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Letter to the Editor

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Precise Editing of a Target Base in the Rice Genome Using a Modified CRISPR/Cas9 System

Dear Editor,

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated Cas9) has been widely used in genome editing in a variety of organisms, including rice (Cong et al., 2013; Feng et al., 2013). The majority of the editing events reported in plants typically result in an abundance of random insertions and deletions (Indels) at the target locus through the error-prone non-homologous end joining pathway (NHEJ) (Feng et al., 2013; Mao et al., 2013; Shan et al., 2013), making genome editing an extremely valuable tool for plant functional genomics research. Most known agriculturally important traits, however, are conferred by point mutations (Huang et al., 2010; Hu et al., 2015). Development of a technique that enables precise and efficient base replacement in the target locus, rather than stochastic disruption of the gene, will greatly facilitate precision plant molecular breeding. Homology-directed repair (HDR) is a potential approach to achieve base replacement by providing a homologous DNA template during genome editing. However, HDR is extremely inefficient due to competition by NHEJ, the dominant pathway to repair double-strand breaks (DSBs) in plants, and Indels are generally much more abundant outcomes, making HDR a rather ineffective method to achieve base replacement in plants (Li et al., 2013; Mao et al., 2013).

Recently, a new approach called "base editing" was developed in mammalian cells, which enables direct and irreversible conversion of one target base into another in a programmable manner, without requiring DSB or a donor template (Komor et al., 2016). In this base-editing system, the rat cytidine deaminase enzyme APOBEC1 is fused to the N-terminus of a Cas9 nickase (Cas9n) that retains the ability to be programmed with a guide RNA (gRNA), and mediates the direct conversion of cytidine (C) to uridine (U), thereby effecting a high frequency (15%–75%) of C→T (or G→A) substitution in human cells (Komor et al., 2016). This method potentially offers an alternative to the HDR-mediated base-replacement approach in plants, and if it works, will greatly facilitate precision plant molecular breeding. Here, we developed a base-editing system in rice using rat APOBEC1, providing a simple and highly efficient base-replacement method for plant research and breeding.

Similar to the mammalian base-editing system, we synthesized rat APOBEC1 and fused it to the N-terminus of Cas9(D10A) using the unstructured 16-residue peptide XTEN (Schellenberger et al., 2009) as a linker (Supplemental Figure 1). A nuclear localization signal (NLS) peptide was added to the C-terminus of Cas9(D10A). The semi-active Cas9(D10A) could nick the non-edited strand and increase the efficiency of base editing by inducing base-excision repair to resolve the U:G mismatch into desired U:A and T:A products (Komor et al., 2016). This APOBEC1-XTEN-Cas9(D10A) fusion sequence was then constructed into a binary vector, under the control of the maize ubiquitin promoter (UBI). Two *Bsa*I endonuclease sites were incorporated to flank the protospacer of gRNA to enable one-step construction of the base-editing plasmid for *Agrobacterium*-mediated transformation (Figure 1A). To test this base-editing system and assess its feasibility for use in plant breeding, two agriculturally important genes of rice, *NRT1.1B* and *SLR1*, were selected for editing. *NRT1.1B* encodes a nitrogen transporter, and it has been shown that a C→T replacement (Thr327Met) in this gene (Figure 1B) could increase nitrogen use efficiency in rice (Hu et al., 2015). *SLR1* encodes a DELLA protein, and amino acid substitutions in or near its TVHYNP motif (Figure 1C) result in reduced plant height (Ikeda et al., 2001; Asano et al., 2009; Hu et al., 2015). In human cells, the efficient "deamination window" (C→T) is approximately 5 nt, typically from positions 4 to 8 within the protospacer, counting the end distal to the protospacer-adjacent motif (PAM) as position 1 (Figure 1D). Accordingly, one CRISPR/Cas9 target site for *NRT1.1B* and one for *SLR1* were designed for the intended editing (Figure 1B and 1C). Rice calli of Zhonghua11 (ZH11) were transformed using *Agrobacterium* and hundreds of hygromycin-resistant calli were obtained after two rounds of selection. Approximately 200 of these hygromycin-resistant calli were mixed together for genomic DNA extraction and genotyped using next-generation sequencing (NGS). As shown in Figure 1D, C→T substitution was achieved within the "deamination window", accounting for 1.4%–11.5% of the total DNA sequences. Similar to the base-editing results in human cells, C→G replacement was also detected at a frequency of 1.6%–3.9%. The C→T/G substitution frequency varied depending on the sequence context, following the same order TC > CC ≥ AC (the second nucleotide C is the target nucleotide) as in human cells (Komor et al., 2016). The base-editing efficiency on *NRT1.1B* was lower compared with that on *SLR1*. This was possibly caused by the lower targeting efficiency of the gRNA for *NRT1.1B*, because the Indel frequency was also much lower at *NRT1.1B* (1.6% versus 7.2%, Figure 1D). These results indicated the feasibility of this base-editing system in plants.

In order to further explore the feasibility of this method in plant breeding, stable transgenic seedlings were regenerated from the hygromycin-resistant calli. As listed in Figure 1E, 37 independent transgenic lines of *NRT1.1B* were genotyped by Sanger sequencing, and the results showed that one of these transgenic seedlings harbored a C→G substitution at

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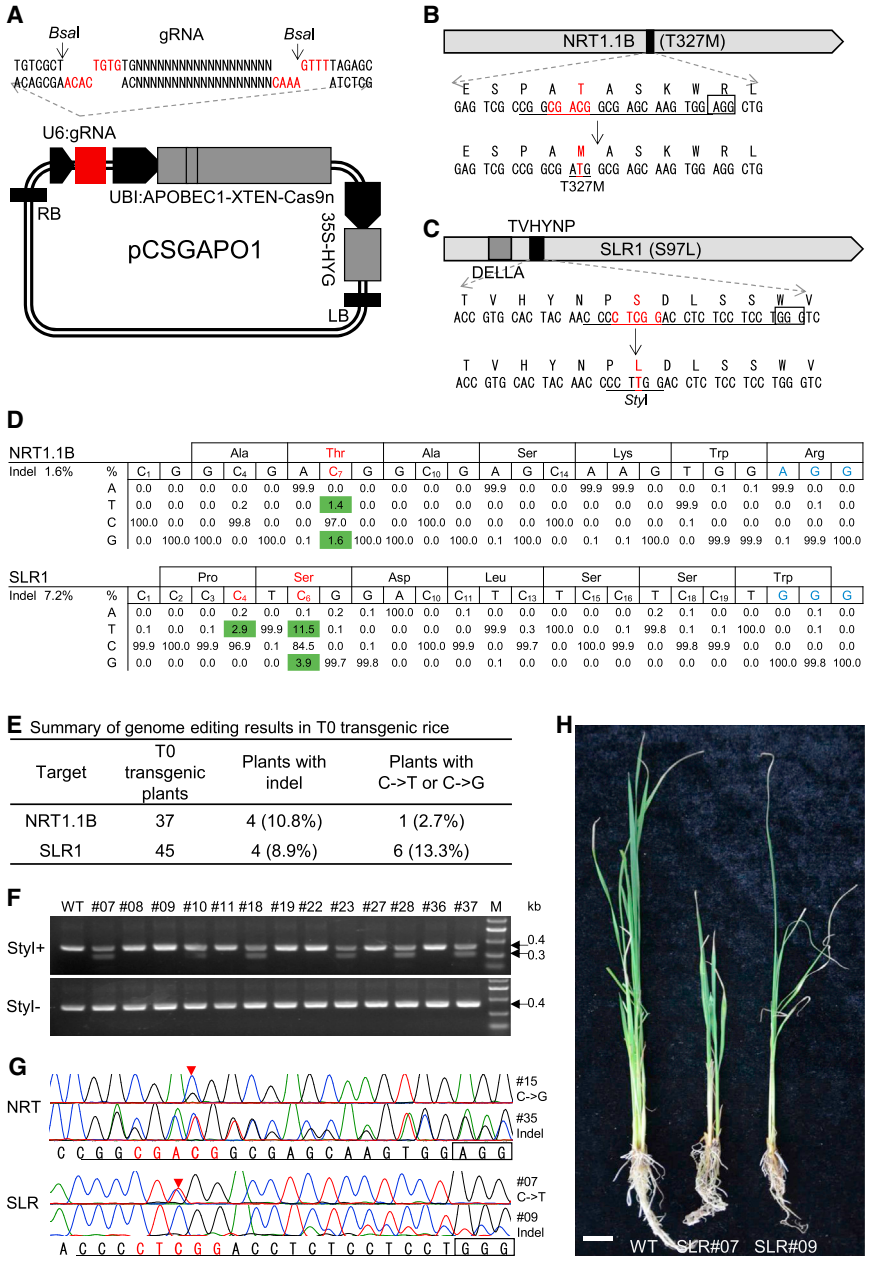

Figure 1. High-Efficiency Editing of a Targeted Base in Rice Using a Modified CRISPR/Cas9 System.

(A) Schematic diagram of the construct for base editing in rice.

(B) The target site for gene *NRT1.1B* of rice.

(C) The target site for gene *SLR1* of rice.

(D) Base-editing results in rice calli identified using next-generation sequencing (NGS). Rice calli of ZH11 were transformed using *Agrobacterium*, and hygromycin-resistant calli were selected for NGS. The sequence of the protospacer is shown with the PAM in blue and the target base in red. The subscripted number indicates the position of the base within the protospacer. The values in green indicate the detected base-substitution rates.

(E) Summary of base-editing results in T0 transgenic rice.

(F) Genotyping of 13 selected T0 transgenic rice for *SLR1* by RFLP analysis. The PCR products were digested with (upper) or without (lower) *Sty*I.

(G) Representative Sanger sequencing chromatograms for transgenic lines containing base substitution (NRT#15, SLR#07) or Indels (NRT#35, SLR#09). The substitution sites are marked with the red arrowheads.

(H) Phenotypes of T0 transgenic seedlings targeting *SLR1*. Wide-type, C→T substituted line #07 and Indel line #09 are shown from left to right. The scale bar represents 1 cm.

For **(B, C, and G)**, the sequence of the protospacer is underlined with the PAM in the box, the deamination window in red, and potential targeted cytidine in bold. For **(F and H)**, WT indicates wide-type rice ZH11 and M stands for DNA marker.

position 7. According to the sequencing chromatogram for this plant (Figure 1G, line 15), the C→G substitution did not occur in all cells, indicating that the plant is chimeric or heterozygous at this site. We did not detect C→T substitution in any of these 37 transgenic seedlings. More transgenic plants will need to be screened in future to identify plant lines harboring a C→T substitution at *NRT1.1B*. For *SLR1*, the C→T substitution generates a *Sty*I endonuclease recognition site (Figure 1C). Therefore, transgenic seedlings for *SLR1* were genotyped using restriction fragment length polymorphism (RFLP) in which successful digestion by *Sty*I cuts the 404 bp PCR product into two fragments (90 bp and 314 bp). The RFLP results showed that six of the 45 transgenic plants genotyped contained a C→T substitution. The presence of an undigested band (404 bp bands) in the base-edited lines suggests that these lines are not homozygous. Consistent with the RFLP results, Sanger sequencing results further confirmed the base replacement in the six transgenic lines (Figure 1E–1G, Supplemental Figure 2). Although a low frequency of C→G replacement was detected by NGS in the calli, C→G replacement was not detected in any of the 45 transgenic seedlings (Figure 1E). In agreement with our NGS results and with the base-editing properties in mammalian cells that the editing efficiency is highest at or near position 7, all the C→T mutations detected in these 45 transgenic lines for editing *SLR1* occurred at position 6, but not position 4 (Figure 1G).

The base replacement (Ser97Leu) near the TVHYNP motif of SLR1 (Figure 1C) is expected to be a dominant mutation, and thus may reduce plant height even in a heterozygous state (Ikeda et al., 2001; Asano et al., 2009). Indeed, the C→T substitution lines gave an obvious semi-dwarf phenotype (Figure 1H, line 07). In contrast, a typical slender phenotype was observed for transgenic lines with Indels in the TVHYNP motif (Figure 1H, line 09). To further investigate the base-editing events, PCR products from the base substitution lines of *NRT1.1B* (line 15) and *SLR1* (line 07) were cloned

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and sequenced. The results revealed that these mutants only contained the wild-type allele and the base substitution allele (Supplemental Figure 3). The base-editing mutations are likely heterozygous, and thus are expected to be transmitted to progeny plants. Taken together, these data show that stable base-edited plants can be efficiently generated using this modified CRISPR/Cas9 system.

In addition to the base substitutions, Indels were also observed in both the NGS and Sanger sequencing results (Figure 1D, 1E, and 1G). The Indel mutations may be caused by the Cas9(D10A) that nicks the non-edited strand. Compared with the Indel frequency in the mammalian base-editing system (typically <1%) (Komor et al., 2016), the frequency was much higher in plants (~10%). This is possibly caused by the stable genome integration of T-DNA that continuously generates Cas9(D10A) in plants, whereas Cas9(D10A) expression in the mammalian cells is transient. In the mammalian base-editing system, uracil DNA glycosylase inhibitor (UGI) was used to increase the base-editing efficiency, since a very high efficiency is necessary for gene therapy (Komor et al., 2016). We chose not to use UGI to increase the base-editing efficiency in plants, because the 1%–10% efficiency values are acceptable for plant breeding purposes and also because UGI may have negative effects on the plant genome when it is stably integrated into the genome with the T-DNA.

Although this base-editing system described here was limited to C→T and C→G (G→A and G→C) substitutions and the base-editing efficiency varied for different targets, the simplicity and effectiveness of this system will make base editing a routine practice for plant research and breeding, especially considering the formidable difficulty of using HDR to achieve base substitution in plants. Since this approach does not involve a DNA donor template, it avoids the occurrence of random integration of donor DNA in the genome of the edited plants. The successful application of rat APOBEC1 here suggests that the fusion of other xenogeneic DNA-modifying enzymes may enable additional types of programmable genome and epigenome base editing in plants. In the future, plant cytidine deaminase homologs (Chen et al., 2016) may also be tested for possible improvements in the efficiency of base-editing.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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