Development of Plant Prime-Editing Systems for Precise Genome Editing

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ABSTRACT
Prime-editing systems have the capability to perform efficient and precise genome editing in human cells. In this study, we first developed a plant prime editor 2 (pPE2) system and test its activity by generating a targeted mutation on an HPT-ATG reporter in rice. Our results showed that the pPE2 system could induce programmable editing at different genome sites. In transgenic T0 plants, pPE2-generated mutants occurred with 0%–31.3% frequency, suggesting that the efficiency of pPE2 varied greatly at different genomic sites and with prime-editing guide RNAs of diverse structures. To optimize editing efficiency, guide RNAs were introduced into the pPE2 system following the PE3 and PE3b strategy in human cells. However, at the genomic sites tested in this study, pPE3 systems generated only comparable or even lower editing frequencies. Furthermore, we developed a surrogate pPE2 system by incorporating the HPT-ATG reporter to enrich the prime-edited cells. The nucleotide editing was easily detected in the resistant calli transformed with the surrogate pPE2 system, presumably due to the enhanced screening efficiency of edited cells. Taken together, our results indicate that plant prime-editing systems we developed could provide versatile and flexible editing in rice genome.

Key words: prime editing, CRISPR, rice, precise editing, surrogate system

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INTRODUCTION
Precise editing of the plant genome has long been desired for functional genomic research and crop breeding. Sequence-specific nucleases, especially the widely used clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems, are capable of introducing targeted DNA double-strand breaks (DSBs) in eukaryotic genomes. In the presence of a donor DNA template, programmable sequence deletion, insertion, and replacement can be generated by homology-directed repair (HDR) of DSBs. Although numerous efforts have been made, the HDR-mediated precise genome-editing method is still not well established in plants, largely due to extremely limited recombination frequencies and the delivery barrier of high copy number of exogenous donor.

CRISPR/Cas9-mediated base-editing systems were developed to enable targeted nucleotide substitutions independent of DSB formation or donor template. To date, two types of base-editing tools, cytidine base editor (CBE) and adenine base editor (ABE), have been exploited to enable programmable and irreversible C·G-to-T·A and A·T-to-G·C transitions, respectively. For the CBE tool, cytosine deaminases, such as rat APOBEC1, PmCDA, human AID, or human APOBEC3A, were fused to a SpCas9 D10A nickase (nSpCas9-D10A) to direct cytosine-to-thymine conversion with/without assistance of uracil glycosylase inhibitor (Komor et al., 2016; Nishida et al., 2016; Ren et al., 2018; Wang et al., 2018). For adenine editing, Escherichia coli transfer RNA adenosine deaminase (TadA) was engineered by directed evolution to enable DNA adenosine deaminase activity. Constructing by an evolved TadA*7.10 and nSpCas9 D10A, the ABE could efficiently convert A to G with negligible unwanted indel mutations (Gaudelli et al., 2017). Both CBE and ABE have been successfully applied in various model plants and crops (Chen et al., 2019). They are widely used to introduce targeted substitutions in major genes to improve important agricultural traits, including plant height, flowering time, disease resistance, and herbicide resistance (Chen et al., 2017; Lu and Zhu, 2017; Shimatani et al., 2017; Kang et al., 2018; Li et al., 2018, 2019a; Tian et al., 2018; Bastet et al., 2019; Zhang et al., 2019; Wu et al., 2020). Base editors were also employed to disrupt genes in plants by creating early stop codons or inducing transcript mis-splicing (Kang et al.,...
In addition, together with a single guide RNA (sgRNA) library, base editors can generate high-density substitutions in the target region, thus facilitating the directed evolution of plant genes (Kuang et al., 2020; Li et al., 2020a; Liu et al., 2020). Base editors have been extensively optimized to achieve better application in plants. To expand the editing scope, we replaced the SpCas9 in the base editors with Cas orthologs or evolved SpCas9 variants with relaxed protospacer adjacent motif (PAM) recognition (Hua et al., 2019a, 2019b; Qin et al., 2019a; Ren et al., 2019; Zhong et al., 2019; Wang et al., 2020). Furthermore, the efficiency of base editors was enhanced by modification of fusion protein structure, utilization of deaminase orthologs, or development of selection-enriched systems (Li et al., 2018, 2020c; Zong et al., 2018; Qin et al., 2019b; Hua et al., 2020; Xu et al., 2020a). Editing by base editors has outperformed HDR-mediated substitutions in plants in terms of efficiency and product purity. However, many mutation types,
such as base transversions and insertions, cannot be processed by current base editors, greatly constraining the application of base-editing systems. Therefore, novel systems that confer multiple types of genome editing are highly desired in plants.

Recently, prime editors (PEs) were developed to generate precise editing without the requirement of DSBs or donor DNA (Anzalone et al., 2019). The major component of PE is a fusion of a Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) and a Cas9 nickase (nCas9). A prime-editing guide RNA (pegRNA) was designed to mediate site-specific nicking by nCas9 and then served as a template for RT to install customizable mutations. PEs could efficiently produce all possible base conversions and small insertions in a wider targeting range with limited by-products in human cells (Anzalone et al., 2019). Here, we present plant prime-editing systems and show that they could provide versatile and precise genome editing in rice.

RESULTS

Establishment of a Prime-Editing System for Plants

The coding sequence of an M-MLV RT mutant (D200N/L603W/T330P/T306K/W313F) was codon-optimized for rice expression and synthesized (Supplemental Sequence). The engineered RT was fused to the C terminus of the SpCas9 H840A nickase (nSpCas9-H840A) with a 33-amino-acid linker. Following instructions obtained from plant ABE optimization (Li et al., 2018), one copy and three copies of nuclear location signal were further fused to 5’ and 3’ termini of the nSpCas9-RT. The hybrid molecule, namely plant prime editing 2 (pPE2), was then inserted downstream of the maize ubiquitin (ZmUBI) promoter in a pHUC411 backbone by replacing SpCas9 (Figure 1A), generating a pHUC411-PE2 binary vector (Supplemental Figure 1).

To intuitively validate the activity of the pPE2 system, we replaced the hygromycin phosphotransferase (HPT) gene in pHUC411-PE2 with the null allele with an ACG substitution at the start codon (HPT-ATG) (Xu et al., 2020a). To enable CRISPR-mediated editing, we inserted an artificial sequence immediately in front of the HPT-ATG as a target. This sequence originated from the PDS-1 target in the OsPDS gene (Qin et al., 2020), which was robustly mutated by SpCas9 and its variants with high efficiency in rice. The peHPT1 pegRNA was assembled by combining a 13-nt primer binding site (PBS) sequence and a 10-nt RT template to 3’ termini of the sgRNA (Figure 1B). The peHPT1 will introduce a C-to-T conversion at position +1 (5’ to 3’ counting from the nick site). This mutation will generate a start codon for HPT, thus recovering hygromycin resistance in the edited cells. The pHUC411-PE2 binary vector (Supplemental Figure 1).

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calli of japonica rice via agrobacteria. As expected, no resistance event was observed in the calli that transformed the vector without pegRNA. In contrast, 97 resistant events out of 162 total calli (59.9%) were obtained in the pHNUC411-PE2-peHPT1 trans-formant after 4 weeks of hygromycin selection (Figure 1C and 1D), suggesting that the mutation at the start codon was corrected by the pPE2 system. Next, 32 independent events were randomly selected to examine the target region by Sanger sequencing. In all tested samples, the position +1 C-to-T conversion was detected (Figure 1D and 1E). After regenerating under selection pressure, 28 lines of transgenic plants were obtained. In all T₉ regenerated plants, the HPT-ATG reporter was corrected and homozygous mutation was detected in five lines (Figure 1D), confirming that the pPE2 can efficiently mediate precise editing in plants. Another pegRNA, peHPT2, was designed to edit the same target at HPT-ATG. peHPT2 used a different protospacer with peHPT1 (Figure 1B). By using a 13-nt PBS sequence and a 34-nt RT template, peHPT2 would allow the position +20 C-to-T conversion at the pseudo-start codon of HPT-ATG. Under hygromycin selection, the resistant calli emerged with 12% frequency (18 out of 150, Figure 1C and 1D). Subsequent sequencing indicated that the editing was also obtained in resistant calli (Figure 1D). Only three mutated plants were regenerated, suggesting variable efficiency of pPE2 system with different pegRNAs.

### Precise Genome Editing with the pPE2 System in Transgenic Plants

To test whether pPE2 edits rice genome, we selected three genomic sites, OsPDS, OsACC1, and OsWx, as targets. A pePDS1 pegRNA was designed to introduce the position +2 insertion of T at the fifth exon of the OsPDS gene to generate an early stop codon in transcript (Figure 2A). After transformation, no albino plants were observed in the pPE2-pePDS1 transgenic rice, indicating the lack of homozygous or biallelic mutants. The OsPDS target site was genotyped in 96 independent events. We found that seven plants (7.3%) were precisely edited (Figure 2B and 2C). Consistent with phenotypic observations, all mutants were putative homozygous or chimera. A peACC1 pegRNA was designed to mediate G-to-C transversion to generate the aryloxyphenoxypropionate herbicide tolerance-related W2125C mutation in the OsACC1 gene (Figure 2A) (Li et al., 2020a). In 96 events of hygromycin-selected transgenic plants, 14 lines (14.6%) showed the expected position +1 G-to-C mutation in the OsACC site (Figure 2B and Table 1). Similar to the editing in pePDS1 plants, no homozygous mutation was detected at the OsACC1 site, implying limited efficiency of the pPE2 system in plants. Furthermore, the herbicide tolerance of W2125C mutant was examined in rooting medium supplied with 5 µM haloxyfop-R-methyl. As indicated in Figure 2D, no significant growth inhibition was observed in edited plants. The obvious enhancement in herbicide tolerance suggests that the prime-editing system can be applied to produce gain-of-function mutants in crops. In addition, a 31-nt RT template and a 15-nt PBS sequence were used to construct a peWX1 pegRNA for inducing T-to-G transversion at the splice site of Wx intron 1 (Figure 2A) (Cai et al., 1998). However, after examining 96 lines of regenerated plants, no mutation was detected at position +22 of the OsWx site.

To test whether pPE2 can install different types of mutations in the plant genome, we designed five additional pegRNAs (pePDS2 to pePDS6) for prime editing at the OsPDS target site. The 2- to 3-bp insertions, a 28-bp deletion, and base transversions were introduced at position +1 of the OsPDS site by these pegRNAs with the same protospacer, the same PBS sequence, and different customized RT templates (Figure 2C). For each pPE2 vector, 96 independent events were examined. The mutants of small insertions at position +1 (by pePDS2 and pePDS3) were identified in 12.5% and 19.8% of plants, respectively (Figure 2C). The targeted deletion induced by pePDS4, however, was not obtained in transgenic population. The A-to-T transversion induced by pePDS5 was obtained in up to 30 plants (achieving 31.3% frequency of mutants) while the pePDS6-induced A-to-C transversion was failed to be generated in plants (0 out of 96, Figure 2C).

The efficiency of prime editing was suggested to be closely related to pegRNA structure in human cells (Anzalone et al., 2019). At the OsACC1 site, three more pegRNAs (peACC2 to peACC4) were designed with the same protospacer of peACC1. Different from the 13-nt PBS sequence and the 10-nt RT template of peACC1, peACC2 pegRNA has a 10-nt PBS sequence and a 13-nt RT template (Supplemental Figure 2). peACC3 has the same 13-nt PBS sequence as peACC1 but a longer RT template (20 nt). Furthermore, an extended 15-nt PBS sequence and a 34-nt RT template were tested in the peACC4 pegRNA. Only 3.1% (3 out of 96) and 1.0% (1 out of 96) of lines were edited by pPE2 combining peACC2 and peACC3, respectively, while no peACC4-induced mutants were found in plants (0 out of 96, Table 1).
Prime Editing in Rice

Figure 3. The Surrogate pPE2 System Eases the Screening of Prime-Edited Plants.
(A) Schematic illustration of pPE2, pPE3/3b, and surrogate pPE2 system. The wheat TaU3 promoter was used for the expression of gRNA or pegHPT1, and the pegRNA of the genomic target was driven by the rice OsU3 promoter.
(B) Editing efficiency of pePDS2 and peWX1. After 2 weeks of selection, ~100 newly emerged resistant calli were selected together as one sample. The target region was then amplified to evaluate editing frequency by sequencing 32 randomly selected T-A clones. SD was generated from three biological repetitions. Significance was determined by two-tailed t-test (*p < 0.05).

Precise Genome Editing with PE3 and PE3b Systems in Transgenic Plants

To optimize efficiency in human cells, we developed a PE3 system by nicking the unedited strand to increase the chance of fixing DNA mismatches according to edited strands (Anzalone et al., 2019). We also tested same strategy in rice. To construct the plant PE3 (pPE3) system, we expressed an additional second guide RNA (gRNA) targeting the complementary strand under the control of a TaU3 promoter in the pPE2 system (Figure 3A). To increase the editing efficiency of peWX1, we co-expressed a gRNA for nicking at position +59 in the pHUC411-PE2 vector. After examining 96 lines of T0 transgenic plants, we found that the same target at the HPT-ATG reporter gets. The activity of the PE2 system is believed to be related to the limited efficiency of the pPE2 system in plant genomic targets. However, the most efficient editing in our study was achieved in ~17.6% of clones by peWX1. By contrast, the enhancement of the surrogate pPE2 system was not statistically significant (p = 0.05724), a targeted mutation could be generated in ~7.3% of clones, enabling mimicry of the critical single-nucleotide polymorphism (SNP) between Wxα and Wxβ allele in rice plants.

DISCUSSION
Prime-editing systems induce a broader spectrum of genome editing than base-editing systems in human cells. Here, we showed that programmable base transitions, base transversions, and 1- to 3-bp insertions were introduced in rice genome by the pPE2 system, indicating the versatility of prime editing in plants. Efficiency would be a major concern in the application of plant prime-editing systems. Generally, more than half of transgenic plants could be edited by well-optimized base editors in rice. However, the most efficient editing in our study was achieved in only 31.3% of transgenic plants (pePDSS5), which suggested the limited efficiency of the pPE2 system in plant genomic targets. The activity of the PE2 system is believed to be related to the pegRNA sequence in human cells (Anzalone et al., 2019). In plants, we found that the same target at the HPTATG reporter could be corrected with highly varied frequencies by using different pegRNAs in the pPE2 system. Differentiations in mutant frequency were also found in the transgenic population of peACC1 to peACC4. These four pegRNAs have an identical protospacer and would induce the exact same +1 mutation at the OsACC1 site. The lengths of the PBS sequence and RT template of the peACC pegRNAs should be a major source of variation in frequency of mutant occurrence (from 0% to 18.8%). Therefore, factors of pegRNA design for each target, including nick positions, lengths of PBS, and RT template, may have to be comprehensively optimized to achieve an ideal
efficiency of pPE2 at specific targets in plant genome. Interestingly, an obvious difference in mutant occurrence frequency was observed between pPDSS5 and pPDSS6 (31.3% compared with 0%), whose sequence differs only in the nucleotide for the base transversion. These data suggest that even a SNP may result in different pegRNA structure and lead to great variation in editing efficiency.

In human cells, the efficiency of prime editing could be optimized by the PE3 and PE3b system. However, at least in the genomic targets we tested in this study, no significant enhancement in mutant occurrence frequency was observed (Table 1). Unlike the numerous copies of PE delivered during human cell transfection, very limited prime-editing molecules can be obtained in plant cells of Agrobacterium-mediated stable transformation. The nicking on different strands thus may be less synchronized in a single target-editing and mismatch-repair process. A previous report indicated that the selection-assisted enrichment of base-edited cells would increase the screening efficiency of edited plants (Xu et al., 2020a). Similarly, our results showed that the prime-editing cells could also be enriched by the same strategy in rice calli (Figure 3). During the preparation and submission of this paper, four independent groups have reported successful plant prime editing at exogenous or endogenous targets by transiently or stably expressing PEs in rice and other plant species (Li et al., 2020b; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020b). Together with future optimizations, we believe that the prime-editing system will provide an efficient, versatile, and flexible genome-editing approach for plant research and crop improvement.

**METHODS**

**Vector Construction**

The rice codon-optimized M-MLV RT RT200NL603W/T330P/T306K/W313F mutant was synthesized (Genscript, Nanjing, China). The H840A mutation was introduced into a plant codon-optimized SpCas9 by the Fast Mutagenesis System (TransGen, Beijing, China). The pPE2 was assembled by a Gibson cloning kit (NEB, Ipswich, USA). The pPE2 was then inserted into a pHUC411 vector to replace the assembled by a Gibson cloning kit (NEB, Ipswich, USA). The pPE2 was selected under 50 mg/l hygromycin. In each resistance event, 1–5 small yellowish calli were selected as a single event to regenerate plants under selection pressure by 25 mg/l hygromycin for 4–5 weeks. The regenerated plants were then transferred to the rooting medium for genotyping after 2–3 weeks of growth. The tissue cultures were incubated at 26°C–28°C.

**Genotyping of Mutants**

To determine the prime editing in T0 transgenic plants, we selected at least three leaves of each event together as a single sample for genotyping. The genomic DNA was extracted by the CTAB (cetyl trimethylammonium bromide) method for use as PCR templates. The target regions were amplified with site-specific primers (Supplemental Sequence). Mutants were identified by the Hi-TOM assay with 5% threshold and/or Sanger sequencing (Liu et al., 2019).

**SUPPLEMENTAL INFORMATION**

Supplemental Information is available at Plant Communications Online.

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**AUTHOR CONTRIBUTIONS**

P.W. conceived this study, supervised the research, and wrote the manuscript with input from all authors. R.X. performed all experiments and analyzed data with help from the authors. J.L. and R.Q. contributed to vector construction and genotyping. X.L. contributed to rice transformation. T.S. contributed to surrogate pPE2 vector construction.

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