Chloroplast and mitochondrial DNA editing in plants

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Abstract

Plant organelles, including mitochondria and chloroplasts, contain their own genomes, which encode hundreds of genes essential for respiration and photosynthesis, respectively. Gene editing in plant organelles, an unmet need for plant genetics and biotechnology, has been hampered by the lack of appropriate tools for targeting DNA in these organelles. In this study, we developed a Golden Gate cloning system, composed of 16 expression plasmids (8 for delivery of the resulting protein to mitochondria and the other 8 for delivery to chloroplasts) and 424 TALE sub-array plasmids, to assemble DddA-derived cytosine base editor (DdCBE) plasmids and used the resulting DdCBEs to promote point mutagenesis in mitochondria and chloroplasts efficiently. Our DdCBEs induced base editing in lettuce or rapeseed calli at frequencies of up to 25% (mitochondria) and 38% (chloroplasts). We also showed DNA-free base editing in chloroplasts by delivering DdCBE mRNA to lettuce protoplasts. Furthermore, we generated lettuce calli resistant to streptomycin, an antibiotic that binds to 16S ribosomal RNA (rRNA) irreversibly, leading to inhibition of protein synthesis, by introducing a point mutation in the chloroplast 16S rRNA gene.

Main

Programmable genome editing tools, which include zinc-finger nucleases (ZFNs)[1], transcription activator-like effector nucleases (TALENs)[2], clustered regularly interspaced short palindromic repeat (CRISPR) systems[3–6], and base editors[7–9] composed of the catalytically-deficient CRISPR-associated protein 9 (Cas9) variant and a nucleobase deaminase protein, have been developed for plant genetic studies and crop improvements through manipulation of genomic DNA sequences. However, these tools come short of editing DNA sequences in plant organelles, including mitochondria and chloroplasts, possibly because there are no efficient DNA double-strand break (DSB) repair systems in organelles and also because it is difficult to deliver both guide RNA and the Cas9 protein to organelles or to express the two components in organelles simultaneously. Plant organelle genomes encode hundreds of genes essential for photosynthesis and respiration. Methods or tools for editing these genes in organelles are highly desired for studying the functions of these genes and improving crop productivity. Recently, Mok et al.[10] demonstrated that CRISPR-free DddA-derived cytosine base editors (DdCBEs) enable targeted C•G-to-T•A base substitutions in mitochondrial DNA in mammalian cells. DdCBEs composed of non-toxic split domains of the bacterial cytidine deaminase toxin, $DddA_{tox}$, a custom-designed transcription activator-like effector (TALE) array, and a uracil glycosylase inhibitor (UGI), function as heterodimers to catalyze cytosine deamination, inducing C-to-T conversions, within a spacer region between the two TALE protein binding sites in target DNA. In this study, we present a rapid and convenient system to assemble DdCBE plasmids for expression in mitochondria and chloroplasts and use the resulting DdCBEs to edit mitochondrial DNA and chloroplast DNA, respectively, demonstrating highly efficient organelle base editing in plants for the first time.

To this end, we first developed a Golden Gate assembly system to construct chloroplast-targeting DdCBE (cp-DdCBE) plasmids or mitochondrial-targeting DdCBE (mt-DdCBE) plasmids (Fig. 1). Our expression

plasmids encode fusion proteins composed of a chloroplast transit peptide (CTP) or a mitochondrial targeting sequence (MTS), the TAL effector N- or C-terminal domains, split-DddA$_{tox}$ halves (G1333N, G1333C, G1397N and G1397C) and UGI, which are codon-optimized for expression in dicot plants, under the control of the parsley ubiquitin (PcUbi) promoter and pea3A terminator. DdCBE plasmids with custom-designed TALE DNA-binding arrays can be constructed in a single sub-cloning step by mixing an expression vector and six TALE sub-array plasmids in an Eppendorf tube. A total of 424 (= 6 x 64 tripartite + 2 x 16 bipartite + 2 x 4 monopartite) modular TALE sub-array plasmids[11] are available for making cp-DdCBEs or mt-DdCBEs that recognize DNA sequences of 16–20 base pairs (bps) in length, including a conserved T at the 5′ terminus. As a result, a functional DdCBE heterodimer recognizes 32- to 40-bp DNA sequences.

To assess whether our DdCBEs can promote base editing in chloroplasts, we constructed four pairs of cp-DdCBE plasmids specific to the chloroplast 16S ribosomal RNA (rRNA) gene encoding the RNA component of the 30S ribosomal subunit, co-transfected each pair into lettuce and rapeseed protoplasts, and measured base editing efficiencies using targeted deep sequencing at day 7 post-transfection (Fig. 2a, 2b). The best-performing cp-DdCBE pair (Left-G1397-N + Right-G1397-C) induced C•G-to-T•A conversions in the 15-bp spacer region between the two TALE array-binding sites at frequencies of 30.2% in lettuce protoplasts and 8.6% in rapeseed protoplasts (Fig. 2b). In line with the previous results in mammalian cells, cytosines (C_9 and C_{13}) in a 5′-TC motif were converted to thymine preferentially by this cp-DdCBE. Interestingly, a cytosine (C_7) in a 5′-AC context was changed to thymine at a frequency of 4.0% in lettuce protoplasts by another cp-DdCBE (Left-G1333-N + Right-G1333-C).

We also tested base editing in two additional chloroplast genes, psbA and psbB, which encode the photosynthetic proteins, D1 and CP-47, respectively, of Photosystem II (PSII) (Fig. 2c, d, Supplementary Fig. 2). Among four cp-DdCBEs targeted to the psbA gene, the most active one (Left-G1397-C + Right-G1397-N) was able to induce C•G-to-T•A conversions in lettuce protoplasts with frequencies of up to 24.2 % (Fig. 2d). Only the two cytosines (C_{11} and C_{12}) in a 5′-TCC context were efficiently converted to thymines by this base editor. It is possible that 5′-TCC was first converted to 5′-TTC and then to 5′-TTT. In rapeseed protoplasts, the other split pair (Left-G1333-N + Right-G1333-C) was most active at four cytosine positions (C_3, C_4, C_{11}, and C_{12}) with editing efficiencies of up to 3.5% (C_3). Note that C_3 and C_4 are in a 5′-TCC context in the rapeseed gene, whereas they are in a 5′-ACC context in the lettuce counterpart, owing to a single nucleotide polymorphism, which is responsible for efficient editing of the two cytosines (C_3 and C_4) in the rapeseed gene but not in the lettuce gene by this DdCBE. Likewise, the cp-DdCBE pair targeted to the psbB gene catalyzed conversion of two cytosines in a TCC context at editing frequencies of 1.2–4.1% in rapeseed protoplasts (Supplementary Fig. 2). Taken together, these results suggest that editing efficiencies are dependent on cytosine positions and contexts within a spacer region as well as DddA$_{tox}$ split positions (G1333 vs. G1397) and orientations (Left-G1333-N vs. Left-G1333-C) and demonstrate that our cp-DdCBEs enable efficient base editing in the chloroplast genome in plants.

Next, we sought to achieve base editing in plant mitochondrial DNA using our custom-designed mt-DdCBEs. To this end, we constructed mt-DdCBE-encoding plasmids, using our Golden Gate cloning system, targeted to the ATP6 and RPS14 genes in the rapeseed mitochondrial genome, transfected the resulting plasmids into rapeseed protoplasts, and measured base editing frequencies using targeted deep sequencing at day 7 post-transfection (Fig. 2e, f, Supplementary Fig. 3). Our mt-DdCBEs were able to catalyze C•G-to-T•A conversions at a frequency of 13.2% in ATP6 (Fig. 2f) or 9.5% in RPS14 (Supplementary Fig. 3). These results show that mitochondrial DNA in plants is amenable to base editing with mt-DdCBEs.

To investigate whether DdCBE-mediated edits in cpDNA and mtDNA were maintained during regeneration, we collected lettuce and rapeseed calli regenerated from DdCBE-treated protoplasts, four weeks after transfection (Fig. 3a), and measured base editing efficiencies in each callus using targeted deep sequencing and Sanger sequencing (Fig. 3b and Supplementary Fig. 4, 5). Base edits induced by the DdCBE specific to the chloroplast or mitochondrial genes were detected in 22 out of 26 lettuce calli or 7 out of 14 rapeseed calli with frequencies of up to 38.4% or 24.9%, respectively (Fig. 3c). Also, base edits in the chloroplast psbA gene were observed with frequencies of up to 3.9% in lettuce calli (Supplementary Fig. 4). Likewise, mitochondrial base edits were detected in rapeseed calli with frequencies of up to 24.9% and 1.9% in the ATP6 and RPS14 target sites, respectively (Supplementary Fig. 5). These results show that DdCBE expression in plant protoplasts is not cytotoxic and that organelle base edits induced by DdCBEs in protoplasts remain intact during regeneration.

Encouraged by the stable maintenance of organelle edits in calli regenerated from protoplasts, we investigated whether the chloroplast DNA edits in the 16S rRNA gene could confer resistance to streptomycin, an antibiotic that binds to 16S rRNA irreversibly, leading to inhibition of protein synthesis. Several SNPs in the 16 s rRNA gene are commonly observed in streptomycin-resistant prokaryotes and eukaryotes; in particular, the 16S rRNA C860T (*Escherichia coli* coordinate C912) mutation endows *Nicotiana tabacum* (tobacco) with resistance to streptomycin[12]. The nucleotide affected by the C860T point mutation in tobacco and the equivalent nucleotide in lettuce correspond to the C_9 position in Fig. 2b and 3b. We transferred lettuce calli regenerated from DdCBE-treated protoplasts to medium containing streptomycin (Fig. 3d). Mock-treated calli turned white, indicative of protoplast dysfunction, upon exposure to streptomycin. In contrast, DdCBE-treated calli remained greenish, showing resistance to streptomycin. Apparently, the heteroplasmic state harboring the C860T mutation was enough to confer resistance to the lettuce calli. Taken together, these results show that plant organelle heteroplasmy generated by DdCBEs in protoplasts can be maintained after cell division and callus development.

Last but not least, we sought to demonstrate DNA-free base editing in organelles using in vitro transcribed cp-DdCBE mRNA rather than expression plasmids. We transfected in vitro transcripts encoding the cp-DdCBE targeted to the 16S rRNA gene into lettuce protoplasts and analyzed base editing frequencies at the target site (Fig. 3a). C-to-T mutations were detected in protoplasts with frequencies of up to 19.0% (Fig. 3e and Supplementary Fig. 6). This method can avoid potential integration of plasmid DNA fragments in the host genome.

In conclusion, we have developed a Golden Gate cloning system, which employs a total of 424 TALE sub-array plasmids and 16 expression plasmids, to assemble DdCBE-encoding plasmids for organelle base editing in plants. Our DdCBEs custom-designed to target three genes in chloroplast DNA and two genes in mitochondrial DNA achieved C-to-T conversions at high frequencies in lettuce and rapeseed protoplasts. Importantly, the edits in plant organelles were maintained during cell division and callus development. Furthermore, we were able to obtain streptomycin-resistant lettuce calli by inducing a C860T mutation in the chloroplast 16S rRNA gene. We expect that our Golden Gate cloning system will be a valuable resource for organelle DNA editing in plants.

Methods

Construction of plasmids for expression in plant protoplasts. DdCBE Golden Gate destination vectors were constructed using Gibson assembly (New England Biolabs). Sequences encoding the TAL N-terminal domain, HA tag, FLAG tag, TAL C-terminal domain, split-$DddA_{tox}$ and UGI were codon-optimized for expression in dicot (*A. thaliana*) plants, and synthesized by Integrated DNA Technology. The sequence encoding the CTP from *AtinfA*[13] and *AtRbcS*[14] and the MTS from the ATPase delta subunit[15] and ATPase gamma subunit[16] were amplified from *A. thaliana* cDNAs. The PcUbi promoter and pea3A terminator were used to replace the mammalian CMV promoter in a backbone plasmid for plant expression[11]. To construct the vector for in vitro DdCBE mRNA transcription, a T7 promoter cassette was cloned into the DdCBE Golden Gate destination vector between the PcUbi promoter and the DdCBE coding region.

TALE array genes were cloned by one-way Golden Gate assembly[11]. Plasmids expressing DdCBE were constructed by BsaI-digestion and T4 DNA ligation of Golden Gate assembly products using 424 TAL effector array plasmids and destination vectors. One-way Golden Gate cloning was performed using the following steps: 20 cycles of 37 ˚C and 50 ˚C for 5 min each, followed by final incubations at 50 ˚C for 15 min and 80 ˚C for 5 min. All vectors for plant protoplast transfection were purified using Plasmid Plus Midiprep kits (Qiagen). DNA and amino acid sequences used in vector construction are provided in Supplementary Sequences.

mRNA in vitro transcription. DdCBE DNA templates were prepared by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). DdCBE mRNAs were synthesized and purified using an in vitro mRNA synthesis kit (Enzynomics). Primers for DNA template PCR amplification are listed in Supplementary Table 1.

Protoplast isolation and transfection. Lettuce (*Lactuca sativa* cv. Cheongchima) seeds were surface sterilized in 70% ethanol for 30 sec and in a 0.4% hypochlorite solution for 15 min, washed three times in distilled water. Lettuce seeds were germinated on 0.5x Murashige and Skoog (MS) medium supplemented with 2% sucrose under conditions of 16 h light and 8 h dark condition at 25 ˚C. Rapeseed (*Brassica napus* cv. Halla) seeds were surface sterilized in 70% ethanol for 3 min, and in a 1.0% hypochlorite solution for 30 min, after which they were washed three times with distilled water. Rapeseed

seeds were germinated on 1x MS medium supplemented with 3% sucrose under a 16 h light and 8 h dark condition at 23 ˚C.

Protoplasts isolation and transfection were performed as described previously[6, 9]. Cotyledons from 7-day old lettuce and 14 day old rapeseed plants were digested with enzyme solution (1% viscozyme, 0.5% celluclast, 0.5% novozyme, 3 mM MES, 9% mannitol and CPW salts, pH 5.8) during incubation with shaking (40 r.p.m) in the dark at room temperature for 3 h. The protoplast- enzyme mixture was washed with an equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 1.5 mM MES, pH 5.6) and intact protoplasts were harvested on a sucrose gradient (21%) by swing-out centrifugation at 80 g for 7 min. The protoplasts were incubated in W5 solution for 1 h at 4 ˚C before polyethylene glycol (PEG)-mediated transfection.

Lettuce protoplasts ($5x10^5$) and rapeseed protoplasts ($2x10^5$) resuspended in MMG solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES, pH 5.7) were transfected with plasmids (30 µg per construct) or mRNAs (40 µg per transcript) by PEG (40% [w/v] PEG 4,000, 0.2 M mannitol and 0.1 M $CaCl_2$)-mediated transfection and incubated for 20 minutes at room temperature. The PEG-protoplast mixture was washed 3 times with an equal volume of W5 solution with gentle inverting and incubated for 10 minutes. Subsequently, the protoplasts were pelleted by swing-out centrifugation at 100xg for 5 minutes.

Protoplast culture. Lettuce protoplasts transfected with DdCBE-encoding plasmids were re-suspended in lettuce protoplast culture medium[17] (LPCM, 0.5x B5 culture medium supplemented with 375 mg/L $CaCl_2•2H_2O$, 18.35 mg/L NaFe-EDTA, 270 mg/L sodium succinate, 103 g/L sucrose, 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 mg/L 6-benzylaminopurine (BAP) and 0.1 g/L MES). The protoplasts in LPCM were mixed 1:1 with LPCM containing 2.4 % low-melting point agarose (Agarose type Ⅶ) and immediately plated on a 6-well plate immediately. After the mixture solidified, the embedded protoplasts were overlaid with 1 ml of LPCM and incubated at 25 ˚C for 1 week in the dark. After this initial incubation, the overlaid LPCM medium was replaced with fresh LPCM every week and the embedded protoplasts were incubated at 25 ˚C under conditions of 16 h dim light and 8 h dark for 1 week and then of 16 h light and 8 h dark for 2 weeks. Micro-calli induced from protoplasts were cultured on regeneration medium (1x MS regeneration medium supplemented with 30 g/L sucrose, 0.1 mg/L α-naphthalaneacetic acid (NAA), 0.5 mg/L BAP and 0.7 % plant agar) for 4 weeks under conditions of 16 h light and 8 h dark at 25 ˚C. In preparation for analysis of the base editing efficiency, transfected protoplasts were cultured in LPCM medium at 25°C in the dark for 1 week without embedding. To examine streptomycin resistance, calli were cultured on regeneration medium containing 50 mg/L streptomycin.

Rapeseed protoplasts transfected with DdCBE-encoding plasmids were re-suspended in rapeseed protoplast culture medium[18] (RPCM, 1x B5 culture medium supplemented with 0.6 g/L $CaCl_2$, 20 g/L glucose, 70 g/L mannitol, 1 mg/L NAA, 1 mg/L BAP and 0.25 mg/L 2.4-D). The protoplast-RPCM mixture was transferred into a 6-well plate and incubated at 25 ˚C for 2 weeks in the dark. After 2 weeks, the

protoplasts were incubated at 25 ˚C under conditions of 16 h dim light and 8 h dark for 3 weeks. The RPCM was replaced with fresh RPCM every week.

DNA extraction. Total DNAs were extracted from cultured cells in liquid medium and transgenic calli using a DNeasy Plant Mini Kit (Qiagen). Cultured cells and calli were harvested by centrifugation at 10,000 r.p.m for 1 minute.

Targeted deep sequencing. Target regions were amplified using Phusion High-Fidelity DNA Polymerase with appropriate primers (Supplementary Table 1). Three rounds of PCR were performed (1st nested PCR, 2nd PCR and 3rd indexing PCR) to make a DNA sequencing library. Equal amounts of the DNA libraries were pooled and sequenced using MiniSeq (Illumina). The paired-end sequencing files were analyzed by CRISPR RGEN Tools (http://www.rgenome.net)[19].

Declarations

Acknowledgements

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Author contributions

B.-C. K. and J. -S. K. designed the study. B. -C. K., S. -J. B., S. L., J. S. L., A. K., H. L., G. B., H. S., and J. K. performed the experiments. B. -C. K. and J. -S. K. wrote the manuscript. J. -S. K. supervised the project.

Competing interests

J-S. K. is a cofounder of, and holds stock in, ToolGen, Inc.

Data availability

Supplementary information is available in the online version of the paper.

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Figures

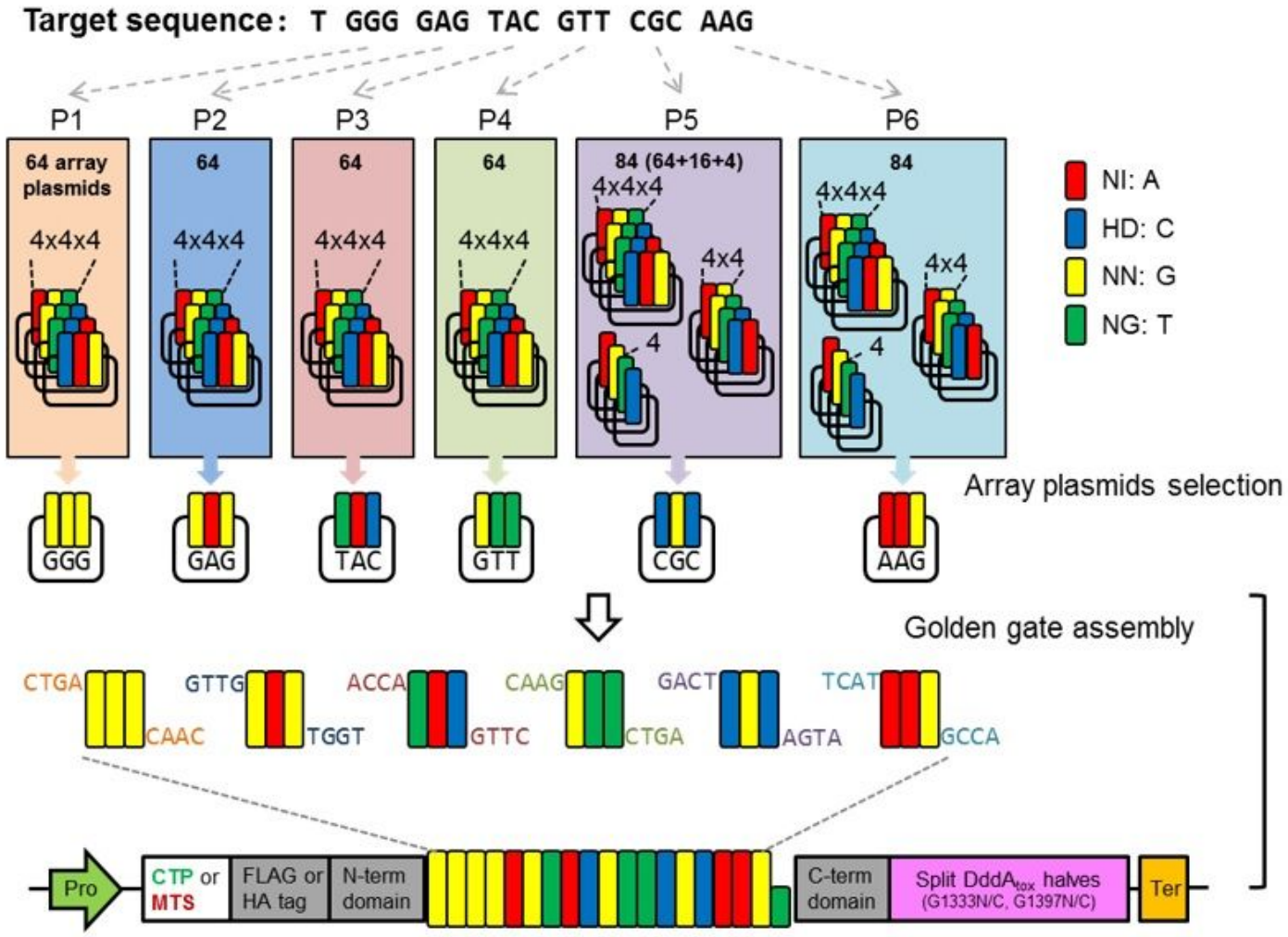


Figure 1

Golden Gate assembly system of plant organelle base editor. Schematic illustration of Golden Gate assembly for cp-DdCBE and mt-DdCBE construction. For each position in a target sequence, TALE sub-array plasmids were selected from a total set of 424 (= 6 x 64 tripartite + 2 x 16 bipartite + 2 x 4 monopartite), and mixed with a destination vector to generate plasmids encoding DdCBEs targeted to specific sequences.

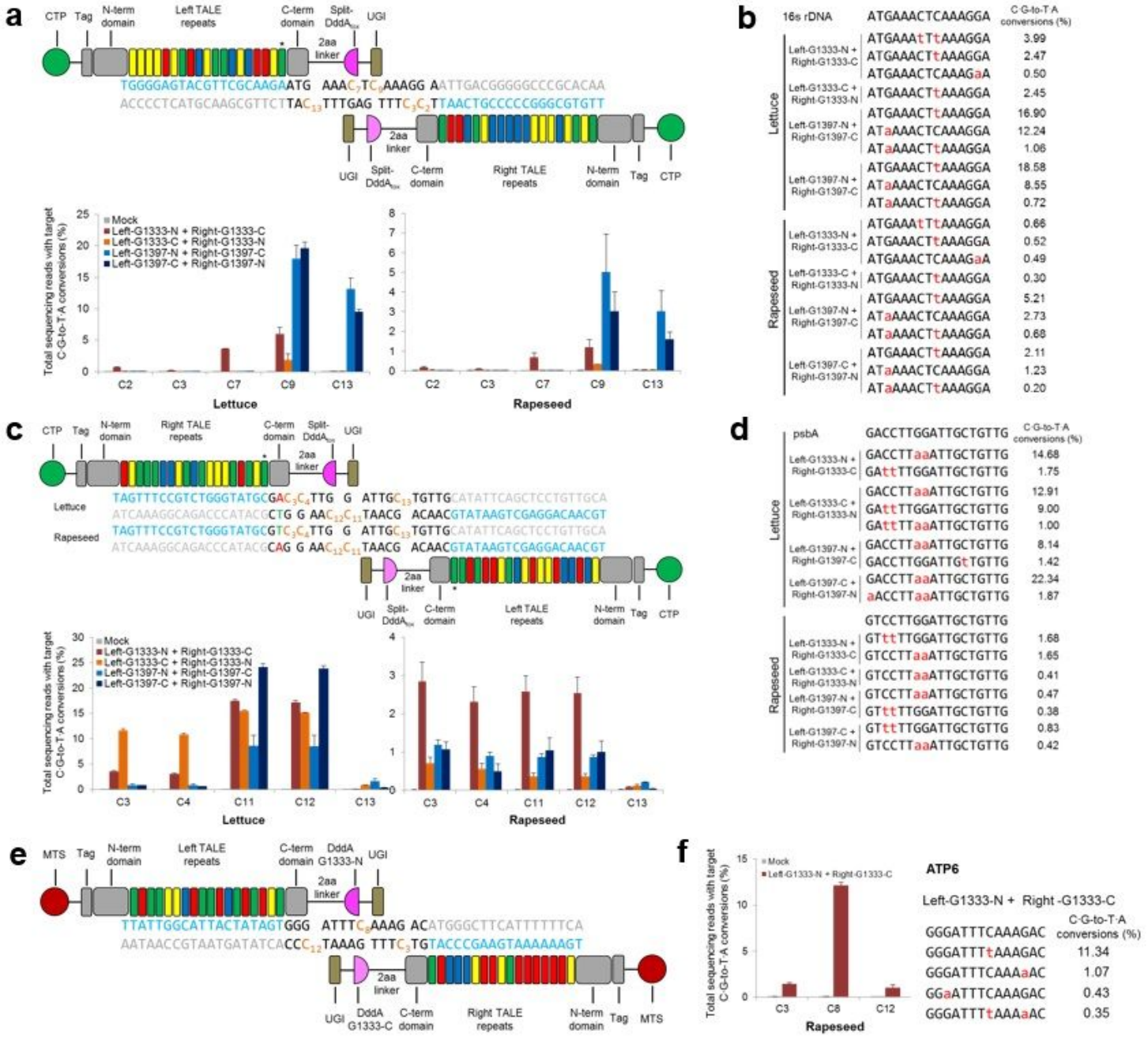
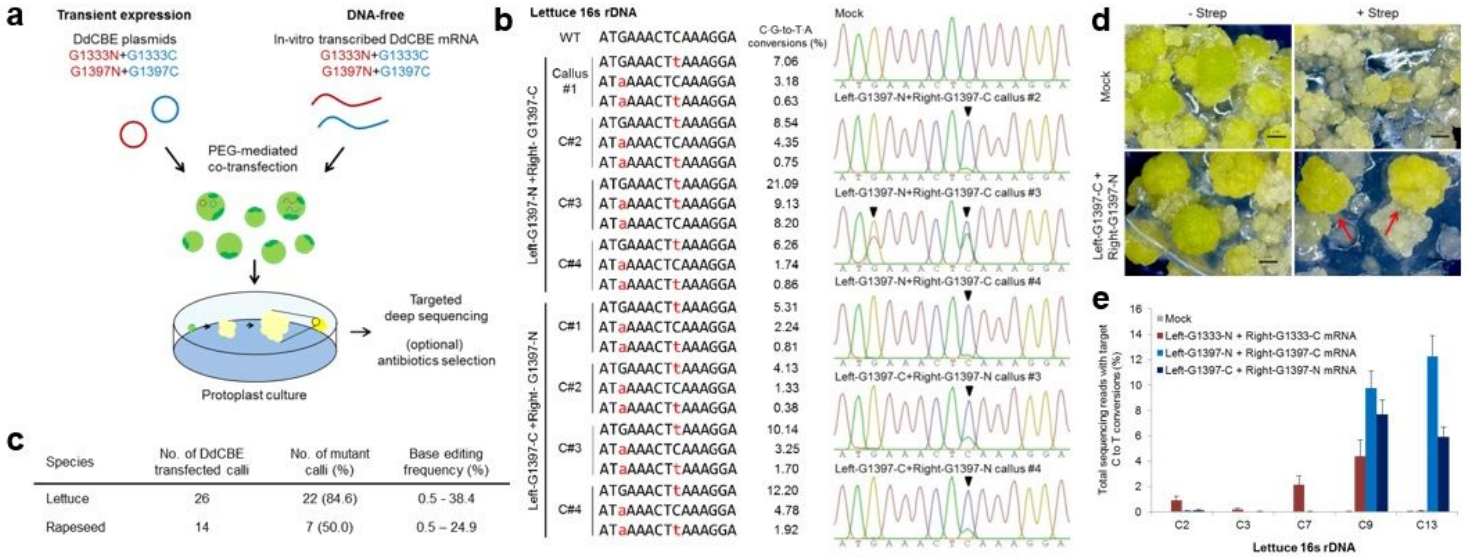


Figure 2

Chloroplast and mitochondrial base editing in plants. a, b, c, d, Frequencies and patterns of chloroplast base editing induced by cp-DdCBE in 16s rDNA (a, b) and psbA (c, d). Split DdCBE G1333 and G1397 pairs were transfected into lettuce and rapeseed protoplasts. e, f Editing efficiencies and patterns of mitochondrial base editing induced by mt-DdCBE in the ATP6 gene. Split DdCBE G1333 pairs were transfected into rapeseed protoplasts. a, c, e, The TALE-binding regions are shown in blue and the cytosines in the spacer are shown orange. b, d, f, Converted nucleotides are shown in red. In all graphs, error bars are the mean±s.d. of n=3 independent biological replicates.



c

Species	No. of DdCBE transfected calli	No. of mutant calli (%)	Base editing frequency (%)
Lettuce	26	22 (84.6)	0.5 - 38.4
Rapeseed	14	7 (50.0)	0.5 – 24.9

Figure 3

Plant organelle genome mutation generated via DdCBE-mediated base editing. a, Diagram of the scheme used to generate chloroplast and mitochondrial genome edited plants. b, The efficiencies of C•G-to-T•A conversions in cp-DdCBE tranfected calli, with representative Sanger sequencing chromatograms. Converted nucleotides are shown in red in the sequences on the left. The arrowheads indicate the substituted nucleotides in the chromatograms. c, DdCBE-induced base editing frequencies in regenerated calli. d, Selection for 16s rDNA mutants. The red arrows indicate streptomycin-selected calli. e, Frequencies of C-to-T conversions induced following transfection of mRNA encoding cp-DdCBE targeted to 16s rDNA into lettuce protoplasts. Error bars are the mean±s.d. of n=3 independent biological replicates.

Supplementary Files

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- DddAplantSI210112.docx