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Brief Communication

Broad range plastid genome editing with monomeric TALE-linked cytosine and dual base editors

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Editing of the plastid genome helps understand the molecular functions of plastid genes and engineer desired traits in crops (Maliga, 2022). The DddA-derived cytosine base editors (DdCBEs) enable C-to-T editing in mitochondrial and plastid genomes (Kang *et al*., 2021; Li *et al*., 2021; Mok *et al*., 2020; Nakazato *et al*., 2021). Recently, Cho *et al*. (2022) developed TALE-linked deaminases (TALED) that can catalyse A-to-G base conversions in human mitochondria.

Harnessing the discovery of $DddA_{tox}$ (Cho *et al*., 2022), we generated new monomeric TALE-linked CBEs for plastid editing by exploring two cytidine deaminases: a human APOBEC3A variant (hA3A-Y130F) with broad editing windows (Ren *et al*., 2021) and an improved cytidine deaminase based on TadA (Lam *et al*., 2023), generating mTCBE and mTCBE-T respectively. Also, we explored a TadA-derived deaminase that can simultaneously deaminate cytosines and adenines (Lam *et al*., 2023) towards engineering a dual base editor, named mTCABE-T. None of these deaminases have been previously investigated for organellar genome editing in either plants or humans.

We first assembled the left or right TALE arrays targeted to three rice plastid genes encoding core components of photosystem II (*OsPsbA*), photosystem I (*OsPsaA*), and RNA component of the 30S ribosome subunit (*Os16SrRNA*). The three monomeric plastid base editors, along with DdCBE and Split-TALED controls, were constructed for expression in rice (Figure 1a). We evaluated base editing efficiency in regenerated rice calli through targeted amplicon deep sequencing. Impressively, mTCBE induced high-efficiency C-to-T conversions, with average editing frequencies of 42.3%, 21.6%, and 19.4% at *OsPsbA*, *OsPsaA*, and *Os16SrRNA* respectively (Figure 1b–d). DdCBE catalysed C-to-T conversions with average editing efficiencies of 7.8%, 33.5% and 34.2% at these target sites (Figure 1b–d). By contrast, mTCBE-T was less efficient than mTCBE, displaying C-to-T editing efficiencies of only 5.8%, 3.3%, and 0.8% in these targets (Figure 1b–d). Overall, mTCBE appears to be a robust base editor alongside DdCBE (Figure 1e).

Remarkably, mTCABE-T exhibited C-to-T substitutions with average efficiencies of 33.2%, 21.3%, and 5.0% as well as A-to-G substitutions with average efficiencies of 17.5%, 7.2%, and 7.8%. The Split-TALED induced C-to-T editing with average efficiencies of 2.7%, 15.0%, and 8.9% as well as A-to-G editing with average efficiencies of 12.9%, 4.8%, and 3.3% in the same targets (Figure 1b–d), consistent with Mok *et al*. (2022) report, which noted that UGI-free split can induce C-to-T and A-to-G edits. mTCABE-T is a better dual base editor with higher C-to-T and A-to-G editing efficiencies than Split-TALED (Figure 1e,f). Further sequence analysis confirmed frequent simultaneous C-to-T and A-to-G editing by six mTCABE-T editors at the three target loci (Figure 1g, Figure S1).

We further examined plastid base editing profiles by different editors. As expected, DdCBE favoured editing cytosines of a 5′-TC context within spacer regions (Figure 1h, Figure S2). Notably, mTCBE often generated distinct editing patterns and efficiently converted cytosines in a 5′-TCC, AC, and GC context at the *OsPsbA* target (Figure 1h). Similarly, at *OsPsaA*, mTCBE converted cytosines in TC and TCC context (Figure S2). At *Os16SrRNA*, four cytosines were converted to T (Figure S2). Although editing frequently happened in the spacer regions, both mTCBE and mTCABE-T also showed base editing at positions upstream or downstream of the spacer region, resulting in only partly overlapping editing profiles by the left and right TALEs (Figure 1h,i, Figure S2). Our data suggest that these monomeric base editors have broader editing ranges than DdCBEs.

We next investigated these monomeric TALE-linked base editors (mTBEs) in T_0 transgenic plants. At *OsPsbA*, targeted deep sequencing revealed that C-to-T base editing occurred at multiple positions, with editing frequencies reaching up to 81% and 86% based on 16 and 26 T_0 plants by mTCBE and mTCABE-T respectively (Figure 1j). Significantly, such high-frequency targeted mutagenesis by mTCBE and mTCABE-T translated to a loss-of-function albino phenotype (Figure 1k). Hence, we genetically confirmed the role of *OsPsbA* in photosynthesis in rice. Consistent with the calli data, mTCBE-T exhibited much lower efficiency, and none of the 29 T_0 seedlings plants showed an albino phenotype (Figure 1k). Our data suggest that high-efficiency editing of plastid genome copies at multiple editable positions could effectively knockout gene function (Figure 1l, Figure S3). Sanger sequencing showed consistent mutation types in different leaves of each edited plant, including homoplasmic edits, which suggests high probability of germ-line transmittable inheritance (Figure S4). At *Os16SrRNA*, C-to-T editing efficiency of up to 80% was also observed in T_0 plants with mTCBE and mTCABE-T (Figure S5). Together, our data demonstrates mTCBE

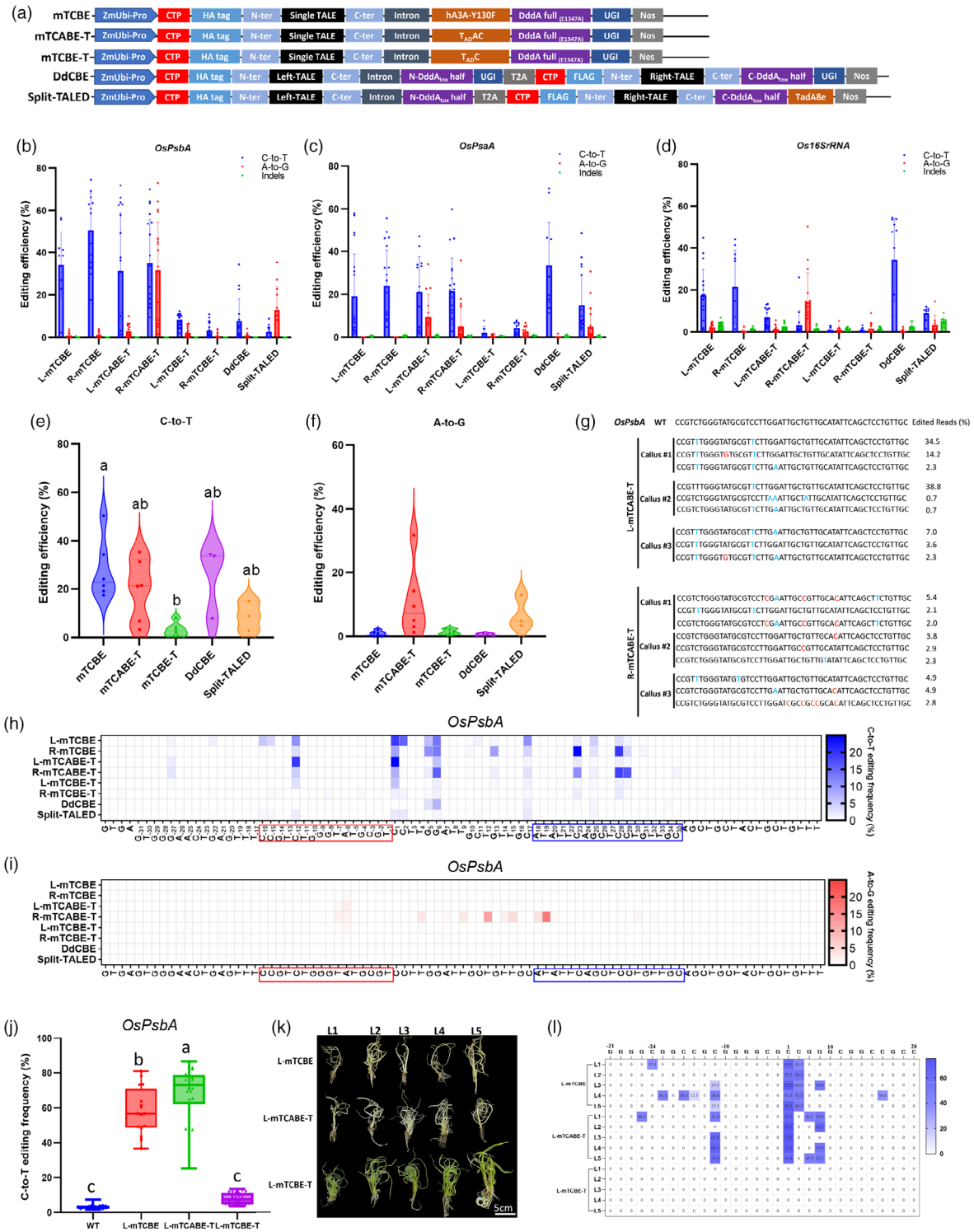

Figure 1 Monomeric TALE-linked cytosine and dual base editors for plant plastid genome editing. (a) Schematic of five TALE-based plastid base editors driven by the dual RNA polymerase II promoter. (b–d) The five base editors in regenerated rice calli were assessed at *OsPsbA*, *OsPsaA*, and *Os16SrRNA*. Error bar represents mean ± standard deviation (SD) of 8–20 independent calli. (e–f) Editing efficiency comparison of five base editors across these three target sites. (g) Editing efficiencies and patterns of mTCABE-T at *OsPsbA*. A-to-G and C-to-T base edits are shown in red and blue. (h, i) The heat map illustrates frequencies of C-to-T and A-to-G substitutions at *OsPsbA*. The colour gradations on the heat map are derived from data gathered from 8 to 20 independent calli. Left and right TALE binding sites are depicted as blue and red rectangles, denoted by 'L' and 'R' respectively. (j) Assessment of L-mTCBE, L-mTCABE-T, and L-mTCBE-T in transgenic rice plants at *OsPsbA*. The value is generated from 16 to 29 transgenic rice seedlings. T_0 lines with lower than 10% C-to-T mutation frequency were considered unedited. (k) The phenotype of five representative transgenic lines generated from L-mTCBE, L-mTCABE-T, and L-mTCBE-T base editors. Bar = 5 cm. L, line. (l) Heat map showing C-to-T conversions at *OsPsbA* site from five representative transgenic lines by different base editors. Different letters indicate significant differences ($P < 0.05$; one-way ANOVA, Tukey's test) in (e), and (j).

and mTCABE-T are potent plastid base editors in transgenic rice plants.

We analysed off-target activity of mTCBE and mTCABE-T at the *OsPsbA* site. Off-target mutations were induced at two of the four top candidate sites, only in few edited lines (Figure S6). Thus, sequences of TALE binding sites and expression of the editors can both affect the off-target editing outcomes.

In summary, we demonstrated two novel mTBEs, mTCBE, and mTCABE-T, for efficient plastid genome editing in rice. These mTBEs offer several advantages over DdCBEs. First, use of single TALE protein streamlines the vector construction process. Second, these mTBEs have broad editing windows. Third, mTCABE-T is an efficient dual base editor. Since mTCABE-T edits both strands of DNA, it can simultaneously induce four different types of base conversions on a single strand such as a coding sequence: C-to-T, A-to-G, T-to-C, and G-to-A. Thus, mTCABE-T in theory could potentially edit every base within its editing window. Hence, mTCABE-T would be a powerful high-density-targeted mutagenesis tool for protein evolution. Although we only demonstrated mTCABE-T in plastids, it is anticipated that mTCABE-T, when equipped with mitochondrial localization signals, could enable base editing in mitochondria in plants and beyond.

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

Y.Q. and X.W. designed the study. X.W., T.F., and J.L. conducted experiments. X.W. and Y.Q. analysed the data. Y.Q., X.W., and L.T. wrote the article with input from all authors.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Editing efficiencies and patterns induced by mTCABE-T in *Os16SrRNA* (A) and *OsPsaA* (B).
Figure S2 Heatmaps showing the frequencies of C-to-T and A-to-G substitutions across different positions targeted to *OsPsaA* (A, B), and *Os16SrRNA* (C, D).
Figure S3 Heatmap showing A-to-G conversions at *OsPsbA* from five representative transgenic lines generated from indicated base editors separately.
Figure S4 Representative genotypes of mTCBE (A) and mTCABE-T (B) induced edits in five randomly selected leaves of T_0 plants.
Figure S5 Evaluation of L-mTCBE, L-mTCABE-T, and L-mTCBE-T in transgenic rice plants at the *Os16SrRNA* site.
Figure S6 Analysis of off-target mutations induced by mTCBE and mTCABE-T in transgenic T_0 lines.
Table S1 PCR primers used in this study.

