



# Prime genome editing in rice and wheat

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**Prime editors, which are CRISPR-Cas9 nickase (H840A)-reverse transcriptase fusions programmed with prime editing guide RNAs (pegRNAs), can edit bases in mammalian cells without donor DNA or double-strand breaks. We adapted prime editors for use in plants through codon, promoter, and editing-condition optimization. The resulting suite of plant prime editors enable point mutations, insertions and deletions in rice and wheat protoplasts. Regenerated prime-edited plants were obtained at frequencies of up to 21.8%.**

Introduction of genome modifications such as substitutions, insertions, and deletions that improve agronomic traits can accelerate crop improvement and breeding<sup>1,2</sup>. In plants, nuclease-initiated homology-directed repair (HDR) is limited by low efficiency and the difficulty of DNA template delivery<sup>3–6</sup>. Cytosine and adenine base editors (CBEs and ABEs) install C•G-to-T•A and A•T-to-G•C transitions<sup>7–9</sup>, and have been successfully used in plants<sup>4</sup>. However, base editors are unable to install transversions, insertions, or deletions<sup>7–10</sup>. Prime editing uses engineered Cas9 nickase–reverse transcriptase (RT) fusion proteins paired with a pegRNA that encodes the desired edit<sup>11</sup>. The RT domain uses a nicked genomic DNA strand as a primer for the synthesis of an edited DNA flap templated by an extension on the pegRNA. Subsequent DNA repair incorporates the edited flap, permanently installing the programmed edit<sup>11</sup>.

To optimize prime editing for plants, we first compared three plant prime editor systems (PPEs): PPE2, PPE3, and PPE3b<sup>11</sup> (Fig. 1a). PPE2 consists of a nCas9(H840A) fused to an engineered M-MLV RT, and a pegRNA composed of a primer binding site (PBS) and an RT template<sup>11</sup>. PPE3 adds an additional nicking single guide RNA (sgRNA) to cleave the non-edited strand, which facilitates favorable DNA repair. In PPE3b, this nicking sgRNA targets the edited sequence, thereby preventing nicking of the non-edited strand until after editing occurs, resulting in fewer indels in mammalian cells<sup>11</sup>.

We codon-optimized PPE genes for cereal plants and expressed them using the maize *Ubiquitin-1* (*Ubi-1*) promoter (Fig. 1b). We used the *OsU3* (or *TaU6*) and *TaU3* promoters to drive pegRNA and nicking sgRNA transcription, respectively. To test whether other RTs support prime editing, we replaced the engineered M-MLV RT with either the CaMV RT (RT-CaMV) from cauliflower mosaic virus<sup>12</sup> or a retron-derived RT (RT-retron) from *E. coli* BL21 (ref. 13) (Fig. 1b).

We first used our previously described<sup>14</sup> rice protoplast reporter system to test the PPE system for blue fluorescent protein (BFP) to green fluorescent protein (GFP) conversion, which requires changing codon 66 from CAC (histidine) to TAC (tyrosine)

(Supplementary Fig. 1). We designed pOsU3-BFP-peg01 with an RT template for changing ACCCAC (threonine-histidine) to ACGTAC (threonine-tyrosine), with the edited bases at positions +1 and +2, counting from the first base 3' of the pegRNA-induced nick. We introduced PPE, pUbi-BFP, pOsU3-BFP-peg01, and a nicking sgRNA into rice protoplasts. Flow cytometric analysis revealed 4.4% GFP-positive cells with PPE3b, and no GFP-positive cells in the absence of the RT (PPE3b( $\Delta$ M-MLV)) (Fig. 1c,d). Although PPE3b was less effective than an optimized plant base editor (PBE, 6.6%) (Fig. 1c,d), these results show that PPE3b can introduce desired prime edits in plants. We observed that the editing efficiency when using RT-CaMV (3.7%) was comparable to that when using the engineered M-MLV RT, but the efficiency was lower when using the RT-retron (2.4%) (Fig. 1c,d). These results indicate that the engineered M-MLV RT can be replaced by other RTs in PPEs.

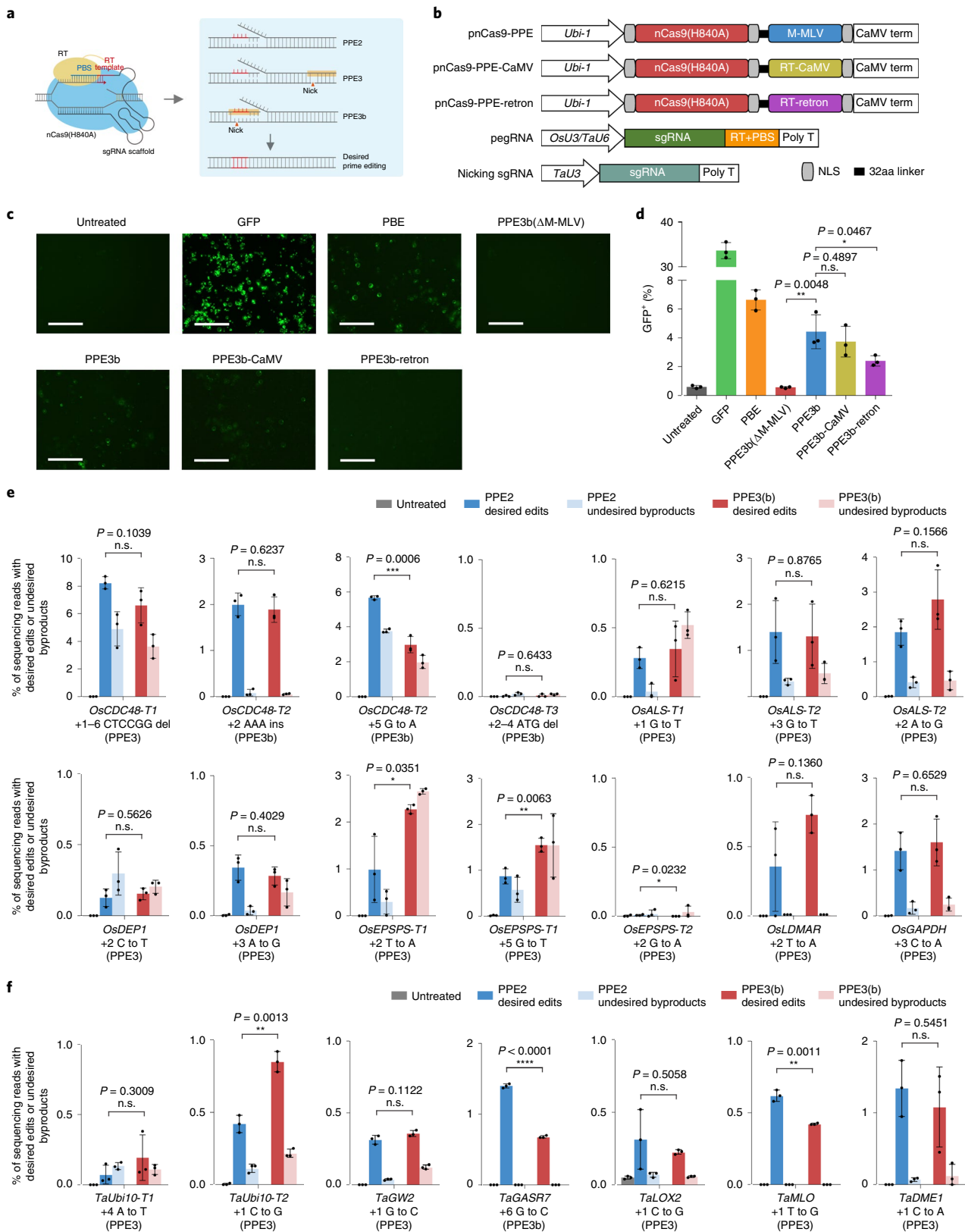
To examine prime editing of endogenous genes in rice and wheat, we chose six rice genes and six wheat genes, and constructed 21 pegRNAs to test the PPE2 and PPE3 (or PPE3b) systems (Supplementary Table 1). We measured the efficiencies of editing in protoplasts by deep amplicon sequencing. The PPE systems were found to induce 6-bp deletions at a frequency of 8.2% at *OsCDC48-T1*, 3-bp insertions at a frequency of 2.0% at *OsCDC48-T2*, and the six types of single nucleotide substitutions including C-to-T, G-to-T, A-to-G, G-to-A, T-to-A, and C-to-A at frequencies of up to 5.7% at the tested target sites in rice (Fig. 1e); in wheat, the frequencies of single nucleotide substitutions, including A-to-T, C-to-G, G-to-C, T-to-G, and C-to-A, reached 1.4% (Fig. 1f). PPE3 and PPE3b had a similar editing efficiency to PPE2 in the protoplast systems (Fig. 1e,f), indicating that the nicking sgRNA does not necessarily enhance prime editing efficiencies in plants, in contrast with observations in mammalian cells<sup>11</sup>. We also found that the PPE systems were less effective at the *OsCDC48-T3* and *OsEPSPS-T2* target sites despite the fact that indel frequencies generated by Cas9 nuclease at those sites were high (Fig. 1e and Supplementary Fig. 2), indicating that prime editing activity may not parallel Cas9 nuclease cleavage activity at some targets.

We observed PPE editing byproducts at 6 out of 21 tested targets at frequencies ranging from 0.5% to 4.9% (Fig. 1e,f). The main byproducts were pegRNA scaffold insertions or replacements (Supplementary Fig. 3), consistent with previous observations in mammalian cells<sup>11</sup>.

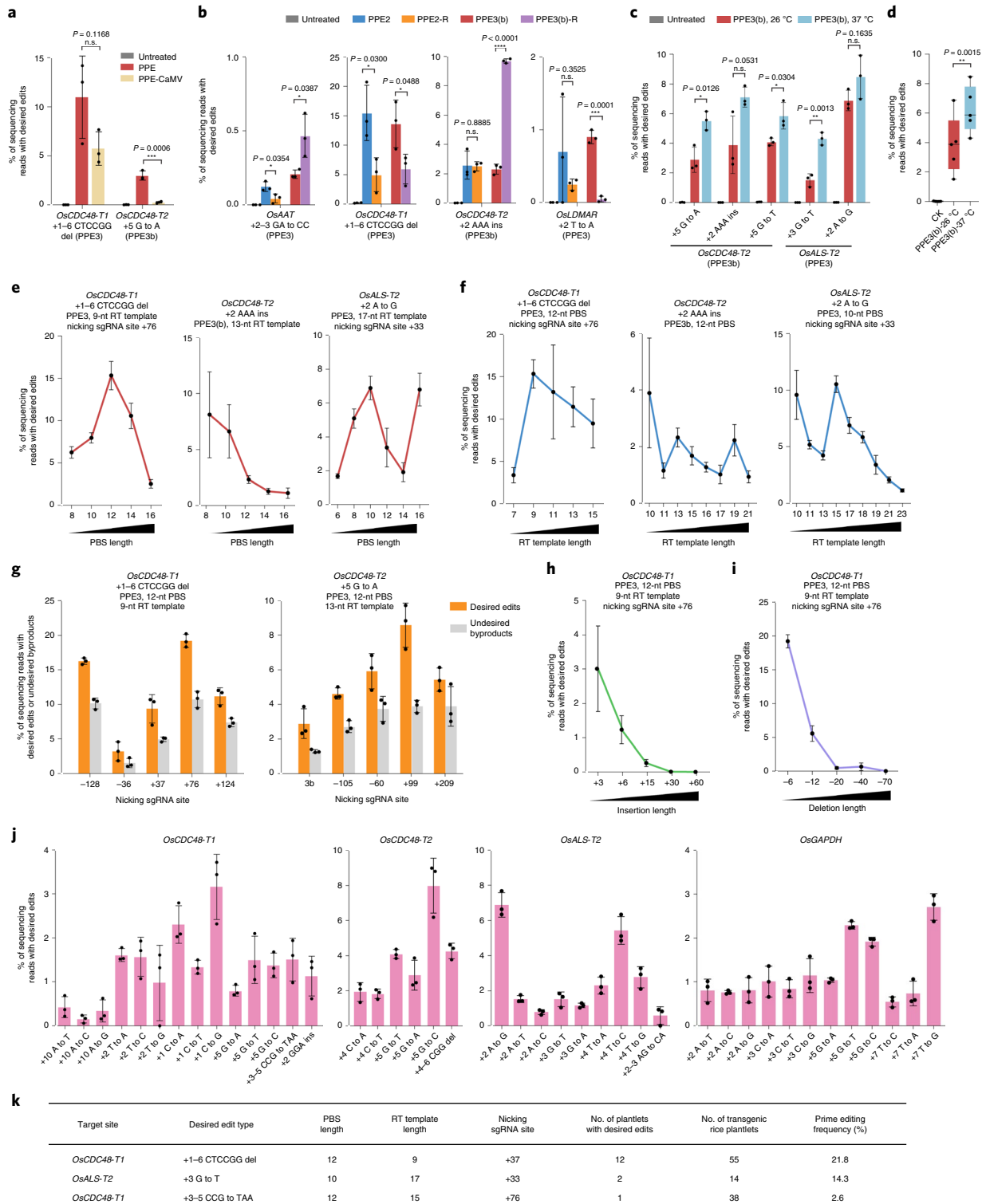
When we examined prime editing of endogenous genes by the PPE-CaMV system, we found that PPE-CaMV generated the desired 6-bp deletion with 5.8% efficiency at the *OsCDC48-T1* site,

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**Fig. 1 | Frequency of prime editing in rice and wheat protoplasts.** **a**, Diagram of the PPE system. A nicking sgRNA is required for PPE3 or PPE3b only. **b**, Schematic representation of the three PPE vectors, pegRNA, and nicking sgRNA vectors. **c**, Comparison of various BFP-to-GFP editing efficiencies in rice protoplasts by microscopy ( $n = 3$  independent experiments). An untreated protoplast sample served as a control. Scale bars, 200  $\mu\text{m}$ . **d**, Frequencies (%) of BFP-to-GFP conversion in rice protoplasts measured by flow cytometry (FCM). **e**, **f**, Frequencies of prime editing and undesired byproducts induced by PPE2 and PPE3 (or PPE3b) at 14 rice target sites (**e**), and seven wheat target sites (**f**). An untreated protoplast sample served as control in **d-f**. Frequencies (mean  $\pm$  s.e.m.) were calculated from three independent experiments ( $n = 3$ ).  $P$  values were obtained using the two-tailed Student's  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . n.s.,  $P > 0.05$ .



**Fig. 2 | Optimized prime editors for precise genome editing in rice.** **a**, Frequencies of prime editing induced by PPE3(b) and PPE3(b)-CaMV at *OscDC48-T1* and *OscDC48-T2* target sites in rice protoplasts. **b**, Frequencies of prime editing induced by the PPE and PPE-R systems at four target sites in rice protoplasts. **c**, Frequencies of prime editing at 26 °C and 37 °C at tested target sites in rice protoplasts. An untreated protoplast sample served as control in **a-c**. Frequencies (mean ± s.e.m.) were calculated from three independent experiments ( $n = 3$ ).  $P$  values were obtained using the two-tailed Student's  $t$ -test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . n.s.,  $P > 0.05$ . **d**, The average frequencies of prime editing at 26 °C and 37 °C at tested target sites in rice protoplasts. An untreated protoplast sample served as control. Data are presented as box plots (center line, median; box limits, 25th and 75th percentiles of the data; lower and upper whiskers, highest and lowest values). Data in each box plot included three independent experiments ( $n = 15$ ). The  $P$  value was obtained using the two-tailed Student's  $t$ -test. \*\* $P < 0.01$ . **e-g**, Effects of PBS length (**e**), RT template length (**f**), and nicking sgRNA location (**g**) on the efficiency of prime editing in rice protoplasts. **h,i**, Rates of prime editing of insertions of different length (**h**) and deletions of different length (**i**) at the *OscDC48-T1* target site in rice protoplasts. **j**, Rates of prime editing of different types of desired base substitutions, insertions and deletions in rice protoplasts. Frequencies (mean ± s.e.m.) were calculated from three independent experiments ( $n = 3$ ) in **e-j**. **k**, Frequencies of prime editing induced by PPE3 in regenerated rice plantlets.

and the desired G-to-A substitution with 0.3% efficiency at the *OsCDC48-T2* site. However, its efficiency was lower than that of M-MLV PPE (11.0% and 3.0% at the *OsCDC48-T1* and *OsCDC48-T2* targets, respectively) (Fig. 2a). We also developed a PPE-ribozyme (PPE-R) system, in which the prime editor protein transcript is coupled with polymerase II (Pol II)-expressed and ribozyme-processed pegRNA<sup>15</sup> (Supplementary Fig. 4). PPE-R increased the efficiency of the desired edit at *OsCDC48-T2*, but decreased efficiencies at *OsCDC48-T1* and *OsLDMAR* (Fig. 2b). In addition, we compared PPE efficiencies at 26°C and 37°C in rice protoplasts, and found that PPE activity was significantly higher at 37°C (average 6.3%) than at 26°C (average 3.9%) (Fig. 2c,d). Our observations suggest that it may be possible to increase the PPE efficiency of endogenous genes in plants by testing different conditions.

In addition, we compared the single base editing efficiencies of cytosine base editing by PBE<sup>14</sup> or adenine base editing by plant adenine base editor (PABE)<sup>16</sup> with that of PPE. We found that the PPEs (PPE2, 0.1%, and PPE3, 0.1%) were less efficient than PBE (2.7%) at *OsDEP1*, whereas PPE2 and PPE3 were more efficient than PABE at *OsALS-T2* (PPE2, 1.8%, PPE3, 2.6%, and PABE, 0.8%) (Supplementary Fig. 5). These results suggest that the relative efficiencies of PPEs, and PBE or PABE can vary by target site.

We then targeted three targets to test the effects of varying the pegRNA PBS length (6–16 nt), RT template length (7–23 nt), and nicking sgRNA position (−128 to +209). We found that editing frequencies were strongly affected by these parameters, and optimal values were different between the sites (Fig. 2e–g). The ratio of desired to undesired edits did not vary with the PBS length or the nicking site (Supplementary Fig. 6), but was affected markedly by the length of the RT template (Supplementary Fig. 7). These results highlight the importance of thoroughly testing a variety of pegRNAs and sgRNAs when editing at new target sites.

We also compared the production of insertions and deletions of different lengths at *OsCDC48-T1* using the PPE3 system. The frequencies of desired insertions of 3 nt, 6 nt, 15 nt, 30 nt and 60 nt were 3.0%, 1.2%, 0.3%, 0.0% and 0.0%, respectively, while for desired 6-nt, 12-nt, 20-nt, 40-nt and 70-nt deletions, they were 19.2%, 5.6%, 0.5%, 0.7% and 0.0%, respectively (Fig. 2h,i). Therefore, editing efficiency decreases with increasing length of insertion or deletion, but PPE can install small DNA insertions and deletions into genomic sites with useful efficiencies.

Next, we examined the capability of PPEs to produce all 12 kinds of base substitution, as well as multiple base substitutions, insertions, and deletions. We selected four rice targets, and found that PPE3 generated all 12 kinds of base-to-base substitutions with efficiencies of 0.2–8.0% (Fig. 2j). PPEs can also create multiple base substitutions (frequency of 1.5% at *OsCDC48-T1*, frequency of 0.6% at *OsALS-T2*), desired insertions (frequency of 1.1% at *OsCDC48-T1*) and desired deletions (frequency of 4.3% at *OsCDC48-T2*) (Fig. 2j). Thus, PPEs can create all types of single base substitutions, as well as multiple base substitutions, insertions, and deletions.

To regenerate prime-edited plants, we constructed a binary expression vector, pH-nCas9-PPE3, and introduced it into rice calli by *Agrobacterium*-mediated transformation (Supplementary Fig. 8a). Desired 6-nt deletions at *OsCDC48-T1* were observed in regenerated plantlets (21.8%, 12 out of 55), G-to-T base substitutions at *OsALS-T2* (14.3%, 2 out of 14), and GGC-to-TAA multi-nucleotide substitutions at *OsCDC48-T1* (2.6%, 1 out of 38) (Fig. 2k and Supplementary Fig. 8b,c). Sanger sequencing revealed that these rice prime-edited plants were chimeras (Supplementary Fig. 8c,d).

The PPEs reported here can efficiently produce a wide variety of edits at genomic sites in rice and wheat, especially when pegRNA designs and editing conditions are optimized. PPEs provide a complementary method for generating changes that cannot be made with other genome editing tools in plants. Although plant prime editing, like mammalian prime editing, is less efficient than base editors for making transition point mutations<sup>11</sup>, our study shows that PPEs can generate transversions, mixtures of different substitutions, insertions, and deletions. The versatility of plant prime editing thus has the potential to advance both plant breeding and functional genomics research.

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41587-020-0455-x>.

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## Methods

**Plasmid construction.** To construct vectors pnCas9-PPE, pnCas9-PPE-CaMV and pnCAs9-PPE-retron, NLS, 32aa linker, engineered M-MLV reverse transcriptase, RT-CaMV, and RT-retron reverse transcriptase and Cas9 (H840A) were codon-optimized for cereal plants, synthesized commercially (GENEWIZ), and the fusion protein sequences were cloned into the vector pJIT163 backbone yielding the various Cas9 (H840A)-RT fusion plasmids. To construct the pegrRNA expression vectors, sgRNAs were amplified using primer sets containing the target sgRNA sequences in the forward primer and the PBS+RT sequences in the reverse primer, and cloned into the OsU3-sgRNA or TaU6-sgRNA vectors<sup>17,18</sup>. To construct the Pol II-expressed and ribozyme-processed pegrRNA, the hammerhead ribozyme sequence, pegrRNA sequence, and the hepatitis delta virus ribozyme sequence were fused and cloned into the vector pJIT163 backbone. To construct the nicking sgRNA expression vectors, we amplified the TaU3-sgRNA fragments and introduced Esp3I restriction sites into the sgRNA constructs by overlap PCR from pCBC-MT1T2 (ref. <sup>19</sup>), and cloned them into the pMOD backbone<sup>20</sup>, yielding the plasmid pQPM-sgR. The nicking sgRNAs constructs were made as reported previously<sup>17,18</sup>. PCR was performed using TransStart FastPfu DNA Polymerase (TransGen Biotech).

To construct the binary vector pH-nCas9-PPE3 for *Agrobacterium*-mediated rice transformation, PPE, pegrRNA and nicking sgRNA expression cassettes were cloned into the pHUE411 backbone<sup>19</sup> by using a ClonExpressII One Step Cloning Kit (Vazyme).

All of the plasmid sequences are listed in Supplementary Sequences. All of the primer sets used in this work are listed in Supplementary Tables 2–7 and were synthesized by the Beijing Genomics Institute (BGI).

**Protoplast transfection.** We used the *Japonica* rice (*Oryza sativa*) variety Zhonghua11 and the winter wheat variety Kenong199 to prepare protoplasts. Protoplast isolation and transformation were performed as described previously<sup>21</sup>. The plasmids (10 µg per construct) were introduced by PEG-mediated transfection. The mean transformation efficiency was 28–45%. The transfected protoplasts were normally incubated at 26 °C for 48 h. For high temperature treatment, the transfected protoplasts were successively incubated for 12 h at 26 °C, 8 h at 37 °C, and 28 h at 26 °C. After incubation, the genomic DNA was extracted with the Plant Genomic DNA Kit (Tiangen Biotech) used for deep amplicon sequencing.

**Flow cytometry analysis.** Flow cytometry analysis was carried out using a FACSAria III (BD Biosciences) as reported previously<sup>21</sup>. Rice protoplast cells were transfected with guide RNA expression plasmids, fluorophore expression plasmids, and editor expression plasmids. Both samples were sorted for GFP-positive cells. Gating for all samples can be found in the Supplementary Data.

**DNA extraction.** Genomic DNA was extracted with a DNA Quick Plant System (Tiangen Biotech). The targeted sequences were amplified with specific primers, and the amplicons were purified with an EasyPure PCR Purification Kit (TransGen Biotech) and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

**Deep amplicon sequencing.** In the first round PCR, the target region was amplified from protoplast genomic DNA with site-specific primers. In the second round, both forward and reverse barcodes were added to the ends of the PCR products for library construction. Equal amounts of PCR product were pooled and sequenced commercially (Novogene) using the NovaSeq platform, and the sgRNA target sites in the sequenced reads were examined for desired edits and indels. Amplicon sequencing was repeated three times for each target site using genomic DNA extracted from three independent protoplast samples. Analyses of prime-editing processivity and indels were performed as described previously<sup>7</sup>.

***Agrobacterium* transformation of rice callus cells.** The pH-nCas9-PPE3 binary vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Callus cells of Zhonghua11 were transformed as reported previously<sup>22</sup>. Hygromycin (50 µg ml<sup>-1</sup>) was used to select transgenic plants.

**Mutant identification by PCR-RE assays and Sanger sequencing.** Rice mutants were identified by PCR restriction enzyme digestion (PCR-RE) assays (Source Data

and Sanger sequencing as described previously<sup>22</sup>. T0 transgenic rice plantlets were examined individually.

**Statistical analysis.** All numerical values are presented as means ± s.e.m. Statistical differences between the control and the treatments were tested using two-tailed Student's *t*-tests.

**Reporting Summary.** Further information on research design is available in the Life Sciences Reporting Summary linked to this article.

## Data availability

All data supporting the findings of this study are available in the article or in Supplementary information files, or are available from the corresponding author upon request. In terms of sequence data, the rice LOC\_Os identifiers (<http://rice.plantbiology.msu.edu/>) are LOC\_Os01g55540 (*OsAAT*), LOC\_Os03g54790 (*OsALS*), LOC\_Os03g05730 (*OsCDC48*), LOC\_Os09g26999 (*OsDEP1*), LOC\_Os06g04280 (*OsEPSPS*), LOC\_Os08g03290 (*OsGAPDH*). The NCBI GenBank identifiers are KJ697755 (*TaGW2*), KF009556 (*TaMLO*), KJ000052 (*TaGASR7*), JF683316 (*TaDME*), GU167921 (*TaLOX2*), FJ459808 (*TaUbi10*). The deep sequencing data have been deposited in an NCBI BioProject database (accession codes PRJNA605069 and PRJNA605074). Plasmids encoding nCas9-PPE, QPM-sgR (for nicking sgRNA construction), and pH-nCas9-PPE (for pH-nCas9-PPE2/3/3b construction) will be made available through Addgene.

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## Author contributions

Q.L., Y.Z., C.X., Y.W., A.V.A., A.R., J.L.D., D.R.L., and C.G. designed the project; Q.L., Y.Z., C.X., S.W., S.J., and Z.Z. performed the experiments; Q.L., A.V.A., A.R., J.L.D., D.R.L., and C.G. wrote the manuscript.

## Competing interests

The authors have submitted a patent application based on the results reported in this paper. D.R.L. is a consultant for and co-founder of Beam Therapeutics, Prime Medicine, Pairwise Plants, and Editas Medicine—companies that use genome editing.

## Additional information

**Supplementary information** is available for this paper at <https://doi.org/10.1038/s41587-020-0455-x>.

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### Software and code

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Data collection

Illumina NovaSeq platform was used to collect the amplicon deep sequencing data. BD FACSAriaIII was used to do flow cytometry.

Data analysis

Graphpad prism 7 was used to analyze the data. Amplicon sequencing data of prime-editing processivity was analyzed using the published code as previously described in reference 7. The custom Python script to analyze types of mutational reads and amino acid substitutions will be made available upon request. Graphpad prism 7 was used to analyze the data. FACSDiva Version 6.1.3 software was used for flow cytometry result analysis.

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## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The experiments of protoplasts were performed with three biological repeats. About 500,000 protoplasts were used for each transfection. The number of protoplasts in each transfection was measured by thrombocytometry. The experiment in rice regenerated plants was performed once, all the regenerated seedlings were sampled, the number of mutants were confirmed by Sanger sequencing.
Data exclusions	No data exclusion.
Replication	All attempts for replication were successful. For the experiments in rice and wheat protoplasts, a minimum of three independent experiments were included.
Randomization	Rice and wheat protoplasts were isolated and randomly separated to each transformation.
Blinding	Not applicable. As samples were processed identically through standard and in some cases automated procedures (DNA sequencing, transfection, DNA isolation) that should not bias outcomes.

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

### Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Antibodies
<input checked="" type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Human research participants
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

## Flow Cytometry

### Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

Sample preparation	Rice protoplasts were isolated from the stem of rice seedlings, transfected as described in the Methods and incubated in 2 ml WI solution for 2 days.
Instrument	BD FACSAriaIII
Software	FACSDiva Version 6.1.3 software was used for analysis.
Cell population abundance	The abundance of cells for flow cytometry analysis was 10,000 for each sample.

Gating strategy

Negative control (untreated) and fluorophore-positive cells were used to establish gates for each cell type. Gates were drawn to collect cells expressing either fluorophore. See the provided examples for gates used.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.