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Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs)

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Prime editors (PEs), which can install desired base edits without donor DNA or double-strand breaks, have been used in plants and can, in principle, accelerate crop improvement and breeding. However, their editing efficiency in plants is generally low. Optimizing the prime editing guide RNA (pegRNA) by designing the sequence on the basis of melting temperature, using dual-pegRNAs and engineering PEs have all been shown to enhance PE efficiency. In addition, an automated pegRNA design platform, PlantPegDesigner, has been developed on the basis of rice prime editing experimental data. In this protocol, we present detailed protocols for designing and optimizing pegRNAs using PlantPegDesigner, constructing engineered plant PE vectors with enhanced editing efficiency for prime editing, evaluating prime editing efficiencies using a reporter system and comparing the effectiveness and byproducts of PEs by deep amplicon sequencing. Using this protocol, researchers can construct optimized pegRNAs for prime editing in 4–7 d and obtain prime-edited rice or wheat plants within 3 months.

Introduction

Introducing desired genetic modifications into plant genomes plays an important role in agricultural improvement[1,2]. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based genome editing toolbox allows researchers to modify targeted genomic DNA in a flexible and precise manner[3]. Prime editors (PEs), which are composed of an engineered Cas9 nickase (H840A)-reverse transcriptase (RT) fusion protein and a prime editing guide RNA (pegRNA), can be used produce changes, including base substitutions, insertions and deletions, into targeted genomic sites[4,5]. The pegRNA contains a primer binding site (PBS) and an RT template (RTT). Cas9 nickase (H840A) recognizes and nicks the nontarget DNA strand of the target site and releases a single-strand DNA, and the PBS then hybridizes with the released DNA and serves as a primer for reverse transcription. The desired edits encoded by the RTT are then reverse transcribed and transferred to the nontarget DNA strand, generating a DNA flap that is subsequently incorporated into the target site by DNA repair[1,4,6] (Fig. 1). All 12 types of point mutation, as well as precise insertions and deletions, can be inserted by prime editing without requiring double-strand breaks or donor DNA templates.

Base editors, which are fusions of a Cas9 nickase (D10A) and a deaminase, can catalyze base conversions in target sites, but some base editors showed single-guide RNA (sgRNA)-independent off-target effects[1]. Unlike base editors, PEs do not induce detectable pegRNA-independent off-target effects, while also producing only a low level of pegRNA-dependent off-target effects[7–9]. However, in plants, the editing efficiency of PEs is substantially lower than that of base editors in general and needs to be further improved[1,5]. Several strategies, including optimizing the activities of both the nCas9-RT and pegRNA, have been used to increase PE efficiency. Engineered RTs, fusing functional elements or peptides to nCas9-RT (Cas9 nickase-reverse transcriptase), increasing the binding affinity of the nuclear localization signal sequences of nCas9 and optimizing their composition and number, also increases PE efficiency[10–14]. The efficiency can also be improved by optimizing the pegRNA; for example, by increasing its rate of transcription[15], preventing its circularization or degradation by incorporating Csy4 binding sites or structured RNA motifs[16,17] and incorporating additional RNA aptamers to recruit RTs[18]. In addition, manipulating repair pathways, for instance by downregulating the mismatch repair pathway, also provides a route for improving the efficiency of PEs[19,20].

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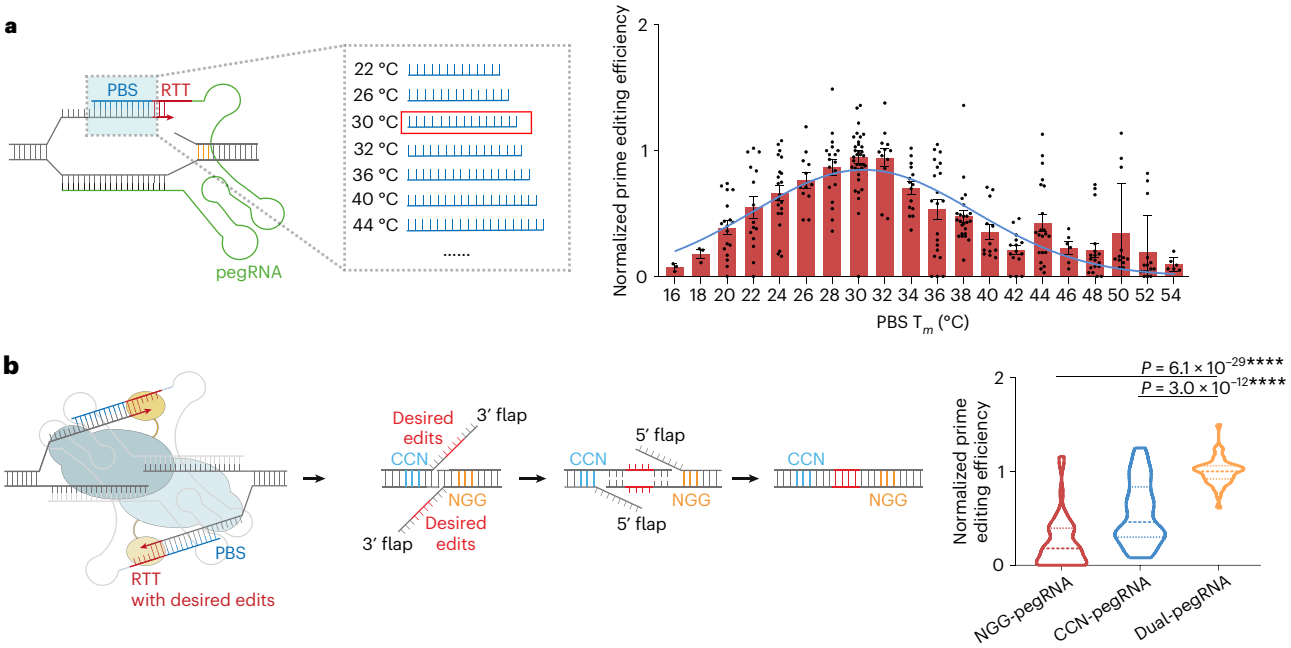
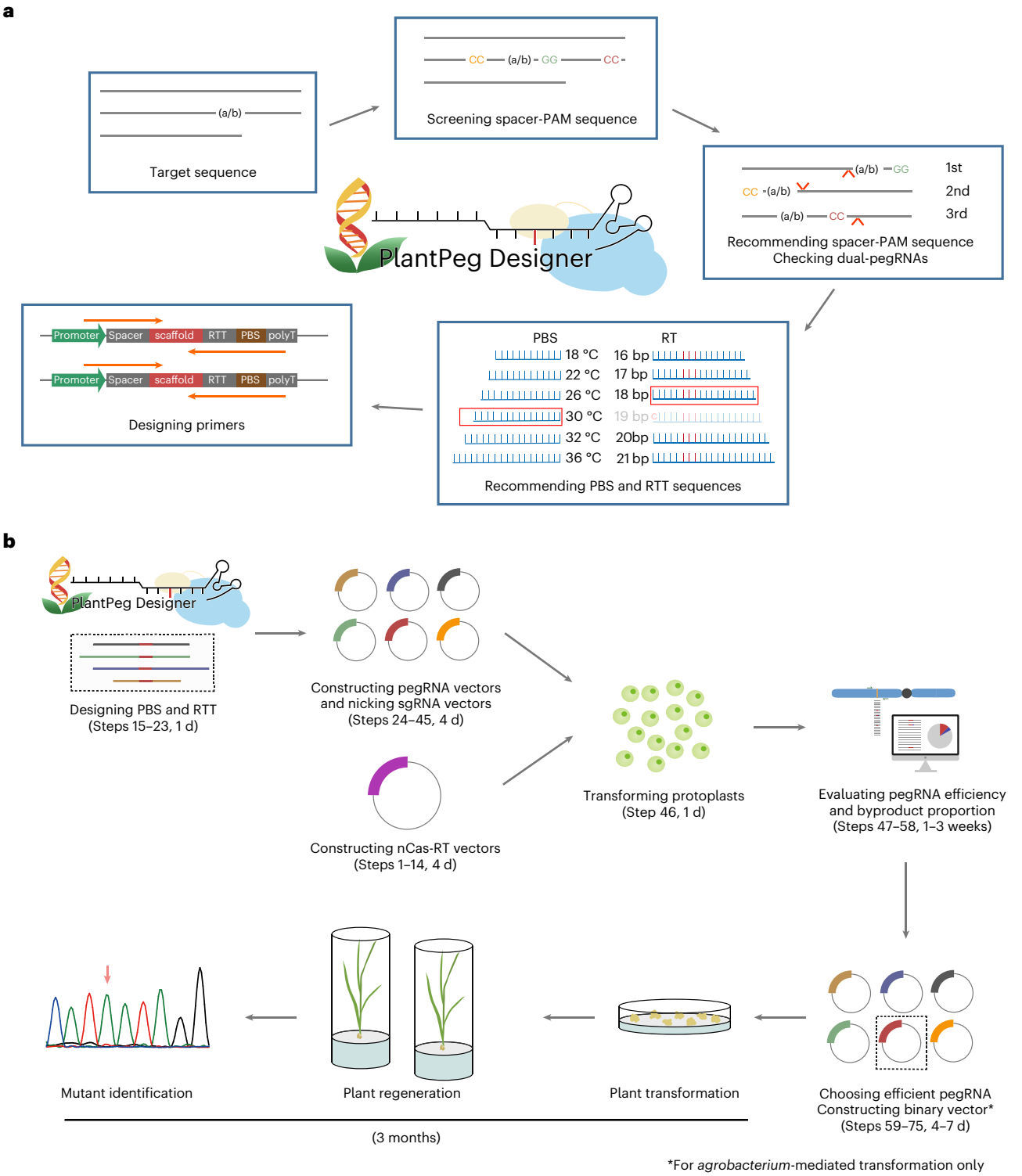


Fig. 1 | The effects on PE in rice protoplasts of varying T_m-directed PBS length and forms of pegRNA. a, Diagram of testing the editing efficiency of PEs with varying T_m-directed PBS lengths (left) and normalized PE frequencies at different PBS melting temperatures (right). The average editing efficiencies of three repeats of the highest editing efficiency obtained at each target was normalized to 1, and the frequencies obtained at the other PBS T_m were adjusted accordingly (prime editing efficiencies in 18 target sites, $n = 3$ independent experiments). The red column represents the average of the normalized editing efficiencies (mean ± standard error of the mean (s.e.m.)). The efficiencies were found to follow a normal distribution (shown by the blue line) using the Kolmogorov–Smirnov test. The fitted curve equation is $y = 0.8523 \times \exp(-0.5 \times ((x - 30.37)/8.415)^2)$ ($R^2 = 0.8101$). **b**, Schematic diagram of prime editing using the dual-pegRNA strategy and overall editing frequencies induced by PPEs containing NGG-pegRNA, CCN-pegRNA and dual-pegRNAs. The editing frequencies using dual-pegRNAs for each target were normalized to 1, and the frequencies using NGG-pegRNA and CCN-pegRNA for each target were adjusted accordingly (15 target sites, $n = 3$ independent experiments). Frequencies in **a** and **b** (mean ± s.e.m., shown as dashed and dotted horizontal lines) were calculated from three independent experiments ($n = 3$). *P*-values were obtained using two-tailed Student's *t*-tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. Panels **a** and **b** (right) reproduced and panel **b** (left) adapted with permission from ref. [25], Springer Nature America.

Although PEs are able to create targeted modifications of short DNA sequences, they cannot easily generate large insertions or deletions, or large replacements. However, several strategies involving incorporating site-specific recombinases and using paired pegRNAs[21,22] have been developed to overcome this problem, thus greatly expanding the versatility of PEs.

Development of the protocol

We have previously shown that the efficiency of PEs is strongly affected by the design of the pegRNA[4,5,23,24]. The protocol described here is an extension of our previous method for plant prime genome editing in rice and wheat[5,10,25]. It is important to thoroughly test a variety of pegRNAs when targeting new sites owing to varying editing efficiencies. However, testing libraries of pegRNAs is time consuming and laborious. Fortunately, using this protocol, we found that, in rice, the editing efficiency of PEs is strongly related to the melting temperature (T_m) of the PBS sequence, and this is likely to be a major factor influencing the design of plant pegRNAs[25]. PBS sequences with a T_m of 30 °C perform optimally in rice (Fig. 1a). Furthermore, using two pegRNAs in trans encoding the same edits for the forward and reverse strands also enhances PE efficiency (Fig. 1b). Combining these two strategies markedly increases the efficiency of PE[25]. By comparing the effects of varying PBS and RTT lengths, and the distance between the desired edits and the nicking site on PE in rice protoplasts, we have identified the optimal values of various parameters for pegRNA design in rice. On the basis of this experimental data, and the above strategies, we developed a web application, PlantPegDesigner, with an experimentally confirmed pegRNA design algorithm (Fig. 2a). Furthermore, we also engineered the M-MLV (Moloney-murine leukemia virus) RT by removing its ribonuclease H domain and incorporated a viral nucleocapsid protein with nucleic acid chaperone activity to develop an engineered plant prime editor (ePPE); this enhanced the frequency of various targeted modifications, including base substitutions, as well as deletions of ≤90 bp and 34 bp insertions, by an average of 5.8-fold (ref. [10]). We coupled the pegRNA design strategy and ePPE in this protocol to obtain high-efficiency prime editing in plant cells.



Applications of the method

Prime editing provides a novel way of inserting various types of edit at targeted sites in eukaryotic cells. The efficiencies of prime editing vary among different pegRNAs. The procedure, including the construction of both nCas9-RT and pegRNA vectors, is straightforward and efficient when applied to rice and other plants. Although the currently recommended optimal parameter values for PlantPegDesigner are set for rice, the tool can be adapted for other organisms by testing and adjusting parameter values.

◀ **Fig. 2 | Overview of prime editing in rice and wheat. a**,**b**, Diagram illustrating the design of the PBS and RTT by PlantPegDesigner (**a**), the construction of pegRNAs, evaluation of the efficiencies of PEs, transformation and regeneration of plants, and identification of prime-edited plants (**b**). **a**, PlantPegDesigner was developed based on data concerning the effect of T_m-directed PBS length variation and the use of a dual-pegRNA strategy. It screens and ranks spacer-PAM sequences for inserting edits in the user-defined PE window, and the dual-pegRNA model is recommended if spacer-PAM sequences can be found in both the forward and reverse strands of the input sequence. For each spacer-PAM sequence PlantPegDesigner reports and recommends PBS sequences and RTT sequences based on PBS T_m and user-defined RT length, respectively. It also specifies primers for constructing pegRNA vectors with appropriate plasmid vector scaffolds. a/b (original sequence/edit sequence). **b**, nCas9-RT vectors are constructed first (Steps 1–14). After PlantPegDesigner designs the PBS and RTT (Steps 15–21), pegRNA vectors can be constructed using PlantPegDesigner-recommended primers (Steps 22–36). The efficiencies of pegRNAs, as well as byproduct frequencies, can be evaluated in transient rice protoplast assays (Steps 37–49). To obtain prime-edited wheat, the chosen vectors are transformed into wheat embryos[49]. To obtain prime-edited rice plants, binary vectors harboring efficient pegRNAs are constructed and transformed into calluses by *Agrobacterium*-mediated transformation (Steps 50–67). Prime-edited plants are identified by Sanger sequencing. Figure 2 adapted with permission from ref. [25], Springer Nature America.

Comparison with other methods

In a previous study, ~13 nt PBS sequences were recommended for prime editing[4]. Compared with manually designed pegRNAs with ~13 nt PBS sequences, PlantPegDesigner-designed pegRNAs had markedly higher editing efficiencies across five targets, and three targets using dual-pegRNA[25] (Fig. 3a,b). Multiple alternative web applications for pegRNA design have been developed[25–34]. However, there was previously a lack of an experiment-based design algorithm. We compared the PE efficiencies of pegRNAs designed with PlantPegDesigner and those designed with three other web applications in rice: multicrispr[27], peg-Finder[28] and PrimeDesign[29]. PlantPegDesigner had higher editing efficiencies at each of the sites tested (Fig. 3c). Furthermore, T_m and dual-pegRNA models are not present in other pegRNA design applications[2]. Moreover, the PlantPegDesigner-recommended dual-pegRNAs were much more efficient than the pegRNAs designed by other web applications when editing the same sites in rice (Fig. 3d). These experimental results highlight the utility of the PlantPegDesigner webtool.

The dual-pegRNAs that we developed uses a pair of pegRNAs encoding the same edit to perform prime editing, and we demonstrated that these had higher editing efficiencies compared with the use of single-pegRNAs[25]. Recently, similar strategies, such as PRIME-Del (programming deletions up to 10 kb and 1–30% editing efficiency)[35], PEDAR (replacement of up to 10 kb with a desired sequences up to 60 bp)[36], HOPE (high prime editing efficiency in human cells)[37], and twinPE (high prime editing efficiency in human cells)[21] have all been introduced that use paired pegRNAs to increase the editing efficiency of PEs in mammalian cells. PlantPegDesigner combines T_m-directed design of PBS length and the use of dual-pegRNAs based on a data design algorithm to optimize prime editing. PlantPegDesigner includes two special models named the T_m and dual-pegRNA models not present in other pegRNA design applications[25]. This approach can be combined with other recent improvements of PEs, such as ePPE and other optimized nCas9-RTs[10,11,14,19] and pegRNAs[15–18].

Limitations of the method

Although PlantPegDesigner has been shown to generate efficient pegRNAs in rice, certain limitations should be noted. The first is that the recommended parameters are suitable for rice, so they must be reevaluated and adjusted for other organisms. In the following protocol, we describe in detail the experimental procedure for testing pegRNA parameters by transient transformation. The second limitation is that PlantPegDesigner was designed for producing high-efficiency pegRNAs but it does not consider off-target effects. We recommend that the specificity of the designed pegRNAs should be further analyzed by Cas-Offinder or other appropriate software[38–41].

Time

About 5–7 d is needed to construct vectors for prime editing and 3–4 months to obtain prime-edited rice or wheat plants.

Experimental design

PegRNA design using PlantPegDesigner

PlantPegDesigner is a simple web application for designing efficient pegRNAs. It is based on published design principles and plant prime editing results, and T_m-directed and dual-pegRNA models[4,5,25]. It provides a variety of parameter choices to meet the needs of users. PegRNA design involves the use of 12 parameters whose default values are optimal for rice but can be changed by the user. These parameters are:

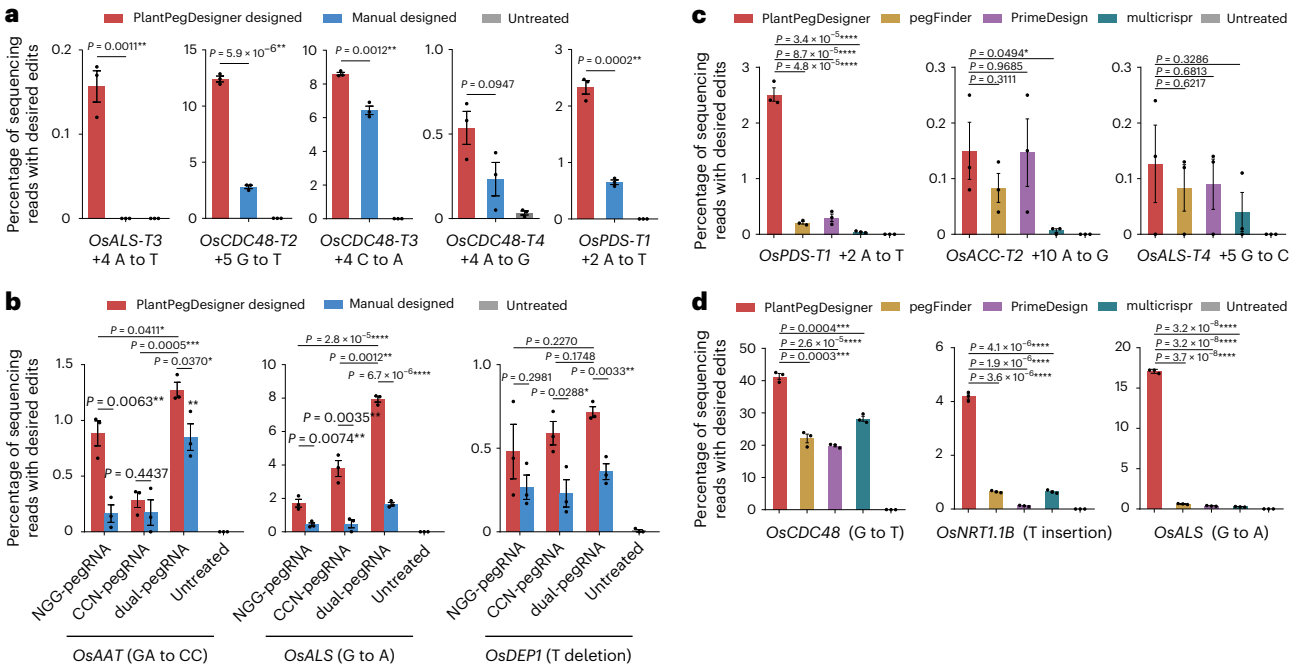


Fig. 3 | Experimental validation of the PlantPegDesigner web application. a,**b**, Comparison of the editing efficiencies of PlantPegDesigner-designed and manually designed NGG-pegRNA and CCN-pegRNA (**a**) and dual-pegRNA in rice protoplasts (**b**). **c**, Editing efficiencies of pegRNAs designed by PlantPegDesigner and three other web applications in rice protoplasts. **d**, Editing efficiencies using PlantPegDesigner-recommended dual-pegRNAs and three web application-recommended single-pegRNAs in rice protoplasts. The edits refer to the base on the DNA forward strand in **b** and **d**. We define the first base 3′ of the pegRNA-induced nick as +1 (the NGG PAM as +4 to +6). Frequencies (mean ± s.e.m.) were calculated from three independent experiments ($n = 3$). *P*-values were obtained using two-tailed Student's *t*-tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. Figure reproduced with permission from ref. [25], Springer Nature America.

1–3: 'PAM sequence' (default to NGG protospacer adjacent motif (PAM)), 'Cut distance to PAM' (default to −3 position) and 'Spacer length' (default to 20 nt): these three parameters depend on the type of Cas protein used (default to SpCas9).

4: 'Spacer GC content' (default to 0–100%): it has been reported that on-target GC content can affect the on-target editing activity of Cas9[42–44]; users can change this parameter if needed.

5: 'Prime editing window' (default to +1 to +15): we define the nicked site as +1 and the NGG PAM as +4 to +6. Previous studies have shown that PEs can work efficiently in an editing window from +1 to +15 in plants[5,15,45–50], but longer editing windows have also been reported[15,45–48,51]. The user can change these parameters.

6–7: 'PBS length' (default to 7–16 bp) and 'PBS GC content' (default to 0–100%): the default values are based on previous reports[4,5,15,45–50].

8: 'Recommended T_m of PBS sequence' (default to 30 °C): we strongly recommend that the PBS T_m be set to 30 °C. If 30 °C is unavailable, 32 °C is recommended.

9–10: 'Homologous RT template length' (default to 7–16 bp) and 'Exclude first C in RT template' (default to true): the default values are based on previous results[4,25].

! CAUTION The homologous RTT length is the length from the desired edits to the 3′ terminus, not the length of the whole RTT.

11–12: 'T_m-directed PBS length model' and 'Dual-pegRNA model': These two models are based on the results of experiments aimed at identifying efficient PBS lengths and dual-pegRNAs (default to turn on). If the two models are turned off, the T_m-based PBS length and use of dual-pegRNA will not be recommended. Also, if the T_m-based PBS length model is turned off, the reverse primer will not be recommended. These two models are described in detail in the following section.

Only one single input sequence comprising the reference and edited sequence is needed for PlantPegDesigner; the format of the input sequence is shown in Box 1. Once the input sequence and parameters have been provided, PlantPegDesigner provides the recommended pegRNAs in several seconds. It first screens all the spacer-PAM sequences in the forward/reverse strands of the input sequence to determine if the desired edits are correctly positioned in the user-defined prime editing window (Fig. 2a). All the available spacer-PAM sequences are ranked by the distance between the

Box 1 | Examples of input sequences for PlantPegDesigner

Designed edits are shown in parentheses in bold in the following format **(original sequence/desired mutant sequence)**.

1 Example of a base substitution (*OsALS-T1*, +2 A to G):
CTCGAGACTCCAGGGCCATACTTGTTGGATATCATCGTCCCGCACCAGGACATGTGCTGCCTATGATCCCA**(A/G)**
GTGGGGGCGCATTCAAGGACATGATCCTGGATGGTGATGGCAGGACTGTGTA

2 Example of a sequence containing an insertion (*OsCDC48-T2*, +2 AAA insertion):
CTTCGCCCAGACTCTGCAGCAGTCTCGTGGGTTCGGCACCGAGTTTAGGTTCGCTGACCAGCCAGCGTCTGGC**(/AAA)**
GCCGGCGCCGCCGCTGACCCCTTCGCATCCGCTGCCGCCGCAGCTGACGATGATGATT

3 Example of a sequence containing a deletion (*OsCDC48-T1*, +1-6 CTCCGG deletion):
CTTCTCAGCTTCTTCAAATGCCTTCCTGAGATTACTCTCACTTTCTCCTGCTAGCTTTGACATAAT**(CTCCGG/)**
GCCATTAATCAGAAAGAAGAAAGCACCTGTTTCATTAGCAACAGCTCTA

4 Example of a sequence containing multiple edits (*OsCDC48-T1*, +1 C to T & +6 G to T):
CTTCTCAGCTTCTTCAAATGCCTTCCTGAGATTACTCTCACTTTCTCCTGCTAGCTTTGACATAAT**(CTCCGG/TTCCGT)**
GCCATTAATCAGAAAGAAGAAAGCACCTGTTTCATTAGCAACAGCTCTA

Cas9-induced nicking site and the designed edits. The dual-pegRNA model is activated if the spacer-PAM sequence can be found on both the forward and reverse strand (Fig. 2a). All the candidate PBS and RTT sequences of variable length will be designed, and one PBS and RTT sequence will be recommended for primer design (Fig. 2a). Those PBS sequences with the user-defined optimal T_m value will be recommended (we strongly recommend default to 30 °C in rice). For the RTT, RT sequences beginning with 'C' are removed and an RTT sequence of length equal to the median length of the remaining RT sequences will be recommended (Fig. 2a). All the other sequences will be shown in case the user wants to test other PBS or RTT sequences. PlantPegDesigner will design the primer sets for a one-step polymerase chain reaction (PCR) strategy for constructing the vector for each pegRNA, including user-defined vectors (details are described in Steps 15–21; Fig. 4).

T_m and the dual-pegRNA model

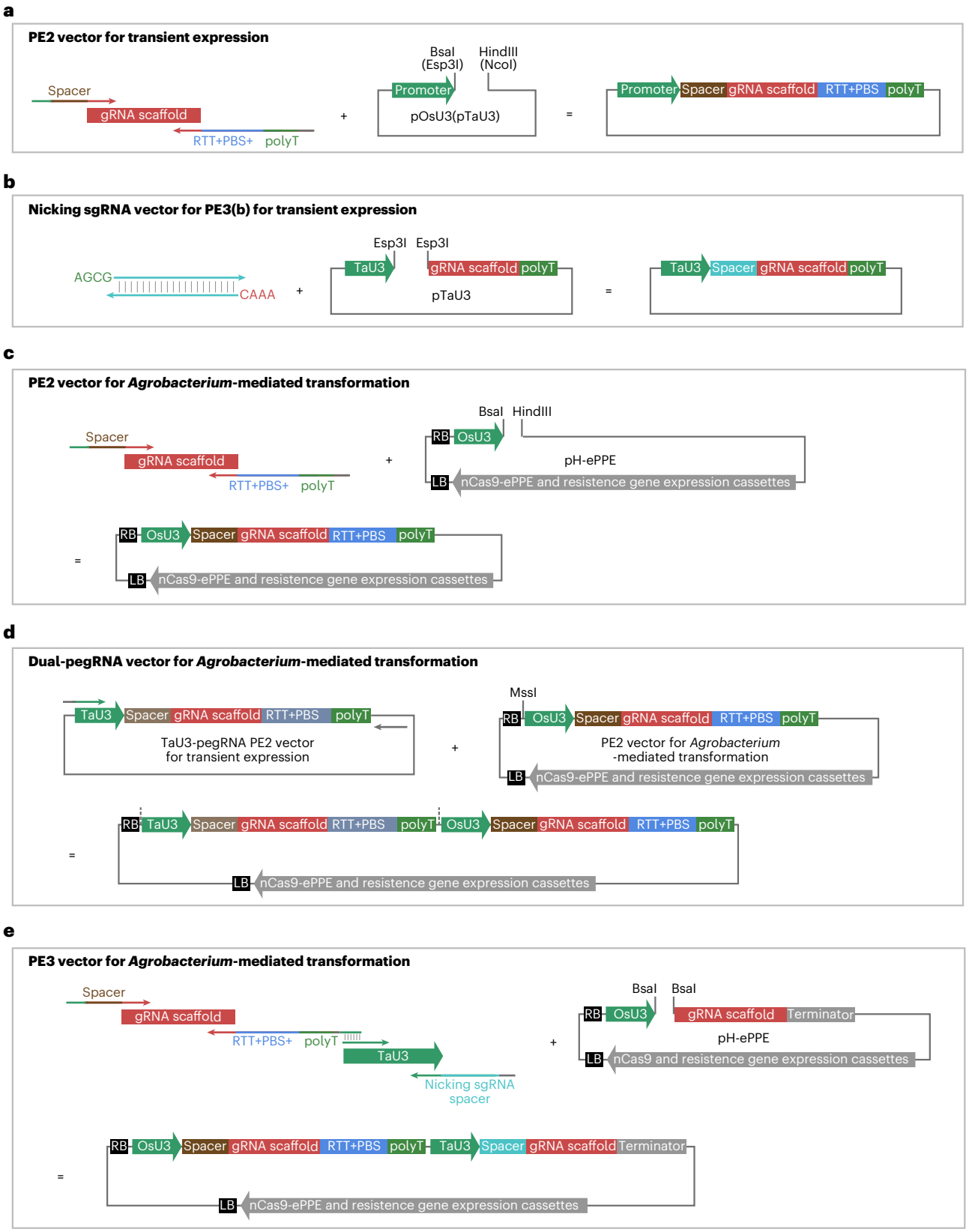
PlantPegDesigner includes two special models named the T_m and dual-pegRNA models, not present in other pegRNA design applications[25]. These two models were established using experimental data from rice protoplasts. First, we identified that the T_m of the PBS sequence was strongly associated with high editing efficiencies[25], and that the most optimal PBS temperature in rice was 30 °C (Fig. 1a). The dual-pegRNA model simultaneously uses two separate pegRNAs in trans encoding the same edits on both the forward and reverse DNA strands. This strategy markedly enhances editing activities in rice protoplast (Fig. 1c). We strongly recommend leveraging these two models.

PE2 and PE3 system

Compared with the PE2 system, the PE3(b) system uses an additional sgRNA to generate a nick on the unedited DNA strand, which can markedly increase prime editing efficiencies in human cells[4]. Since the PE3 system did not have a markedly increased editing efficiency in plants[5,15,45,48–50], the current version of PlantPegDesigner does not design a corresponding nicking sgRNA vector. If the user wants to test PE3(b) editing efficiencies, a nicking sgRNA expression vector can be codelivered with the pegRNA and nCas9-RT in protoplasts. For the nicking sgRNA expression vector, we recommend using a promoter that is different from the one driving the expression of the pegRNA. Steps 29–36 describe how to construct a nicking sgRNA expression vector, using pTaU3 as an example.

Protoplast transformation

Transient protoplast transformation is a widely used assay for evaluating the genome editing efficiency and assessing the frequency of editing byproducts in plants (Fig. 2), such as in rice, *Arabidopsis thaliana*, wheat and more[52–55]. For example, users can design PBS and RTT sequences using the PlantPegDesigner (Steps 15–23) and construct pegRNA vectors (Steps 24–45), then the pegRNA vectors and PE vectors can be simultaneously transformed into protoplasts as an experimental group. Next, users can perform targeted amplicon deep sequencing to evaluate pegRNA editing efficiencies and byproducts, which is more convenient and time efficient than plant transformations (3 months for rice). The off-target prime editing efficiency can also be evaluated in protoplast transformation experiments. Off-target sites can be predicted using Cas-OFFinder and off-target prime editing efficiencies can be evaluated by deep amplicon sequencing. A more detailed off-target evaluation method was described in our previously published study[7].



Fluorescence activated cell sorting (FACS)-based reporter system

We recommend first testing prime editing efficiency using a blue fluorescent protein (BFP) to green fluorescent protein (GFP) reporter system and analysis by microscopy or FACS[5] to evaluate whether

◀ **Fig. 4 | Construction of vectors for plant PEs. a–e**, A one-step PCR strategy is recommended for constructing plant PEs. Construction of PE2 vector for transient expression (Steps 15–28) (**a**), nicking sgRNA vector (Steps 29–36) (**b**), PE2 vector for *Agrobacterium*-mediated transformation (Steps 50–56) (**c**), dual-pegRNA vector for *Agrobacterium*-mediated transformation (Step 57) (**d**) and PE3 vector for *Agrobacterium*-mediated transformation (Steps 58–66) (**e**). To construct the pegRNA vector in **a**, **c** and **e**, the spacer-scaffold-RTT-PBS-polyT sequence is generated by PCR using the gRNA scaffold as template, then cloned into the enzyme-digested vector backbone. To construct nicking sgRNA vectors (**b**), guide sequence oligos containing overhangs for ligation into the pair of Esp3I sites are cloned into pTaU3 vector. For dual-pegRNA vector in **d**, *TaU3* and *OsU3* promoters drive two separate pegRNAs in trans encoding the same edits for the forward and reverse DNA strands. For PE3 vectors for *Agrobacterium*-mediated transformation in **e**, the spacer-scaffold-RTT-PBS-polyT and TaU3-nicking sgRNA spacer are fused by overlap PCR, and cloned into pH-ePPE vector.

Box 2 | Evaluation of PE efficiency using the BFP-to-GFP reporter system

Procedure

1 Transform the plasmids of the reporter system (pUbi-BFP(M) and pTaU3-BFP-PE2) and the PE construct to test (nCas9-PPE) into protoplasts (Supplementary Fig. 1). Transform the plasmids of Ubi-GFP into protoplasts as positive control. Transform 10 μl ddH_2O into protoplasts as negative control. Isolate and transform rice and wheat protoplasts using previously published detailed protocols[52].
2 After 2 d, transfer the cultured protoplasts into FACS tubes.
3 Open the BD FACSDiva software and flow cytometer following standard procedures.
4 Generate a worksheet with suitable gates using the positive (Ubi-GFP) and negative (untreated) controls. The gating strategy is shown in Supplementary Fig. 3.
5 Place the FACS tubes in appropriate positions in the flow cytometer and click the 'load' button in the BD FACSDiva software.
6 Adjust the flow rate to one or two, and click the 'Record' button in the BD FACSDiva software to start to count the numbers of cells with rescued GFP fluorescence. Generally, 2–5 min are needed for each sample. At least 5,000 events should be detected.
! CAUTION If the protoplast concentration is low (<2×10^6/ml), more time is needed (>10 min). The flow rate can be adjusted to >2 to save time, but we recommend a maximal flow rate of 5.
7 Perform flow cytometry analysis for each sample.
8 Save and export the results with BD FACSDiva software. Close the flow cytometer following standard procedures.
9 Caluculate the prime editing efficiency by dividing the number of P2 (GFP^+) cells by the number of P1 (GFP^+ and GFP^-) cells.

the promoters for driving nCas9-RT and pegRNA and overall design are suitable for the selected plant species (Supplementary Fig. 1 and Box 2). This allows one to rapidly evaluate different PE constructs and establish an appropriate PBS T_m for a given plant species. A detailed protocol on the preparation and transformation of rice and wheat protoplasts can be found in our previously published methods[52]. For measuring PE2 editing efficiency, the nCas9-RT and pegRNA vectors should be codelivered into protoplasts. For measuring PE3 editing efficiency, the nCas9-RT, pegRNA and nicking sgRNA vectors should be codelivered into protoplasts.

Choice and construction of vectors

pOsU3, pTaU3 and pTaU6 vectors are recommended for particle bombardment and protoplast transformation experiments. The pH-ePPE vector is recommended for *Agrobacterium*-mediated transformation of monocotyledons. pOsU3 and pTaU3 vectors are recommended for the majority of monocot plants, and pTaU6 is recommended for wheat. Other promoters the user prefers can also be used to drive plant pegRNA transcription.

PlantPegDesigner is capable of designing primers to be used for constructing customized vectors from customized guide RNA scaffolds. Users only need to provide the sequences flanking the spacer sequence for the forward primers and the reverse complements of the sequences flanking the PBS and RTT sequences for the reverse primers (both uppercase and lowercase are accepted) (Fig. 2a). For example, the forward primer and reverse primers for constructing TaU6-driven esgRNA vectors are: forward primer (5′–3′): TCGCTTGCTGCATCAGACTTG + spacer sequence + GTTTAAGAG CTATGCTGG and reverse primer (5′–3′): CTATGACCATGATTACGCCAAGCTTAAAAAAA + PBS and RTT sequence + GCACCGACTCGGTGCCAC.

We recommend one-step PCR cloning for pegRNA construction. Using the pOsU3 vector as PCR template, the desired amplicon is amplified using primer sets containing the target sgRNA sequences in the forward primer and the PBS+RT sequences and poly T sequence in the reverse primer. It is then cloned into the vectors by Gibson assembly. For testing dual-pegRNAs in protoplasts, we recommend using different promoters to drive the expression of the two pegRNAs in a single cell.

Control group

The control group that undergoes the transformation process but without any DNA introduced is needed in this protoplast transformation method (marked as 'Untreated' in Fig. 3) to evaluate the effect of background mutations.

Materials

Biological materials

- Rice variety Zhonghua 11 (seeds are available from the authors upon request)
- Fast-T1 *Escherichia coli* competent cells (Nanjing Vazyme, cat. no. C505-01/02)
- XL10 *E. coli* competent cells (Nanjing Vazyme, cat. no. C505-01/02)

Plasmids and primers

- pOsU3: for constructing plant pegRNA expression vectors that include an OsU3 promoter-driven SpCas9-based sgRNA scaffold (Addgene, cat. no. 170132)
- pTaU6: for constructing plant pegRNA expression vectors that include a TaU6 promoter-driven SpCas9-based sgRNA scaffold (the vector is available from the authors upon request)
- pTaU3: for constructing plant pegRNA expression vectors that include a TaU3 promoter-driven SpCas9-based sgRNA scaffold (Addgene, cat. no. 140448)
- nCas9-ePPE: an nCas9-RT plant expression vector that includes a ZmUbi promoter-driven fusion of synthesized nCas9 (H840A) and optimized M-MLV RT (Addgene, cat. no. 183095); detailed sequence in Supplementary Note 1
- pH-ePPE: a plant nCas9-RT and pegRNA expression vector that includes an OsU3 promoter-driven SpCas9-based sgRNA scaffold and a ZmUbi promoter-driven engineered PE with enhanced editing efficiency in plants (Addgene, cat. no. 183097); detailed sequence in Supplementary Note 2
- pUbi-BFP(M): a mutated GFP plant expression vector that includes a ZmUbi promoter-driven mutated GFP that can be changed to an active form by plant PEs (vector is available from the authors upon request)
- pUbi-GFP: a GFP plant expression vector that includes a ZmUbi promoter-driven GFP (vector is available from the authors upon request)
- pTaU3-BFP-PE2: a pegRNA expression vector for reporter systems, which includes a TaU3 promoter-driven BFP-pegRNA (vector is available from the authors upon request)
- Primers (BGI, custom order; Table 1)

Reagents

For production of vectors

- Agarose (Invitrogen, cat. no. 16500500)
- Tris acetate ethylenediamine tetraacetic acid (TAE) buffer (10×; Cellgro, cat. no. 46-010-CM)
- 2× TransStart FastPfu PCR SuperMix (+dye) (TransGen cat. no. AS221-12)
- ClonExpress II One Step Cloning Kit (Vazyme Biotech, cat. no. C112-02)
- AxyPrep DNA Gel Extraction Kit (Axygen, cat. no. AP-GX-250)
- AxyPrep Plasmid Miniprep Kit (Axygen, cat. no. AP-MN-P-250)
- Wizard Plus Midipreps DNA Purification System (Promega, cat. no. A7640)
- Ethanol (Sigma-Aldrich, cat. no. E7023)
- Kanamycin (TransGen, cat. no. GG201-01)
- Ampicillin (Inalco, cat. no. 1758-9314-100g)
- Tryptone (Oxoid, cat. no. LP0042)
- NaCl (IGDB, cat. no. YC-SJ02027)
- Yeast extract (Oxoid, cat. no. LP0021)
- Commercially synthesized RT and linker sequences (Nanjing GenScript Biotech, and Genewiz, Suzhou, custom order)
- DNAquick Plant System (Tiangen, cat no. DP321-03)
- 2× Rapid Taq Master Mix (Vazyme Biotech cat. no. P222-03)
- NEXTflex RNA-seq barcodes (NEXTflex, cat. no. 512914)

Equipment

- PCR thermocycler (Veriti 96-well thermal cycler; Applied Biosystems, cat. no. 9902)
- Heating water bath (Memmert, cat. no. WNB22L4L5)
- UV–visible light spectrophotometer (Thermo, model no. NanoDrop ND-2000)
- Centrifuge series (Eppendorf, model nos. 5810R, 5424 and 5417R)
- Incubator shaker (Eppendorf, model no. Innova42)
- NanoDrop spectrophotometer (Thermo Scientific)
- MicroPulser (Bio-Rad, model no. 1652100)

Table 1 | Primers used for vector construction

Step	Name	Sequence (5′–3′)	Purpose
2, 10	RT-F	gtctcagctcgggggcgacGAATTCTCCGGGAGCGAGACG (the overlapping sequence to nCas9-PPE vector is shown in lowercase letters)	Forward primer to amplify the synthesized RT sequence
	RT-R	tggtgatttcagcgtaccGCGGCCGCTCACACCTTCCTTTTCTTCTTCGGG (the overlapping sequence to nCas9-PPE vector is shown in lowercase letters)	Reverse primer to amplify the synthesized RT sequence
22	Primer-F	Recommended by PlantPegDesigner (e.g., see bottom of Fig. 5b)	Forward primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence
	Primer-R	Recommended by PlantPegDesigner (e.g., see bottom of Fig. 5b)	Reverse primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence
Box 3	Primer-R1	The proximal region of the corresponding Primer-R sequence	Reverse primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence
	Primer-R0	The distal region of the corresponding Primer-R sequence	Reverse primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence
28	M13-R	caggaaacagctatgac	Reverse primer for colony PCR
28, 53	Det-F	The spacer sequence of corresponding Primer-F	Forward primer for colony PCR
28, 56	M13-F	gtaaaacgacggccagt	Primer for sequencing
29	Oligo-F	AGCGNNNNNNNNNNNNNNNNNNN	Cloning sgRNA into pTaU3
29, 36	Oligo-R	AAACNNNNNNNNNNNNNNNNNNN	Cloning sgRNA into pTaU3; Reverse primer for colony PCR
36	TaU3-200F	GCCATACGAGACTGATGTCTC	Forward primer for colony PCR and sequencing
38	OsCDC48T1-F	AGGTCAACCTGCGACAACAA	Amplifying the OsCDC48T1 for first-round PCR
	OsCDC48T1-R	CTTGTTGAGCTCCCATTGCG	Amplifying the OsCDC48T1 for first-round PCR
39	NGS-OsCDC48T1-F	NNNNNNAGCTTCTTCAAATGCCTTCCTGAGA (NNNNNN represent the 6 nt barcode sequence for distinguishing amplicons originating from different treatments. The barcode sequences correspond to NEXTflex RNA-seq barcodes, which provide 48 available unique 6 nt barcodes)	Amplifying the OsCDC48T1 for second-round PCR
	NGS-OsCDC48T1-R	NNNNNNTTTTCAAGTCTATTGGTGTGAAGCCT (NNNNNN represent the 6 nt barcode sequence for distinguishing amplicons originating from different treatments. The barcode sequences correspond to NEXTflex RNA-seq barcodes, which provide 48 available unique 6 nt barcodes)	Amplifying the OsCDC48T1 for second-round PCR
53, 66	Ubi-90R	ctgcacttcaaacaagtgtgac	Reverse primer for colony PCR
57	Gib-TaU3-F	cctgtcaaacactgatagtttGGCGGCAGGGAGAGTTTTAACA	Forward primer to amplify the TaU3::pegRNA:polyT sequence; Forward primer for colony PCR
	Gib-TaU3-R	tcgtttcccgccttcagtttccttatctgggaactactcacac	Reverse primer to amplify the TaU3::pegRNA:polyT sequence; Reverse primer for colony PCR
	HUE-seq	ggacgaacggataaacctttcacg	Primer for sequencing
58	Agro-Primer-R	GTCAATGTTAAAACTCTCCCTGCCGCCAAAAAAA + PBS and RTT sequence + GCACCGACTCGGTGCCAC	Reverse primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence with TaU3 overlap
58, 64	Agro-Primer-F	TTGTGCAGATGATCCGTGGCG + Spacer sequence + GTTTTAGAGCTAGAAATA	Forward primer to amplify the spacer-scaffold-RTT-PBS-polyT sequence with TaU3 overlap
62, 64	TaU3-PE3-R	ACTTGCTATTTCTAGCTCTAAAAC + reverse complementary of spacer of nicking sgRNA sequence + CGCTTCTTGGTGCCGCGCCT	Reverse primer to amplify the TaU3 promoter-nicking sgRNA spacer sequence
62, 66	TaU3-PE3-F	GGCGGCAGGGAGAGTTTTAACATTG	Forward primer to amplify the TaU3 promoter-nicking sgRNA spacer sequence

- Gel electrophoresis apparatus (Bio-Rad, model no. 1704489EDU or equivalent)
- Digital gel imaging system (BioDoc-It; UVP, cat. no. 97-0256-02)
- Fluorescence microscope (Olympus, model no. SZX16)
- Centrifuge tubes (50 ml)
- Petri dishes (Greiner Bio-One, cat. no. 633180)
- Standard microcentrifuge tubes (1.5 ml; Eppendorf, cat. no. 0030 125.150)
- Single-edge razor blades (Feiying, cat. no. 9875814)
- Nylon mesh (40 μm; BD Falcon, cat. no. F613461)
- Syringe sterilization filter (0.22 μm; Sartorius Stedim Biotech, cat. no. 21423103)
- Eppendorf tubes (1.5 and 2.0 ml; Eppendorf)
- Sanger sequencing system (BGI Genomics)
- Next-generation sequencing system (Illumina, model no. NovaSeq 6000)

Reagent setup

Lysogeny broth (LB) medium (1 L)

Add 10 g tryptone, 5 g yeast extract and 10 g NaCl to 1 L double-distilled water (ddH_2O) and autoclave for 20 min at 121 °C. Store at 4 °C for up to 1 month. After sterilization and cooling to 50 °C, add antibiotics from sterile stock solutions; for example, kanamycin and rifampicin to final concentrations of 50 mg/L and 25 mg/L, respectively ▲ **CRITICAL** Add antibiotics after cooling to 50 °C; otherwise, the antibiotics will be inactivated by high temperature.

LB agar plates (1 L)

Add 14 g agar to 1 L of LB medium and autoclave for 20 min at 121 °C. Adjust volume to 1 L. After sterilization and cooling to 50 °C, add antibiotics from sterile stock solutions; for example, kanamycin and rifampicin to final concentrations of 50 mg/L and 25 mg/L, respectively. Pour ~35 ml of LB agar per sterile Petri dish. Store at 4 °C for up to 1 month ▲ **CRITICAL** Add antibiotics after cooling to 50 °C; otherwise, the antibiotics will be inactivated by high temperature.

Equipment setup

BD FACSAria

For rice protoplast, a 70 μm nozzle is recommended and ddH_2O can be used as sheath fluid. FACSDiva 6 is used for distinguishing GFP^+ and GFP^- cells.

Operating system

Linux or Mac OS.

Software

Perl v5.10 or higher (https://www.perl.org/get.html) or Python v3.5 or higher (https://www.python.org/).

Example datasets

An example dataset has been deposited in Github at https://github.com/ReiGao/GEanalysis/tree/master/ExampleDataAndResutls/cleandata/OsCDC48T1_A.

Web application

The web portal server for PlantPegDesigner is accessible at http://www.plantgenomeediting.net for nonprofit use.

Procedure

Construction of nCas9-RT expression vectors for protoplast transformation ● Timing 3 d

▲ **CRITICAL STEPS** 1–14 describe how to construct an nCas9-RT vector of choice with a customized RT. Previous work has shown that the engineered M-MLV RT can be replaced by other RTs in PEs[5]. Furthermore, an engineered RT without RNase H activity and incorporating a viral nucleocapsid protein acted as an enhanced plant prime editor (ePPE) with a higher editing efficiency than the canonical plant PE in our previous work[10]. We therefore recommend using nCas9-ePPE for measuring plant prime editing efficiencies. nCas9-ePPE includes a ZmUbi promoter-driven fusion of synthesized nCas9

(H840A) and optimized M-MLV RT and is available from the authors upon request.

▲ **CRITICAL** If using nCas9-ePPE or other ready-made vectors, Steps 1–14 can be skipped.

1 Commercially synthesize the RT sequence with an XTEN linker sequence (to provide additional flexibility for nCas9-RT fusion protein) and an SGGS-SV40NLS-TGA sequence (the SGGS linker sequence, SV40 nuclear localization signal and stop codon) at the 5′ terminus and 3′ terminus, respectively. An example is shown below:

TCCGGGAGCGAGACGCCAGGCACCTCCGAGTCGGCCACCCCAGAATCT…(RT sequence)…AGCGGCGGCAGCCCGAAGAAGAAAAGGAAGGTGTGA

2 Amplify the sequence by PCR using the following reaction with primers RT-F and RT-R (Table 1). For the Gibson assembly reaction in Step 8, the overlapping sequences to the digested nCas9-PPE vector can be added at the 5′ terminus of the primers, and then the overlapping sequence can be added at the ends of the products by PCR reaction (Table 1).

Component	Volume (μl)	Final concentration
TransStart FastPfu PCR SuperMix (+dye) (2×)	25	1×
RT-F (10 pmol/μl; Table 1)	1	0.2 μmol
RT-R (10 pmol/μl; Table 1)	1	0.2 μmol
Synthesized fragment	Variable	10 ng
ddH_2O	22	-

3 Perform PCR using the following conditions:

Cycle no.	Denaturation	Annealing	Extension
1	95 °C, 3 min		
2–32	95 °C, 15 s	55 °C, 15 s	72 °C, 2 kb/min
33			72 °C, 5 min

4 Run 50 μl of the PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 10 min. A single bright product band is expected.

■ **PAUSE POINT** Store PCR products until use in Step 7. The products can be stored at 4 °C for up to 1 week.

? TROUBLESHOOTING

5 Linearize the nCas9-PPE vector with StuI and NotI in the following reaction at 37 °C for 1 h.

Component	Volume (μl)	Final concentration
rCutSmart Buffer (10×)	5	1×
StuI, 10 U/μl	2	0.4 U/μl
NotI-HF, 20 U/μl	1	0.4 U/μl
nCas9-PPE plasmid DNA	Variable	1 μg
ddH_2O	Up to 50	-

6 Run 50 μl the enzyme-digested products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 20 min. Bands of ~9,200 bp (digested backbone vector) and ~2,000 bp (digested RT gene fragment) are expected.

7 Purify the ~9,200 bp product from Step 6 and the band from Step 4 with an AxyPrep DNA Gel Extraction Kit according to the manufacturer's instructions. The concentration of the gel-extracted products should be ≥30 ng/μl as measured by NanoDrop spectrophotometer.

■ **PAUSE POINT** The purified products can be stored at −20 °C for at least 2 months.

8 Clone the purified synthesized fragment with sequences that overlapping to the 15–20 nt border of vector backbone (from Step 4, purified in Step 7) into the purified nCas9-PPE vector backbone (from Step 7, purified in Step 7) with the ClonExpress II One Step Cloning Kit in the following reaction incubated at 37 °C for 30 min:

Component	Volume (μl)	Final concentration
CE Buffer (5×)	4	1×
Exonuclease II	2	-
Purified synthesized fragment	Variable	Length × 0.02 ng
Purified vector backbone	Variable	Length × 0.04 ng
ddH_2O	Up to 20	-

9 Mix 10 μl of the product with 100 μl Fast-T1 *E. coli* competent cells and incubate them overnight at 37 °C on LB plates with ampicillin.

10 Screen six to eight clones by colony PCR using the following reaction with primers RT-F and RT-R (Table 1).

Component	Volume (μl)	Final concentration
Rapid Taq Master Mix (2×)	10	1
RT-F (10 pmol/μl)	0.2	0.2 μmol
RT-R (10 pmol/μl)	0.2	0.2 μmol
Single colony of bacteria	-	-
ddH_2O	Up to 20	-

11 Perform PCR using the following conditions:

Cycle no.	Denaturation	Annealing	Extension
1	95 °C, 3 min		
2–32	95 °C, 15 s	55 °C, 15 s	72 °C, 1 min
33			72 °C, 5 min

12 Run 5 μl PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 10 min. A single bright product band is expected, which means this clone is positive.
? TROUBLESHOOTING

13 Select one positive clone from Step 12 and extract plasmid DNA with a Plasmid Miniprep Kit, following the manufacturer's instructions. Perform sanger sequencing of the plasmid DNA (200 ng) using primers RT-F and RT-R to double check whether the clone is correct.

14 Culture the positive clone from Step 13 on 100 ml LB liquid medium containing 50 μg/ml ampicillin and incubate at 37 °C with shaking at 200 rpm for 14–16 h. Extract the plasmid DNA with a Wizard Plus Midipreps DNA Purification System, following the manufacturer's instructions. The concentration of the plasmid DNA should be at least 1 μg/μl measured by NanoDrop spectrophotometer.
■ PAUSE POINT The plasmid DNA can be stored at −20 °C for several months.
▲ CRITICAL STEP The nCas9-ePPE using nSpCas9 (Cas9 from *Streptococcus pyogenes*) can be replaced by other Cas9 variants. Cas9 variants can be synthesized and cloned into HindIII- and EcoRI-digested nCas9-PPE vector. A start codon and nuclear localization sequence should be added at the 5′ terminus of the Cas9 variant. An example is: ATGCCTAAGAAAAAGAGAAAAGTG… (nCas9 variant sequence) Detailed protocols for vector construction and plasmid extraction can be found in Steps 1–14.

Design and construction of PE2 pegRNA expression vectors for protoplast transformation

● Timing 3 d

▲ CRITICAL We strongly recommend using PlantPegDesigner to identify efficient plant pegRNAs and primers. Steps 15–21 describe how to design a pegRNA vector of choice using PlantPegDesigner.

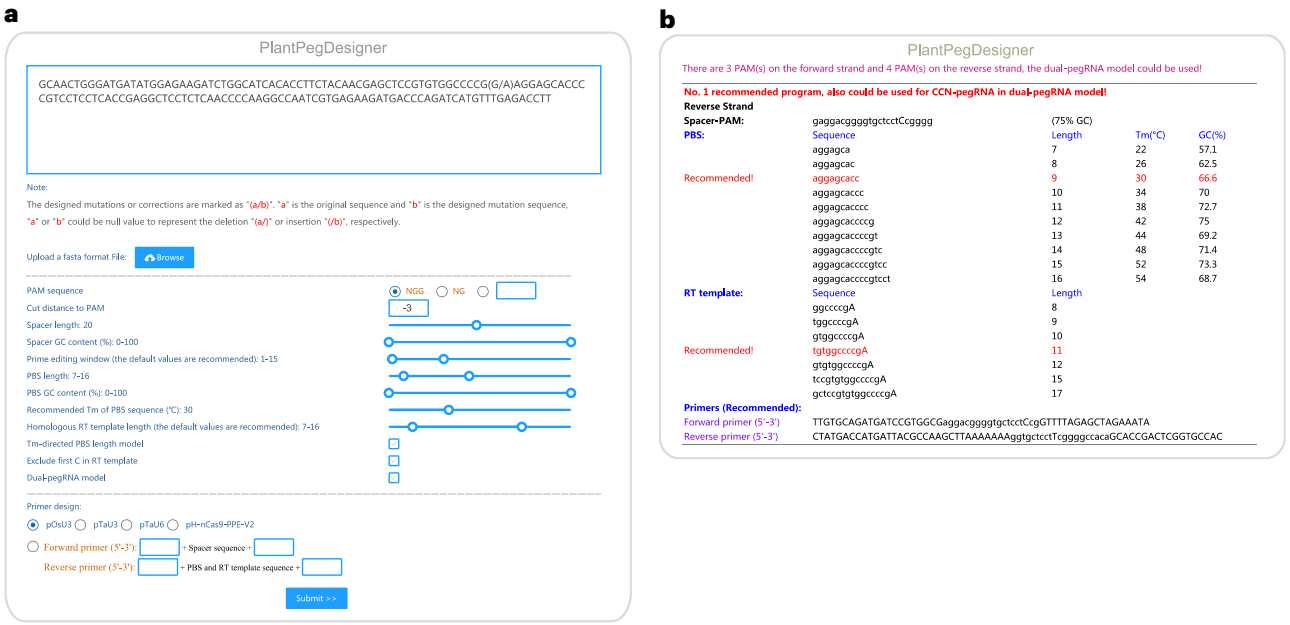


Fig. 5 | Input and output pages of PlantPegDesigner. a, Input page of PlantPegDesigner including the input textbox, link for uploading the pooled input sequence file, user-defined parameters and plasmid vector backbones. **b**, Output page. For each spacer-PAM sequence, PlantPegDesigner reports all the possible PBS and RTT sequences, and recommends the best ones. Primers for user-chosen plasmid vector construction are also recommended. Figure adapted with permission from ref. [25], Springer Nature America.

▲ **CRITICAL STEPS** 22–36 describe how to construct a pegRNA vector of choice, using pOsU3 as an example (Fig. 4). In this protocol, we recommend the use of one-step PCR cloning for constructing pegRNAs, as described in Steps 24–36.

In some cases, such as for insertion or replacement of a fragment >150 bp by PE, the RTT may be too long for pegRNA construction. Having the sequences synthesized commercially is also an easy and reliable way to construct vectors. If the user commercially synthesized pegRNA sequences, Steps 24–36 are not needed.

15 Access the PlantPegDesigner web application (http://www.plantgenomeediting.net/) via the Chrome browser. Figure 5a displays the input page.

? TROUBLESHOOTING

16 Prepare the input sequence. The input sequence contains both the reference sequence and the desired edited sequence, with designed edits marked as '(a/b)', 'a' being the original sequence and 'b' the desired mutant sequence (Box 1). Both uppercase and lowercase are accepted.

! CAUTION 'a' and 'b'may be null values representing insertions '(/b)'and deletions '(a/)', respectively (see examples of base conversions, insertions and deletions below). PlantPegDesigner also supports introducing multiple edits in one pegRNA (Box 1).

17 Choose the optimal parameters for 'PAM sequence', 'Spacer GC content', 'Prime editing window', 'PBS length', 'Recommended T_m of PBS sequence', 'Homologous RT template length', 'T_m-directed PBS length model' and 'Dual-pegRNA model' (for further explanation of parameters, see 'Experimental design').

! CAUTION The optimal values are based on rice protoplast prime editing data. Users need to obtain the optimal values for their specific organism.

18 Choose the vectors to be used for pegRNA expression according to 'Experimental design'.

19 Click the 'Submit' button and wait for several seconds. The output page will be displayed as Fig. 3b. All the available spacer-PAM sequences will be ranked by the distance between the Cas9-induced nick and the desired edit (Fig. 2a).

? TROUBLESHOOTING

20 Check whether the dual-pegRNA model can be used, as indicated by the notice message in the first line (Fig. 3b).

21 PlantPegDesigner will give many programs for designing pegRNA, including different spacer, scaffold and polyT sequences (subsequently abbreviated as spacer-scaffold-RTT-PBS-polyT), and will recommend one program for the user (according to optimal PBS or RT seqeunce[25]) (Fig. 3b).

Box 3 | Examples of two-primer mixture to reduce expense

The reverse primer contains the PBS + RT sequences and the poly T sequence. Ordering a long primer (longer than 60 bp) is usually more expensive than two short primers. Thus, to reduce expense, Primer-R can be replaced by a two-primer mixture, Primer-R1 and Primer-R0 (Table 1), comprising, respectively, the proximal and distal sequences of Primer-R, with an overlap of at least 20 nt. Primer-R0 should be diluted 20-fold for PCR use.

For example, for *OsALS-T1*, +2 A to G edits, the sequence of Primer-R recommended by PlantPegDesigner is: CTATGACCATGATTACGCCAA GCTTAAAAAAACTATGATCCCAGGTGGGGGCGCATGCACCGACTCGGTGCCAC and this primer could be replaced by Primers R1 and R0, as follows:

Primer-R1: CTATGACCATGATTACGCCAAGCTTAAAAAAACTATGATCCCAGGTGGG

Primer-R0: AAAAAAACTATGATCCCAGGTGGGGGCGCATGCACCGACTCGGTGCCAC

The components of the PCR reaction should be:

Component	Volume (μl)	Final concentration
TransStart FastPfu PCR SuperMix (+dye)	25	1×
Primer-F (10 pmol/μl; Table 1)	1	0.2 μmol
Primer-R1 (10 pmol/μl; Table 1)	1	0.2 μmol
Primer-R0 (0.5 pmol/μl; Table 1)	1	0.01 μmol
Template with guide RNA scaffold	1	-
ddH_2O	21	-

Commercially synthesize the Forward primer and Reverse primer (subsequently abbreviated as Primer-F and Primer-R) of the recommended program (Fig. 3b) for use in Step 22. Optionally, if the dual-pegRNA model can be used, save the primer information for the recommended programs using both an NGG-pegRNA and CCN-pegRNA.

22 PCR-amplify the spacer-scaffold-RTT-PBS-polyT sequence using Primer-F and Primer-R (Table 1) as recommended by PlantPegDesigner. Any vectors composing sgRNA scaffold sequence, such as pOsU3, pTaU3 or pH-ePPE vectors, can be used as templates for amplifying fragment of pegRNAs. For the Gibson assembly reaction in Step 28, the overlapping sequences to the digested vector have to be added at the 5′ terminus of Primer-F and Primer-R. The sequence of primers with overlapping sequences to the digested vector are recommended by PlantPegDesigner.

Component	Volume (μl)	Final concentration
TransStart FastPfu PCR SuperMix (+dye)	25	1×
Primer-F (10 pmol/μl; Table 1)	1	0.2 μmol
Primer-R (10 pmol/μl; Table 1)	1	0.2 μmol
PCR template	1	-
ddH_2O	22	-

▲ **CRITICAL STEP** We recommend using a high-fidelity DNA polymerase to decrease the PCR error rate.

! CAUTION To reduce expense, the reverse primer can be replaced by a two-primer mixture (Box 3).

23 Perform the PCR as follows:

Cycle no.	Denaturation	Annealing	Extension
1	95 °C, 3 min		
2–32	95 °C, 15 s	T_m °C, 15 s	72 °C, 1 min
33			72 °C, 5 min

24 Run 50 μl of the PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 10 min. A single bright band of ~150 bp is expected.

? TROUBLESHOOTING

25 Linearize the pOsU3 vector with BsaI and HindIII in the following reaction at 37 °C for 1 h.

Component	Volume (μl)	Final concentration
rCutSmart Buffer (10×)	5	1×
BsaI-HFv2, 20 U/μl	1	0.4 U/μl
HindIII-HF, 20 U/μl	1	0.4 U/μl
Plasmid DNA	Variable	1 μg
ddH_2O	Up to 50	-

26 Run 50 μl of the enzyme-digested products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 20 min. For the pOsU3 vector, bands of ~3,000 bp (desired vector backbone band) and ~120 bp (sequence between BsaI and HindIII), are expected.

27 Purify the ~150 bp product from Step 24 and the ~3,000 bp product from Step 26 with an AxyPrep DNA Gel Extraction Kit according to the manufacturer's instructions. The concentration of the gel-extracted products should be ≥30 ng/μl as measured by NanoDrop spectrophotometer.

■ **PAUSE POINT** The purified products can be stored at −20 °C for at least 2 months.

28 Clone the pegRNA (the purified ~150 bp PCR product from Step 27) into the pOsU3 vector backbone (the purified ~3,000 bp enzyme-digested product from Step 27) with a ClonExpress II One Step Cloning Kit, and screen and sequence positive clones by repeating Steps 8–14, using primers Det-F and M13-R (Table 1) in Step 10 and the M13-F primer (Table 1) to Sanger sequence the plasmids to confirm correct clones in Step 13.

■ **PAUSE POINT** The plasmid DNA can be stored at −20 °C for several months.

Construction of nicking sgRNA expression vectors of PE3 for protoplast transformation

● **Timing 3 d**

29 Commercially synthesize the forward and reverse sgRNA oligos (BGI, Beijing) Oligo-F and Oligo-R (Table 1).

30 Dilute them to final concentrations of 10 μM and set up the following annealing reaction:

Component	Volume (μl)	Final concentration
Oligo-F (10 pmol/μl; Table 1)	10	5 pmol/μl
Oligo-R (10 pmol/μl; Table 1)	10	5 pmol/μl

31 Anneal the mixture in a PCR machine, using the following program:

Cycle no.	Condition
1	95 °C, 5 min
2	From 90 °C to 10 °C, −10 °C min^{-1}, 8 min
3	10 °C, hold

▲ **CRITICAL STEP** The annealed guide sequence oligos contain overhangs for ligating them into the pair of Esp3I sites in the pTaU3 vector

■ **PAUSE POINT** The product can be stored at −20 °C for at least 2 months.

32 Linearize the pTaU3 vector with Esp3I in the following reaction at 37 °C for 1 h.

Component	Volume (μl)	Final concentration
rCutSmart Buffer (10×)	5	1×
Esp3I, 10 U/μl	2	0.4 U/μl
Plasmid DNA	Variable	1 μg
ddH_2O	Up to 50	-

33 Run 50 µl the enzyme-digested product on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 20 min. For the pTaU3 vector, a ~2,800 bp band is expected.

34 Purify the ~2,800 bp products with an AxyPrep DNA Gel Extraction Kit. The concentration of the gel-extracted products should be ≥30 ng/µl as measured by a NanoDrop spectrophotometer.
PAUSE POINT The purified product can be stored at −20 °C for at least 2 months.

35 Ligate the annealed products from Step 30 to the purified vector backbone from Step 34 by setting up the reaction below and incubating at 25 °C for 10 min:

Component	Volume (µl)	Final concentration
T4 DNA ligase buffer (10×)	1	1×
T4 DNA ligase, 40 U/µl	0.5	2 U/µl
Annealed product	1	1 pmol/µl
Purified pTaU3 vector backbone	Variable	50 ng
ddH_2O	Up to 10	-

! CAUTION The T4 DNA ligase buffer should be thawed and resuspended at room temperature (~20–25 °C) before use.

36 Repeat Steps 9–14. The primers used for colony PCR are primers TaU3-200F and Oligo-R (Table 1) and the expected PCR product should be ~240 bp.
PAUSE POINT The plasmid DNA can be stored at −20 °C for several months.

Measurement of efficiency and byproducts of prime editing constructs by deep sequencing in rice protoplasts ● Timing 1–3 weeks

37 We recommend using a transient protoplast assay for measuring PE editing efficiency and the frequency of byproducts according to Box 2. Collect the protoplasts 2 d post-transfection by centrifuging at 12,000*g* for 2 min at room temperature, and discarding the supernatant. Extract genomic DNA by the cetyltrimethyl ammonium bromide method[56].

38 For deep amplicon sequencing, PCR amplify 350–1,000 bp amplicons that contain the target site in or near their center with primers in a first-round PCR. Here, we used OsCDC48T1 site as an example, the genomic DNA sequence is shown in Supplementary Note 3. Perform first-round PCR using the primers OsCDC48T1-F and OsCDC48T1-R (Table 1) by repeating Steps 2–4.

39 Perform the second-round PCR using the product of Step 38 as template by repeating Steps 2–4 again. In the second round, the amplicons containing the target site roughly centrally should be ~110–200 bp. Barcodes are added to both ends of the amplicons using primers with built-in barcode sequences (Table 1). The forward and reverse PCR primers each contain a unique 6 nt barcode[25].
▲ CRITICAL STEP The barcode sequences correspond to NEXTflex RNA-seq barcodes, which provide 48 available unique 6 nt barcodes. The barcodes can be used to distinguish amplicons originating from different treatments.
! CAUTION We recommend using a high-fidelity DNA polymerase to decrease the PCR error rate.

40 Pool 5 µl PCR products (up to 100 amplicons in one sample) for next-generation sequencing.
▲ CRITICAL STEP The products of different treatments, with different 'barcode + primer' sequences, can be mixed and sequenced simultaneously. Different target sites can be distinguished by the different barcodes accompanying the target-specific sequences. For each 5 µl PCR product, ~100,000 reads are enough to detect the mutations.

41 Perform amplicon deep sequencing of the mixed pools using a NovaSeq 6000 (Novogene).

42 Create a file named '1.csv' in the Linux/Mac operating system containing the primer sequences for separating the products of different treatments from the combined amplicon deep sequencing data. The file contents are in the following format:
OsCDC48T1_A GAGTGGTTTTCAAGTCTATTGGTGTGAAGCCT GGTAGCAGCTTCTT CAAATGCCTTCCTGAGA
▲ CRITICAL STEP The treatment name follows the format 'target name' + '_' + 'A/B/C….'. The 'A/B/C….' means different treatments, such as pegRNAs with different PBS or RTT. The 'target name' should be identical to the one in Step 44. Perl should be installed to run this script in the Linux/Mac operating system.

43 Use the script 'splitbarcodeCLEANdataPE.pl' and '1.csv' to separate the different treatment products with the following command:

sh splitbarcodeCLEANdataPE.pl

44 Prepare a 'gene2.fa' file to provide reference sequences as in the following:

>OsCDC48T1
TTTCTTCT
TTCTGATTAATGGCCCGGAGATTATGTCA
AAGCTAGC

In this example file, the first line represents the target name, the second and fourth lines represent the flanking sequences and the third line gives the sequence for detecting mutations. To evaluate the frequency of desired edits and byproducts, the third line must contains the RTT sequence with at least 3–5 bp of both upstream and downstream sequences.

45 Use the 'globMerge.pl' and 'gene2.fa' commands to merge the paired-end reads, and calculate the frequencies of indels or base changes with the following command:

perl globMerge.pl

46 Prepare a 'list.txt' file to provide the flanking and reference sequences, as in the following:

OsCDC48T1 TTCTGATTAATGGCATTAT TTCTGATTAATGGCCCGGAGATTAT

47 Use 'DesiredandByProductsV2.py' and 'list.txt' to merge the paired-end reads, and calculate the percentages of indels or base changes with the command: python DesiredandByProductsV2.py.

48 Present the prime editing results for all samples as follows:

Group Filename MutatedReferenceSequence All Desired Wildtype Byproducts
OsCDC48T1 1_OsCDC48T1_A.merge.detil.csv TTCTGATTAATGGCATTAT TTCTGATTAATGGCCCGGAGATTAT 10543 3336 5810 1397

49 Calculate prime editing efficiencies (Desired/Total) and byproduct frequencies (Byproducts/All). **? TROUBLESHOOTING**

Construction of binary vectors for PE2 for *Agrobacterium*-mediated transformation

● Timing 3 d

▲ CRITICAL To obtain prime-edited plants, the plasmids determined to bring about efficient prime editing in the transient protoplast assay in the previous procedure section (Step 49) can be used to transform plants by particle bombardment. For *Agrobacterium*-mediated transformation, binary vectors (plasmids with cis-acting T-DNA border sequences and trans-acting virulence function (vir) genes in two separate replicons) should be constructed. Steps 50–66 describe how to construct a PE2 binary vector of choice for rice calluses, using pH-ePPE as an example. We recommend one-step PCR cloning for pegRNA construction, using a construction strategy similar to that for PE2 pegRNA expression vectors (Fig. 4).

50 Design the primer sets using PlantPegDesigner (Choose pH-ePPE for vector construction). A detailed protocol for vector construction is given in Steps 15–21.

51 Clone the pegRNA into the pH-ePPE vector backbone (purified ~18,500 bp HindIII and BsaI-digested product) with a ClonExpress II One Step Cloning Kit in the following reaction, which is incubated at 37 °C for 30 min.

Component	Volume (μl)	Final concentration
CE Buffer (5×)	4	1×
Exonuclease II	2	-
Purified pegRNA fragment	Variable	6 ng
Purified pH-ePPE vector backbone	Variable	400 ng
ddH_2O	Up to 20	-

52 Mix 10 μl of the product with 100 μl XL10 *E. coli* competent cells and incubate at 37 °C overnight on LB plates with kanamycin.

53 Screen six to eight positive clones by colony PCR using the following reaction with primers Det-F and Ubi-90R (Table 1):

Component	Volume (μl)	Final concentration
Rapid Taq Master Mix (2×)	10	1
Det-F (10 pmol/μl; Table 1)	0.2	0.2 μmol
Ubi-90R (10 pmol/μl; Table 1)	0.2	0.2 μmol
Single bacterial colony	-	-
ddH_2O	Up to 20	-

54 Perform PCR using the following conditions:

Cycle no.	Denaturation	Annealing	Extension
1	95 °C, 3 min	-	-
2–32	95 °C, 15 s	55 °C, 15 s	72 °C, 1 min
33	-	-	72 °C, 5 min

55 Run 5 μl pf the PCR products on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 10 min. A ~240 bp band is expected

? TROUBLESHOOTING

56 Select two or three positive clones, and extract plasmid DNA with a Plasmid Miniprep Kit. The concentration of the plasmid should be at least 150 ng/μl as measured with a NanoDrop spectrophotometer. Sequence the plasmids with M13-F primer (Table 1) to identify correct clones.

57 (Optional) To construct a binary vector with dual-pegRNAs, we recommend using pTaU3 as the entry vector (Fig. 4). The pegRNA can be cloned into pTaU3 vector with a ClonExpress II One Step Cloning Kit. The protocol for constructing the pTaU3-pegRNA vector is given in Steps 29–36, using Esp3I for vector digestion. Then, amplify the TaU3::pegRNA:polyT expression cassette using primer sets Gib-TaU3-F and Gib-TaU3-R (the expected band should be ~600 bp; Table 1), and clone it into the vector from Step 56 (with MssI digestion). Use primer HUE-seq for Sanger sequencing (Table 1). The detailed cloning protocols can be found in Steps 50–57.

Construction of binary vectors of PE3 for *Agrobacterium*-mediated transformation

● Timing 3 d

▲ CRITICAL Although the PE2 system is recommended for plants because of the similar efficiencies of PE2 and PE3(b), users may also design and test PE3 systems in plants. Steps 58–66 describe how to construct a PE3 binary vector of choice for rice calluses, using pH-ePPE as an example. One-step PCR cloning by overlap PCR is recommended for constructing pegRNA and nicking sgRNA expression cassettes for binary PE3 vectors (Fig. 4).

58 Select customized primer design and use PlantPegDesigner for designing primers to amplify the pegRNA fragment (Agro-Primer-F and Agro-Primer-R; Table 1).

59 PCR-amplify the pegRNA fragment using primers Agro-Primer-F and Agro-Primer-R (Table 1), following Steps 24–25.

60 Run 50 μl of the PCR product on a 1.2% (wt/vol) agarose gel in TAE buffer at 160 V for 10 min. A single bright band of ~150 bp is expected.

61 Purify the ~150 bp product with an AxyPrep DNA Gel Extraction Kit, following manufacturer's instructions. The concentration of the gel-extracted product should be ≥30 ng/μl as measured by a NanoDrop spectrophotometer.

62 PCR-amplify the TaU3 promoter fragment with the spacer of the nicking sgRNA sequence using primers TaU3-PE3-F and TaU3-PE3-R (Table 1), as follows, with PCR conditions as described in Step 54:

Component	Volume (μl)	Final concentration
TransStart FastPfu PCR SuperMix (+dye)	25	1×
TaU3-PE3-F (10 pmol/μl; Table 1)	1	0.2 μmol
TaU3-PE3-R (10 pmol/μl; Table 1)	1	0.2 μmol
Template (pTaU3 vector)	Variable	10 ng
ddH_2O	Up to 50	-

63 Purify the ~450 bp product with an AxyPrep DNA Gel Extraction kit, following manufacturer's instructions. The concentration of the gel-extracted product should be ≥30 ng/μl as measured with a NanoDrop spectrophotometer.

64 PCR-amplify the sequence for the pegRNA::TaU3::nicking sgRNA, using primers Agro-Primer-F and TaU3-PE3-R (Table 1), following Steps 24–25. A single bright band of ~720 bp is expected.

65 Purify the ~720 bp product with an AxyPrep DNA Gel Extraction Kit, following manufacturer's instructions. The concentration of the gel-extracted product should be ≥30 ng/μl as measured with a NanoDrop spectrophotometer.

66 Clone the fragment from Step 65 into the pH-ePPE vector backbone (purified ~20,000 bp BsaI enzyme-digested product, obtained as described in Step 25) with a ClonExpress II One Step Cloning Kit and screen the positive clones by colony PCR using primers TaU3-PE3-F and Ubi-90R (Table 1), as described in Steps 8–13.

Generating and identifying prime-edited plants ● Timing 3 months

67 Use previously published protocols[57] to obtain prime-edited wheat plants, including isolation of immature embryos, biolistic delivery of vectors for PEs, induction of calluses, regeneration of seedlings and identification of edited wheat plants. Alternatively use previously published protocols[58–60] to obtaing prime-edited rice plants, including transformation of binary vectors into *Agrobacterium tumefaciens*, growing calluses, infection and cocultivation of calluses and *A. tumefaciens*, tissue culture of calluses, regeneration and identification of edited rice plants.

▲ **CRITICAL STEP** PCR/restriction enzyme assay, which can simply and rapidly test whether the desired edits are contained in PCR products, is strongly recommended for identifying prime-edited rice plants[52]. Some products of prime editing may be resistant to restriction enzyme digestion because of the loss of restriction sites and so produce uncleaved bands in agarose gels (Supplementary Fig. 2a). Other prime-edited products may contain novel restriction sites and so produce extra bands upon digestion (Supplementary Fig. 2b).

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Steps	Problem	Possible reason	Solution
4, 24	No PCR product or incorrect length or sequence	Incorrect PCR conditions or PCR primers	Improve PCR conditions
12, 55	No positive clones or incorrect sequence in Gibson joint	The vectors were not completely linearized	Increase the concentration of enzyme and reaction time used for linearization in Step 5 or 51
		Low concentration of the gel-extracted products	Increase the concentration of vectors used for linearization in Step 5 or 51
		Incorrect PCR primers	Check and redesign PCR primers
		Low fidelity of Gibson reaction	Select more clones for sequencing
			Change the Gibson reaction enzyme
15	Web application display error	Incorrect version of browser used	Clean the cache of your browser
			Use the Chrome browser
19	No output message	Incorrect input sequence	Check the input format as Steps 16–17
		Incorrect parameter setting	Check parameter settings
49	No prime editing efficiency is detected	Low protoplast transformation efficiency	Optimize protoplast transformation procedure according to our published protocol
		Incorrect pegRNA or PE construction	Sanger sequence the pegRNA and PE vectors used
		Incorrect deep sequencing analysis	Check the deep sequencing analysis procedure from Steps 42–49

Timing

Steps 1–14, construction of nCas9-RT expression vectors for protoplast transformation: 4 d
Steps 15–28, construction of PE2 pegRNA expression vectors for protoplast transformation: 4 d
Steps 29–36, construction of PE3 nicking sgRNA expression vectors for protoplast transformation: 4 d

Steps 37–49, measurement of prime editing efficiency by deep sequencing in protoplast: 1–3 weeks
Steps 50–66, construction of binary vectors for *Agrobacterium*-mediated transformation: 4–7 d
Step 67, generation of prime-edited rice or wheat: 3 months

Anticipated results

Using this protocol, the users can design and construct prime editing systems for use in rice and wheat plants and evaluate their efficiencies. We assessed the effects of T_m-based PBS length on prime editing efficiency and found that PE were most efficient in rice protoplasts when PBS T_m approached 30 °C (Fig. 1a). We compared the PE activities of NGG-pegRNA alone, CCN-pegRNA alone and the two together at the same sites, and found that the dual-pegRNAs remarkably increased prime editing efficiency (Fig. 2a,b). Combining these two strategies thus significantly increases prime editing efficiency (Fig. 3). Compared with manually designed pegRNAs (using original general design guidelines[4]), PlantPegDesigner-designed pegRNAs were found to have markedly higher prime editing efficiencies across five sites using single-pegRNA (1.3- to 4.4-fold higher; Fig. 3a) and three sites using dual-pegRNA (2.9- to 17.4-fold higher; Fig. 3b)[25]. Compared with other softwares, PlantPegDesigner designed pegRNAs have markedly higher prime editing efficiencies across three sites using single-pegRNA (on average 1.8-, 1.9- and 13.8-fold higher at three tested targets; Fig. 3c) and three sites using dual-pegRNA (on average 1.8-, 16.3- and 45.8-fold higher at three tested targets; Fig. 3d)[25]. And our previous studies[25] found the proportion of byproducts, including undesired indels, was not higher using the dual-pegRNAs than the single-pegRNAs.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All the data shown in this protocol are deposited in NCBI BioProject under accession codes PRJNA605069, PRJNA605074, and PRJNA702010. An example dataset for amplicon deep sequencing analysis used in this protocol is available on GitHub at https://github.com/ReiGao/GEanalysis.

Code availability

All the code used in this protocol is available on GitHub at https://github.com/ReiGao/GEanalysis. The PlantPegDesigner web application code is available at https://github.com/JinShuai001/PlantPegDesigner.

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Author contributions

C.G. S.J., Q.L. and Q.G. wrote the manuscript; Q.L. and S.J. designed figures; C.G. supervised the project;

Competing interests

The authors declare no competing interests.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All the code for amplicon deep sequencing ananlysis used in this protocol is available on GitHub at: https://github.com/ReiGao/GEanalysis. The web portal server is accessible at http://www.plantgenomeediting.net for non-profit use. The PlantPegDesigner web application code is available at https://github.com/JinShuai001/PlantPegDesigner.
Data analysis	Graphpad prism 6 was used to analyze the data.

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The deep sequencing data have been deposited in a National Center for Biotechnology Information BioProject database (accession code PRJNA702010).

Field-specific reporting

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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 400,000 protoplasts were used for each transfection. The number of protoplasts in each transfection was measured by thrombocytometry.
Data exclusions	No data exclusion.
Replication	All attempts at replication were successful.
Randomization	Rice 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.

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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.

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Flow Cytometry

Plots

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Methodology

Sample preparation	Rice protoplasts were isolated from the stem of rice seedlings, transfected as described in the Mehtods and incubated in 1 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.

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