL12 exonuclease, combined with a composite promoter (35S-CmYLCV-*SbU6*) driving epegRNA transcription and evaluated the PE efficiency in sorghum protoplasts and transgenic plants.

We constructed the SbPE vector by fusing M-MLV to nCas9 H840A using a composite promoter for epegRNA transcription (Figure S1, Table S1) and detected mutations in protoplasts, with average efficiencies of 2.16% (*SbALS* W545L), 1.26% (*SbALS* S624I), 1.21% (*SbEPSPS* TAP-IVS), and 1.34% (*SbEPSPS* D208N) (Fig. 1b, c). Then, we replaced nCas9 H840A with the higher-activity variant nCas9 H840A/R221K/N394K to construct the SbPEmax vector, achieving average editing efficiencies of 3.40% (*SbALS* W545L), 1.71% (*SbALS* S624I), 2.35% (*SbEPSPS* TAP-IVS), and 1.78% (*SbEPSPS* D208N), representing a 1.33~1.95-fold increase over SbPE (Fig. 1b, c). We added M-MLV$^{\Delta \text{RNase H}}$ to the N-terminus of nCas9 H840A/R221K/N394K and either the T5E or UL12 exonuclease to its C-terminus, generating SbPEmaxT5E and SbPEmaxUL12 vectors, respectively. For SbPEmaxUL12, the average editing efficiencies were 4.25% (*SbALS* W545L), 3.08% (*SbALS* S624I), 3.12% (*SbEPSPS* TAP-IVS), and 4.24% (*SbEPSPS* D208N), representing 1.96~3.16, and 1.25~2.38-fold improvements over SbPE and SbPEmax, respectively. SbPEmaxT5E greatly improved editing efficiency, with average efficiencies of 9.33% (*SbALS* W545L), 3.47% (*SbALS* S624I), 4.83% (*SbEPSPS* TAP-IVS), and 5.91% (*SbEPSPS* D208N), representing 3.54~4.41, 2.06~3.54, and 1.39~2.20-fold improvements over SbPE, SbPEmax, and SbPEmaxUL12, respectively (Fig. 1b, c). The greatest improvement was detected for the *SbALS* W545L target site, with a 2.20-fold increase in SbPEmaxT5E compared with SbPEmaxUL12.

We transformed the most efficient SbPEmaxT5E-based constructs targeting *SbALS* or *SbEPSPS* in sorghum cultivar "Tx430". Among 16 transgenic plants for the *SbALS* W545L target site, sequencing revealed precise introduction of the desired edit in the heterozygous plant #H8, a precise edit efficiency of 6.25% (1/16; Fig. 1d; Table S3). For *SbALS* S624I, none of the 12 transgene-positive plants had precise editing (Table S3), consistent with low editing efficiency of this target

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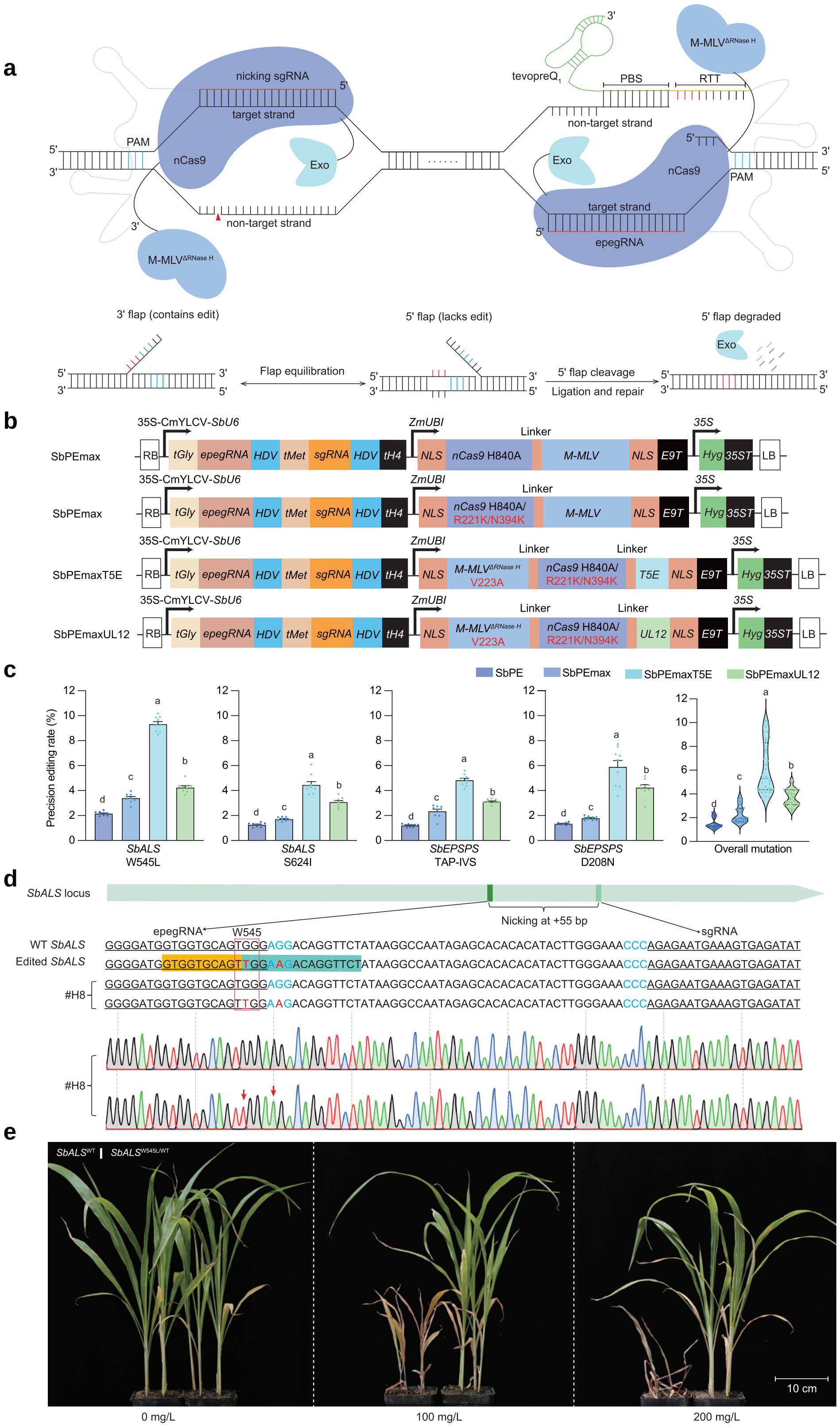

Figure 1 Prime editing of *SbALS* and *SbEPSPS* confers herbicide resistance in sorghum. (a) Prime editing (PE) system and 5′–3′ exonuclease disrupting the balance of 5′ and 3′ flaps. Exo, 5′–3′ exonuclease; epegRNA, engineered prime editing guide RNA; PBS, primer-binding site; RTT, reverse transcription template; tevopreQ$_1$, a protective sequence; nsgRNA, nicking sgRNA that induces a second cut away from epegRNA. (b) SbPE, SbPEmax, SbPEmaxT5E, and SbPEmaxUL12 systems. NLS, nuclear localization signal; LB, T-DNA left border; RB, T-DNA right border; 35S-CmYLCV-*SbU6*, composite promoter comprising 35S, CmYLCV, and native *SbU6* promoters. (c) Efficiency of prime editors in editing targets in sorghum protoplasts; means ± SEM ($n = 9$). Different lowercase letters indicate significant differences by Duncan's test. (d) *SbALS* locus (top) and Sanger sequencing chromatogram (bottom) of targeted region in *SbALS* for W545L edit. Red arrows: desired edits; Underline: epegRNA and nicking sgRNA target sequences; Blue: protospacer adjacent motifs (PAMs); Yellow box: PBS sequence; Cyan box: RTT sequence. (e) *SbALS*$^{\mathrm{WT}}$ and *SbALS*$^{\mathrm{W545L/WT}}$ plants 21 days after treatment with 0, 100, or 200 mg/L bispyribac-sodium in the greenhouse. Scale bar, 10 cm.

in protoplasts (Fig. 1c). For *SbEPSPS* D208N, we identified 13 transgene-positive plants, among which plant #G4 was heterozygous for the desired edit (Figure S2), corresponding to an editing efficiency of 7.69% (1/13; Table S3). For *SbEPSPS* TAP-IVS, none of the 13 transgene-positive plants harbored the desired edits (Table S3). Overall, our strategy enabled the precise editing of several target sites, expanding the application of PE to sorghum.

We found no off-target mutations at five potential *SbALS* W545L epegRNA sites and the corresponding nicking sgRNAs in the T1 generation (Table S4). The mutation was stably inherited, and transgene elements were segregated to generate transgene-free plants. (Table S5). For an unknown reason, the *SbEPSPS* D208N heterozygous T0 plant did not survive, preventing analysis of segregation in its progeny.

We sprayed wild-type (*SbALS*$^{\mathrm{WT}}$) and transgene-free *SbALS*$^{\mathrm{W545L/WT}}$ plants at the 4-leaf stage with 0 mg/L, 100 mg/L or 200 mg/L bispyribac-sodium. All *SbALS*$^{\mathrm{WT}}$ plants became chlorotic and died within 21 days after spraying (DAS). *SbALS*$^{\mathrm{W545L/WT}}$ plants showed minimal growth inhibition at 100 mg/L and mild suppression at 200 mg/L (Fig. 1e). In the field, all *SbALS*$^{\mathrm{WT}}$ plants died by 21 DAS with 100 mg/L bispyribac-sodium, whereas *SbALS*$^{\mathrm{W545L/WT}}$ plants appeared healthy (Figure S3). At maturity, no significant agronomic trait differences existed among groups (Figure S4).

In conclusion, we successfully developed an optimized PE system, SbPEmaxT5E that produces herbicide-resistant sorghum, overcoming a long-standing limitation and facilitating sorghum trait improvement.

Author contributions

B.D. and S.L. designed the experiments. J.Z., J.X., L.S., S.L., W.W., S.W., F.L., J.Z., Z.W., and G.L. performed the experiments. J.Z. wrote the manuscript. B.D. and S.L. revised the manuscript. All authors read and approved the final manuscript for publication.

Supplementary material

Supplementary material is available at *Plant Physiology* online.

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Conflicts of interest

No conflict of interest is declared.

Data availability

All data generated or analyzed in this study are included in this article and its Supplementary material.

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