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Plant Prime Editors Enable Precise Gene Editing in Rice Cells

Dear Editor,

Genome editing is revolutionizing plant research and crop breeding. Sequence-specific nucleases (SSNs) such as zinc finger nuclease (ZFN) and TAL effector nuclease (TALEN) have been used to create site-specific DNA double-strand breaks and to achieve precise DNA modifications by promoting homology-directed repair (HDR) (Steinert et al., 2016; Voytas, 2013). Later, RNA-guided SSNs such as CRISPR-Cas9, Cas12a, Cas12b, and their variants were applied for genome editing in plants (Li et al., 2013; Nekrasov et al., 2013; Tang et al., 2017; Zhong et al., 2019; Ming et al., 2020; Tang et al., 2019). However, HDR relies on simultaneous delivery of SSNs and DNA donors, which has been challenging in plants (Steinert et al., 2016; Zhang et al., 2019). Another challenge for realizing efficient HDR in plants is that DNA repair favors non-homologous end joining (NHEJ) pathways over HDR in most cell types (Puchta, 2005; Qi et al., 2013). Unlike SSN-induced HDR, which is limited by the choice of the donor and DNA repair mechanism, cytidine or adenine base editors that were developed in recent years can convert C to T or A to G within a 3–8 nucleotide targeting window in the protospacer, respectively (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017). Base editors, albeit highly efficient, can only direct certain transition mutations, but cannot perform predefined transversion mutations or insertions and deletions (indels). In all these contexts, the recent breakthrough on the development of so-called prime editors (PEs) in human cells is very exciting (Anzalone et al., 2019). In prime editing, a Cas9H840A nickase is fused with a reverse transcriptase. The fusion protein nicks the editing DNA strand to initiate reverse transcription by priming to the nicked DNA and copying genetic information encoded by a prime editing-guide RNA (pegRNA). The versatile pegRNA is a modified single-guide RNA (sgRNA) that carries a reverse transcription (RT) template and primer binding site (PBS) or primer in sequence at its 3′ end. PEs do not require DNA donors as in HDR. PEs also appear to be more precise and as efficient as base editors at certain target sites (Anzalone et al., 2019).

We were interested in assessing this new prime editing technology in plants and did our investigations in rice cells. According to the seminal article by Liu and coauthors (Anzalone et al., 2019), Prime Editor 3 systems (PE3 and PE3b) are most efficient. We generated a Plant Prime Editor 3 version 1 (PPE3-V01) where the Cas9H840A and the engineered Moloney murine leukemia virus (M-MLV) RT were both codon optimized for plant expression (Figure 1A). The pegRNA and the sgRNA used for nicking the non-edited strand were expressed by OsU3 and OsU6 promoters, respectively (Figure 1A). We first evaluated the PE3 system where the nsgRNA would nick the non-editing strand within 100 bp from the editing site. We chose five target sites in four genes (*OsALS*, *OsKO2, OsDEP1*, and *OsPDS*) and decided to primarily use the 13-nt length for both the PBS and the RT templates. Rice protoplasts were transfected with the resulting T-DNA expression vectors of PPE3-V01 and the editing was analyzed by next-generation sequencing (NGS) of PCR amplicons. Anticipated prime editing outcomes were confirmed at all five sites, although the editing efficiencies at these sites were quite low (0.05%–0.15%) (Figure 1B). The positive reads of the NGS data were validated for precise incorporation of designed edits, and some reads revealed large deletions presumably due to paired nicking when pairing the Cas9H840A nickase with two guide RNAs (a pegRNA and an nsgRNA) in the PB3 system (Figure 1C). To minimize these deletion byproducts, we applied the PB3b strategy where the nsgRNA was designed to match the edited strand, but not the wild'-type sequence. We also wanted to try out different target sites, hoping to see improved editing frequency at any of them. Hence, we targeted five additional sites in four genes (*OsALS*, *OsEPSPS*, *OsGRF4*, and *OsSPL14*) with our PPE3b-V01 system. This time, we also tried variable lengths for RT templates, ranging from 13 nt to 23 nt. Prime editing outcomes were again observed at these five sites, with the highest editing frequencies up to 0.4% (by *OsEPSPS*-pegR01) (Figure 1D). Analysis of the NGS data suggested most of positive reads represented precise prime editing outcomes as expected, and deletion byproducts were indeed greatly diminished with this PB3b strategy (Figure 1E). Interestingly, we found a common type of illegitimate editing outcome due to the insertion at 5′ upstream of the RT template within the pegRNA (Figure 1E; see DNA sequences marked in purple). However, this is not surprising and can be attributed to extensive RT activity.

We were curious whether our PPE3/PPE3b-V01 vector system is responsible for the relatively low prime editing activities at the 10 endogenous sites in rice cells. To address this, we made a separate plant PE system, termed PPE-V02, which is different from PPE-V01 in many aspects, including codon optimization for both Cas9 and M-MLV RT, nuclear localization signal configuration, promoters and terminators for the Cas9H840-M-MLV-RT fusion gene and guide RNAs, the strategy for multiplexing two guide RNAs, and the vector backbone (Figure 1F). We also reasoned that a systematic approach for testing different PBS and RT template pairs of different lengths may be required to achieve higher prime editing frequency at each target site. To this end, we generated a series of PPE3-V02 vectors for prime editing at three independent sites, and we included five different pairs of PBS and RT templates for testing in rice protoplasts. Indeed, our results showed that prime editing frequencies varied at each target site, which was greatly

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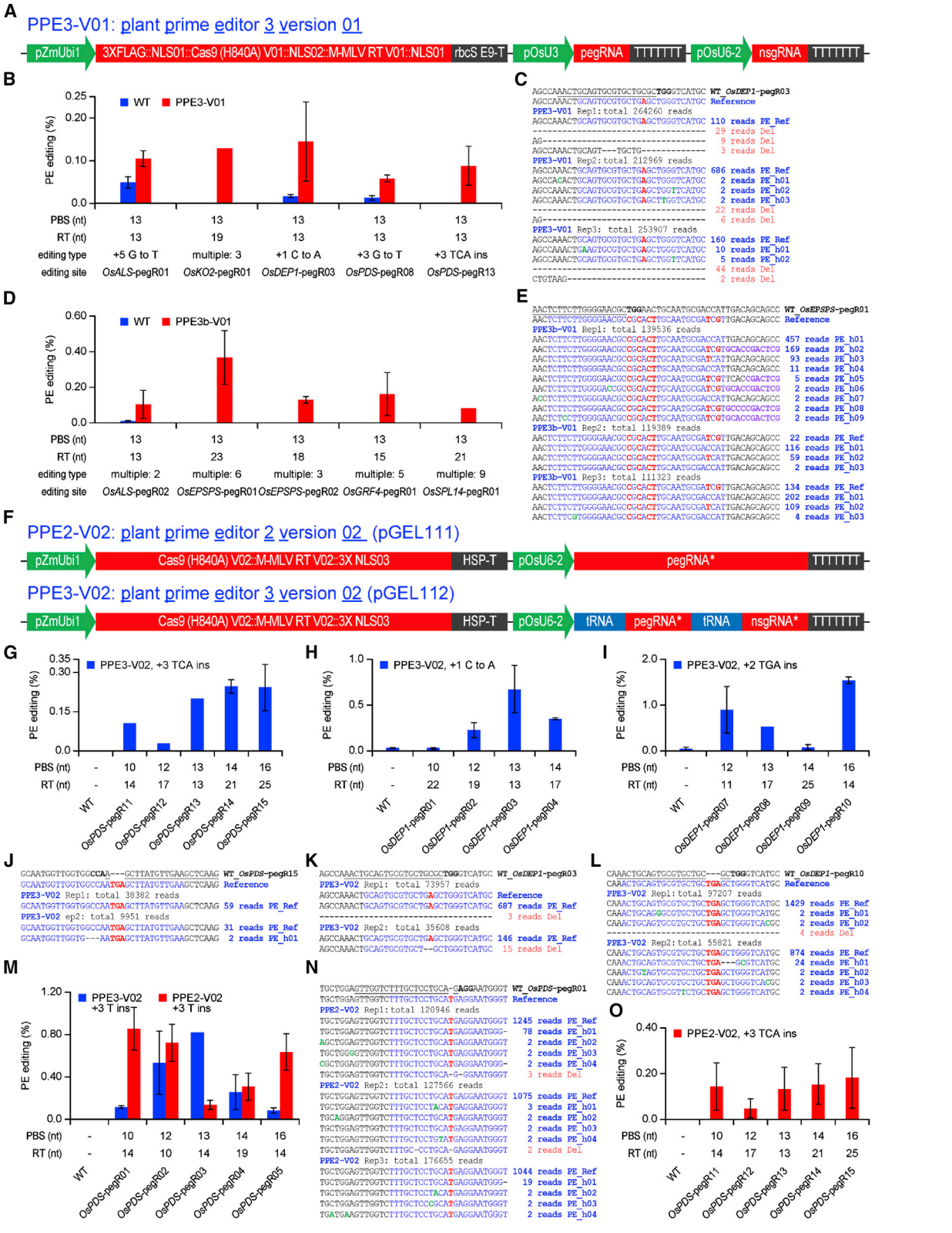

Figure 1. Assessment of Multiple Plant Prime Editors in Rice Cells.

(A) Schematic of the expression vector of Plant Prime Editor 3-Version 1 (PPE-V01).

(B) Quantification of prime editing frequencies by PPE3-V01 at five target sites. Lengths of primer binding site (PBS) and reverse transcription (RT) template, editing type, and editing site are indicated under the x axis.

(legend continued on next page)

impacted by the PBS–RT choices (Figure 1G-1I). As the same three edits in *OsPDS* and *OsDEP1* were pursued earlier by PPE3-V01 (Figure 1B), our data suggested that PPE3-V02 had improved the editing efficiency at these sites with an optimal PBS–RT template combination (Figure 1G and 1H and Supplemental Figure 1). Notably, PPE3-V02 resulted in the highest editing frequency of 1.55% at another site in *OsDEP1* (by *OsDEP1*-pegR10) (Figure 1I). Considering that the M-MLV RT has optimal activity at higher temperatures (Anzalone et al., 2019), we were curious whether prime editing efficiency could be improved by increasing temperatures. We tested our PPE3-V02 system at 37°C but did not find obvious improvement on editing efficiency compared with 32°C, which is the default temperature used in this study (Supplemental Figure 2).

The PE3 systems were previously demonstrated to have higher editing frequency in human cells than PE2, which does not use a second sgRNA to nick the non-edited strand (Anzalone et al., 2019). However, this might not be the case in plants due to the possible differences in RT reactions and DNA repair pathways in plant cells. Hence, we made a plant PE2 (PPE2-V02) system (Figure 1F) and compared it with PPE3-V02 at editing an *OsPDS* site based on different PBS–RT template combinations. Interestingly, the results suggested PE2-V02 had comparable or even higher editing frequency than PE3-P03 at this site in most cases (Figure 1M). Precise editing by PE2-V02 was further validated by the analysis of the NGS reads (Figure 1N). Application of PE2-V02 at another site, however, resulted in an overall low editing frequency even though multiple PBS–RT template combinations were explored (Figure 1O). Taken together, our data suggest that both PE2 and PE3 systems are functional in rice cells, and it is worth trying both for pursuing optimal prime editing for a given site in plants.

In summary, we developed three versions of plant PEs and demonstrated their use for precise editing at many endogenous genes in rice. SNPs and indels have been successfully introduced at these sites at variable frequencies with PE2, PE3, and PE3b strategies. The prime editing frequencies that we achieved in rice cells might be on a par with the SSN-mediated HDR systems. However, they are one order of magnitude lower than the editing frequencies reported in human cells (Anzalone et al., 2019). Hence, significant improvements are required to develop efficient plant PEs before prime editing reaches prime time in plants.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

Y.Z. and Y.Q. designed the experiments. X.T. and S.S. designed and made the constructs for the prime editing systems. X.T., S.S., Q.R., X.J., M.L., T.F. and D.Y. generated all prime editing T-DNA vectors. X.T., Q.R., X.J., and X.Z. conducted rice protoplast isolation and transformation. X.T., Q.R., X.J., M.L., T.F., S.X., Y.G., L.L., and. X.Z. performed NGS sample preparation. X.T., Q.R., and S.S. performed NGS data analysis. Y.Z. and Y.Q. analyzed the data and wrote the manuscript. All authors participated in discussion and revision of the manuscript.

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(C) Prime editing events revealed by next-generation sequencing (NGS).
(D) Quantification of prime editing frequencies by PPE3b-V01 at five target sites.
(E) Editing events revealed by NGS reads.
(F) Schematics of the expression vectors of Plant Prime Editor 3 or 2-Version 2 (PPE3/2-V02).
(G–I) Comparison of multiple PBS–RT pairs of different lengths for directing TCA insertion at the *OsPDS* target site, directing C to A base change at the *OsDEP1* target site and directing TGA insertion at the *OsDEP1* target site, respectively, by PPE3-V02.
(J and K) Validation of prime editing outcomes by NGS at the *OsPDS*-pegR15, *OsDEP1*-pegR03, and *OsDEP1*-pegR10 target sites.
(M) Comparison of PPE3-V02 and PPE2-V02 for precise editing at five target sites.
(N) PPE2-V02 based prime editing events revealed by NGS for the *OsPDS*-sgRNA01 3T ins construct (for insertion of a T 3 nt downstream of the PBS).
(O) PPE2-V02 based prime editing at another site with multiple PBS-RT pairs of different lengths. The experiments were done in rice protoplasts.
Three biological replicates were used to assess the PPE3-V01 system **(B–E)**, and two biological replicates were used to assess PPE3-V02 and PPE2-V02 systems **(G–O)**. Error bars represent standard deviations of the biological replicates. For NGS-based genotyping data presentations **(C, E, J, K, L, and N)**, the sequences (from top to bottom) include the wild-type (WT) sequence (protospacer underlined and PAM in bold), the expected prime editing outcome (Reference), confirmed precise prime editing events matching the expected prime editing outcome (PE_Ref), precise prime editing plus additional single nucleotide polymorphisms (e.g., PE_h01; h stands for haplotype) and deletions resulted from the NHEJ repair. The prime edited DNA nucleotides are highlighted in red.

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